records after the first 2 years of the holding period.

PART 436—TESTS AND METHODS OF ASSAY OF ANTIBIOTIC AND ANTIBIOTIC-CONTAINING DRUGS

Sec.

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Subpart A—Definitions; Interpretations; Requirements

§ 436.1 Sterility requirements of items packaged with sterile antibiotic drugs.

(a) *Diluents packaged in combination with sterile antibiotic drugs.* If a sterile antibiotic drug is packaged in combination with an immediate container of a diluent, the immediate container of diluent shall be sterile when tested by the method prescribed in § 436.20(e)(1).

(b) *Dispensers packaged in combination with sterile antibiotic drugs.* If a sterile antibiotic drug is packaged in combination with a dispenser, such dispenser shall be sterile when tested by the method prescribed in § 436.20(e)(1).

[39 FR 18944, May 30, 1974, as amended at 41 FR 46852, Oct. 26, 1976]

§ 436.2 Alternative assay methods.

Alternative assay methods (including automated procedures) employing the same basic chemistry or microbiology as the official methods described in this part and in the individual monographs of this chapter may be used, provided the results obtained are of equivalent accuracy. However, only the results obtained from the official methods designated in the individual monographs are conclusive.

Subpart B—Sterility Test Methods

§ 436.20 Sterility test methods and procedures.

(a) *Laboratory facilities.* The test must be performed using aseptic techniques in an area as free from contamination as is possible to achieve. Testing should not be conducted under direct exposure to ultraviolet light or in areas under aerosol treatment. Environmental tests to assess the suitability of testing conditions should be made frequently enough to assure the validity of test results.

(b) *Equipment and reagents*—(1) *Bacterial membrane filter.* The filter has a nominal porosity of 0.45 micron±0.02 micron, a diameter of approximately 47 millimeters, and a flowrate of 55 milliliters to 75 milliliters of distilled water passing each square centimeter of filter area per minute with a differential pressure of 70 centimeters of mercury at 25° C.

(2) *Penicillinase solutions.* When the amount of penicillinase to be used is specified in terms of Levy units, use a penicillinase solution standardized in terms of Levy units. One Levy unit of penicillinase inactivates 59.3 units of penicillin G in 1 hour at 25° C. and at a pH of 7.0 in a phosphate buffered solution of a pure alkali salt of penicillin G when the substrate is in sufficient concentration to maintain a zero order reaction.

(c) *Culture media.* Use ingredients that conform to the standards prescribed by the U.S.P. or N.F. In lieu of preparing the media from the individual ingredients, they may be made from dehydrated mixtures which, when reconstituted with distilled water, have the same or equivalent composition as such media and have growth-promoting buffering, and oxygen tension-controlling properties equal to or better than such media. The pH of each medium should be adjusted with 2*N* hydrochloric acid or sodium hydroxide

before sterilization, so that after sterilization and the addition of the penicillinase, if necessary, the pH will fall within the specified range. Dispense 90±10 milliliter quantities of the liquid media into individual test tubes (38 millimeters x 200 millimeters). Close the tubes with suitable closures, and sterilize in an autoclave at 121° C. for 20 minutes. The autoclave temperature should be reached within 10 minutes. After sterilization, cool the medium at once to approximately 25° C. and store at 20° C. to 30° C. The sterility of each lot of tubes of liquid medium may be confirmed by incubating an adequate number of tubes as described in the test procedures in paragraph (e) of this section.

(1) *Medium A.* Use U.S.P. fluid thioglycolate medium I.

(2) *Medium B.* Use U.S.P. fluid thioglycolate medium I, with sufficient sterile penicillinase added to inactivate the penicillin activity in the sample under test. The penicillinase must be added to individual tubes of sterile medium A, using aseptic technique. Prior to use, or at the time of the test, a representative number of the tubes containing added penicillinase are incubated at 30° –32° C. for 24 hours to 48 hours, and are examined for sterility. If the sample contains penicillin as the only antibiotic, the ability of the penicillinase to inactivate all the penicillin in the sample under test is checked as follows: Add to one test tube of medium B the proper amount of penicillin from one of the individual containers under test. Then add 1.0 milliliter of a 1:1.000 dilution of an 18–24 hour culture of *Staphylococcus aureus* (American Type Culture Collection 6538–P)[1] in medium A. Typical microbial growth must be observable after 24 hours incubation at 30°–32° C. If the sample contains a mixture of penicillin plus some other antibiotic or antibacterial agent the ability of the penicillinase to inactivate all the penicillin in the sample is not tested directly on the sample under test, but is determined separately, using an amount of penicillin alone equivalent to the amount of penicillin in the sample or by any other suitable method for standardizing the penicillin-inactivating power of the penicillinase preparation.

(3) *Medium C.* To each liter of medium A add 5.0 milliliters of polysorbate 80 before sterilization. To each tube of sterilized medium add sufficient sterile penicillinase, and proceed as directed for medium B.

(4) *Medium D.* To each liter of medium A add 5.0 milliliters of polysorbate 80 and sufficient 2*N* sodium hydroxide so that the pH will be 7.9±0.1 after sterilization. Then add sufficient sterile penicillinase to each tube and proceed as directed for medium B.

(5) *Medium E.* Use U.S.P. XVIII soybean-casein digest medium.

(6) *Medium F.* To each liter of medium E add 5.0 milliliters of polysorbate 80 before sterilization. To each tube of sterilized medium add sufficient sterile penicillinase to solubilize the penicillin in the sample to be tested.

(7) *Medium G.* Prepare as follows:

Peptic digest of animal tissue .....6.0 gm.
Pancreatic digest of casein .....4.0 gm.
Yeast extract .....3.0 gm.
Beef extract .....1.5 gm.
Dextrose.....1.0 gm.
Agar.....15.0 gm.
Distilled water, q.s.....1,000.0 ml.
pH 6.6±0.1

Suspend the powder in a liter of distilled water. Allow to stand for 5 minutes, then mix thoroughly. Boil for 1 or 2 minutes or until solution is complete. Dispense in suitable flasks and sterilize at 121° C. for 15 minutes. Aseptically pour approximately 25-milliliter quantities into sterile Petri dish bottoms measuring 20 millimeters x 100 millimeters. Cover plates with sterile porcelain tops, glazed on the outside. Allow plates to stand at room temperature for 48 hours prior to use as a control on the sterility of the plates.

(8) *Medium H.* Prepare, sterilize, and dispense as described for medium G, except as follows:

Dextrose .....40.0 gm.
Peptic digest of animal tissue .....10.0 gm.
Agar.....15.0 gm.
Distilled water q.s.....1,000.0 ml.
pH 5.6±0.1 after sterilization

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

(9) *Medium I.* To each liter of Medium A add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol.

(10) *Medium J.* To each liter of Medium E add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol.

(11) *Medium K.* (*Rinse medium*). Prepare as follows:

Peptic digest of animal tissue	5.0 gm.
Beef extract	3.0 gm.
p-tert-octylphenoxy polyethoxyethanol	10.0 gm.
Distilled water, q.s	1,000.0 ml.

pH 6.9±0.2 after sterilization

(12) *Medium L.* To each liter of Medium A add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol and approximately 10,000 Levy units of penicillinase.

(13) *Medium M.* To each liter of Medium E add 1 milliliter of *p-tert*-octylphenoxy polyethoxyethanol and approximately 10,000 Levy units of penicillinase.

(14) *Medium N:*

Pancreatic digest of casein	15.0 gm.
Peptic digest of soybean meal	5.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Water	1,000.0 ml.

pH 7.3±0.2 after sterilization

(d) *Diluting fluids*—(1) *Diluting fluid A.* Dissolve 1 gram of U.S.P. peptic digest of animal tissue or equivalent in sufficient distilled water to make 1,000 milliliters. Dispense in flasks and sterilize as described in paragraph (c) of this section. Final pH=7.1±0.1.

(2) *Diluting fluid B.* To each liter of diluting fluid A add 5.0 milliliters of polysorbate 80 before sterilization.

(3) *Diluting fluid C.* To each liter of diluting fluid A add 0.5 gram of sodium thioglycollate, and adjust with NaOH so that after sterilization the final pH will be pH 6.6±0.6. Dispense in flasks and sterilize as described in paragraph (c) of this section.

(4) *Diluting fluid D.* To each liter of diluting fluid A add 1 milliliter of *p-tert*- octylphenoxy polyethoxyethanol. Dispense in flasks and sterilize as described in paragraph (c) of this section. Final pH=7.1±10.1.

(5) *Diluting fluid E.* Use isopropyl myristate that is sterile and that has a water-extract pH of 5.5 or greater. Determine the water-extract pH of a portion of the isopropyl myristate as follows: Place 100 milliliters of the isopropyl myristate sample and 10 milliliters of distilled water into a centrifuge bottle of approximately 250 milliliters capacity and seal the bottle tightly. Place the centrifuge bottle on a shaker so that its longest dimension is oriented in the direction of shaker movement and shake at 250 cycles per minute for 1 hour. Centrifuge the bottle at 1,800 revolutions per minute for 20 minutes. With a suitable vacuum system, remove and discard the upper layer; then pipet 5 milliliters of the lower water layer into a beaker and determine the pH using a standardized pH meter. If the water-extract pH is less than 5.5, pass the isopropyl myristate through a glass column packed with basic aluminum oxide, activity grade No. 1. Determine the water-extract pH of a portion of the isopropyl myristate that has been passed through the aluminum oxide column. Sterilize isopropyl myristate by filtration through a 0.22-micron membrane filter and aseptically dispense 100-milliliter portions into sterile 250-milliliter flasks.

(6) *Diluting fluid F.* To each liter of diluting fluid A add 20 grams of disodium edetate, and adjust with NaOH so that after sterilization the final pH will be 7.1±0.1. Dispense in flasks and sterilize as described in paragraph (c) of this section.

(7) *Diluting fluid G.* To each liter of sterile diluting fluid A add 10 grams of sterile *L*-lysine.

(8) *Diluting fluid H.* To each liter of diluting fluid A add 10 grams of sodium bicarbonate before sterilization.

(9) *Diluting fluid I.* To each liter of diluting fluid A add 23.4 grams of sterile L-arginine base.

(10) *Diluting fluid J.* Sterilize 2.0 grams of anhydrous sodium carbonate by dry-heating at 180° C for 2 hours. Dissolve in 100 milliliters of diluting fluid A just prior to use.

(e) *Conduct of test*—(1) *Bacterial membrane filter method*—(i) *Sample preparation*—(*a*) Antibiotic drug. From each of 20 immediate containers, aseptically transfer approximately 300 milligrams of solids if it is not a liquid drug, or 1 milliliter by volume if it is a liquid drug, or the entire contents if the container contains less than these amounts; except that if it is a liquid

drug containing penicillin in a concentration greater than 300,000 units per milliliter, use the volume that contains 300,000 units, into a sterile 500-milliliter Erlenmeyer flask containing approximately 200 milliliters of diluting fluid A. (If it is a composite sample packaged in one immediate container in accordance with the requirements of § 431.5(b) of this chapter, transfer the entire contents, or approximately 6 grams, into the Erlenmeyer flask.) Stopper the flask and swirl to dissolve the drug. As soon as the sample has completely dissolved, proceed as directed in paragraph (e)(1)(ii) of this section. If the pooled portions from 20 containers will not dissolve completely in 200 milliliters of diluting fluid or will not filter rapidly, 400 milliliters of diluting fluid may be used or two separate tests may be performed using a pool of 10 containers for each test.

(*b*) Diluent packaged in combination with a sterile drug. Using the entire contents from each of 20 immediate containers, proceed as directed in paragraph (e)(1)(ii) of this section.

(*c*) Sterile dispensers packaged in combination with a sterile drug. Prepare 20 clean, empty containers of approximately the same size as those in which the sterile antibiotic drug is packaged. To each container add diluting fluid A in a volume approximately the same as that of the sterile drug when it is prepared for dispensing. Cap the containers, sterilize by autoclaving at 121° C. for 20 minutes, and then allow to cool to room temperature. Aseptically open each dispenser package and remove each dispenser in turn. Use each aseptically to remove 1 milliliter of the fluid from a separate sterile container prepared as described above. Aseptically transfer the fluid to a 500-milliliter Erlenmeyer flask containing approximately 200 milliliters of diluting fluid A. Stopper the flask and proceed as directed in paragraph (e)(1)(ii) of this section.

(ii) *Test procedure.* Aseptically filter the solution through a bacteriological membrane filter. All air entering the filtering system is filtered through air filters capable of removing microorganisms. Filter three 100-milliliter quantities of diluting fluid A through the membrane. For the penicillin and cephalosporin classes of antibiotics, add sufficient penicillinase to diluting fluid A to inactivate the residual antibiotic activity on the membrane after filtration. By means of a sterile circular blade, paper punch, or any other suitable sterile device, cut a circular portion (approximately 17.5 millimeters in diameter) from the center of the filtering area. Transfer the cut center area to a sterile 38 by 200 millimeter (outside dimensions) test tube containing 90±10 milliliters of sterile medium A. Incubate the tube for 7 days at 30°–32° C. Using sterile forceps, transfer the remaining outer portion of the membrane into a second similar tube containing 90±10 milliliters of medium E. Incubate the second tube for 7 days at 22°–25° C.

(2) *Direct method.* From each of 20 immediate containers, transfer approximately 300 milligrams of solids if it is not a liquid drug, or 1 milliliter by volume if it is a liquid drug, or the entire contents if it contains less than these amounts, except if it is a liquid drug containing penicillin in a concentration greater than 300,000 units per milliliter use that volume that contains 300,000 units, into individual sterile test tubes (38 millimeters × 200 millimeters) containing 90±10 milliliters of medium A. Incubate all tubes at 30° C. to 32° C. for 7 days. Gently agitate the tubes every 1 to 3 days or until complete solubilization occurs. At intervals, examine all tubes for visible growth. If growth is observed in any tube, confirm by microscopic examination. From each of the same 20 immediate containers, transfer a second portion (equivalent to that portion initially transferred to the tubes containing medium A) to individual sterile test tubes (38 millimeters × 200 millimeters) containing 90±10 milliliters of medium E, except when each container does not have sufficient material to provide for the two similar-size portions, obtain the second portion from 20 additional immediate containers. Incubate all tubes at 22° C. to 25° C. for 7 days. Gently agitate the tubes every 1 to 3 days or until complete solubilization occurs. At intervals, examine all tubes for visible growth. If growth is observed in any tube, confirm by microscopic examination.

(3) *Bacterial membrane filter method for ophthalmic ointments*—(i) *Ointments that do not contain penicillin.* From each of 10 immediate containers aseptically transfer 0.1 gram of the product into a sterile 250-milliliter flask containing 100 milliliters of diluting fluid E which has previously been heated to a temperature of 47° C. Repeat the process, using 10 additional containers. Swirl both of the flasks to dissolve the ointment. Immediately aseptically filter each solution through a separate bacteriological membrane filter previously moistened with approximately 0.2 milliliter of medium K. Filter all air entering the system through air filters capable of removing microorganisms. Remove any residual antibiotic from the membranes by rinsing each filter five times with 100 milliliters of medium K. The membranes should be covered with fluid throughout each step of the filtration procedure until the end of the last filtering step. By means of a sterile circular blade, paper punch, or other suitable sterile device, cut a circular portion (approximately 17.5 millimeters in diameter) from the center of the filtering area of each membrane. Transfer the center portion of the filtering area of each filter to a sterile test tube 38 millimeters × 200 millimeters (outside dimensions) containing 90 milliliters±10 milliliters of sterile medium I. Incubate the tube for 7 days at 30° C. to 32° C. Using sterile forceps transfer the outer portion of each filter to a similar test tube containing 90 milliliters ±10 milliliters of sterile medium J. Incubate this tube for 7 days at 22° C. to 25° C.

(ii) *Ointments containing penicillin.* Proceed as directed in paragraph (e)(3)(i) of this section, except in lieu of sterile medium I use sterile medium L for the center portion of the filtering area of each filter and in lieu of sterile medium J use sterile medium M for the remaining outer portion of each filter.

(f) *Evaluation of results*—(1) *Bacterial membrane-filter method.* The batch, or the part of the batch represented by a particular filling operation meets the requirements of the test if no sample tube shows growth. If growth is observed in any sample tube, run a second test in the appropriate medium, except perform it in duplicate, using 40 immediate containers. If in the original test, growth is observed in only one of the two media, test both portions of the cut filter membrane by placing each into a separate tube of the same medium. The batch meets the requirements if no tube on the second test shows growth. If growth is observed in any of the control tubes as well as in the sample tubes in either the original or the second test such test is invalid and must be performed again. In any event, further tests may be justified if there is sufficient reason to believe that the results obtained in the first and second tests may not be valid. In such instances, the batch is satisfactory if on the final test no tube shows growth.

(2) *Direct method.* The batch, or the part of the batch represented by a particular filling operation, meets the requirements of the test if no tube shows growth after incubation. If growth is observed in any sample tube, run a second test in the appropriate medium using 40 immediate containers. The batch is satisfactory if, on the second test, no tube shows growth. If growth is observed in any of the control tubes (except inoculated tubes, if the sample is penicillin) as well as in the sample tubes in either the original or the second test, such test is invalid and must be performed again. In any event, further tests may be justified if there is sufficient reason to believe that the results obtained on the first and second tests may not be valid. In such instances the batch is satisfactory if in the final test no tube shows growth.

[39 FR 18944, May 30, 1974, as amended at 41 FR 46852, Oct. 26, 1976; 42 FR 27228, May 27, 1977; 43 FR 43457, Sept. 26, 1978; 49 FR 25846, June 25, 1984; 49 FR 40006, Oct. 12, 1984; 50 FR 48397, Nov. 25, 1985; 52 FR 4611, Feb. 13, 1987; 53 FR 13401, Apr. 25, 1988]

Subpart C—Biological Test Methods

§ 436.31 Equipment and diluents for use in biological testing.

(a) *Equipment*—(1) *Temperature-measuring devices.* Use an accurate clinical

thermometer or any other temperature-measuring device of equal sensitivity that has been tested to determine the time necessary to reach the maximum reading.

(2) *Pyrogen-free glassware.* Render all glassware free from pyrogens by heating at 250° C. for not less than 30 minutes or by any other suitable method.

(3) *Pyrogen-free syringes and needles.* Render all syringes and needles free from pyrogens by heating at 250° C. for not less than 30 minutes or by any other suitable method.

(4) *Pyrogen-free sodium chloride.* Heat sodium chloride for not less than 2 hours at 200° C.

(5) *Pyrogen-free sodium carbonate.* Heat anhydrous sodium carbonate for not less than 4 hours at 170° C.

(b) *Diluents.* (1) Diluent 1 (pyrogen-free water): Prepare pyrogen-free water by collecting freshly distilled water and sterilizing it in an autoclave at 121° C. for not less than 20 minutes. Pyrogen-free water meets the requirements for the absence of pyrogens as described in §436.32(a)(3) when 10 milliliters per kilogram are administered as described in §436.32(a)(2). In testing water for the absence of pyrogens, the aliquot to be tested is made isotonic by the addition of pyrogen-free sodium chloride.

(2) Diluent 2 (pyrogen-free saline solution): Prepare an isotonic solution of sodium chloride by dissolving 9.0 grams of pyrogen-free sodium chloride (prepared as described in §436.31(a)(4)) in pyrogen-free, distilled water (diluent 1) to make 1,000 milliliters. Sterilize in an autoclave at 121° C. for not less than 20 minutes. Pyrogen-free saline solution meets the requirements for the absence of pyrogens as described in §436.32(a)(3) when 10 milliliters per kilogram are administered as described in §436.32(a)(2).

(3) Diluent 3 (sterile distilled water): Prepare freshly distilled water. Sterilize in an autoclave at 121° C. for 20 minutes.

(4) Diluent 4 (sterile saline solution): Dissolve 9.0 grams of sodium chloride in distilled water to make 1,000 milliliters. Sterilize in an autoclave at 121° C. for 20 minutes.

(5) Diluent 5 (10 percent gum acacia): Dissolve 10 grams of gum acacia in approximately 50 milliliters of distilled water. Allow to stand overnight at room temperature and dilute to 100 milliliters with distilled water. Filter through cotton. Store under refrigeration.

(6) Diluent 6 (0.5 percent gum acacia in distilled water). 11132

(7) Diluent 7 (1.0N hydrochloric acid).

(8) Diluent 8 (0.1N hydrochloric acid).

(9) Diluent 9 (0.05N sodium hydroxide).

(10) Diluent 10 (1 percent U.S.P. methylcellulose (4,000 centipoises) solution): Dissolve 1 gram of U.S.P. methylcellulose (4,000 centipoises) in 100 milliliters of distilled water. Allow to stand overnight at room temperature or until solution is complete. Store under refrigeration.

(11) Diluent 11 (0.12*N* sodium hydroxide).

(12) Diluent 12 (0.5 percent methylcellulose (4,000 centipoises) in distilled water). Proceed as directed in paragraph (b)(10) of this section, except use 0.5 gram of methylcellulose (4,000 centipoises).

(13) Diluent 13 (pyrogen-free sodium carbonate solution). Dissolve 25.6 grams of anhydrous pyrogen-free sodium carbonate (prepared as described in paragraph (a)(5) of this section) in 1,000 milliliters pyrogen-free, distilled water (diluent 1). Pyrogen-free, sodium carbonate solution meets the requirements for the absence of pyrogens as described in §436.32(a)(3) when 1.0 milliliter per kilogram is administered as described in §436.32(a)(2).

(14) Diluent 14 (0.07M sterile sodium carbonate solution). Dissolve 7.3 grams of sodium carbonate in distilled water to make 1,000 milliliters. Sterilize in an autoclave at 121° C. for 20 minutes.

(15) Diluent 15 (pyrogen-free sodium carbonate solution): Dissolve 9.9 grams of anhydrous pyrogen-free sodium carbonate (prepared as directed in paragraph (a)(5) of this section) in 1,000 milliliters of pyrogen-free, distilled water (diluent 1). Pyrogen-free sodium carbonate solution meets the requirements for the absence of pyrogens as described in §436.32(a)(3) when 1.0 milliliter per kilogram is administered as described in §436.32(a)(2).

(16) Diluent 16 (0.13*M* sterile pyrogen-free sodium carbonate solution). Dissolve 14.0 grams of anhydrous pyrogen-free sodium carbonate (prepared as described in paragraph (a)(5) of this section) in 1,000 milliliters pyrogen-free, distilled water. Sterilize in an autoclave at 121 °C for 20 minutes.

[39 FR 18944, May 30, 1974, as amended at 40 FR 51625, Nov. 6, 1975; 50 FR 48397, Nov. 25, 1985; 53 FR 13401, Apr. 25, 1988]

§ 436.32 Pyrogen test.

(a) *Method 1*—(1) *Test animal.* Use healthy, mature rabbits weighing not less than 1,800 grams each that have maintained their weight on an antibiotic-free diet for at least 1 week under the environmental conditions specified in this section. House the animals individually in an area of uniform temperature (±3° C.) and free from disturbances likely to excite them. Do not use animals for pyrogen tests more frequently than once every 48 hours or prior to 2 weeks following their having been given a test sample that was adjudged pyrogenic. Before using an animal that has not been used for a test during the previous 2 weeks, condition it 1 to 3 days prior to pyrogen testing by conducting a sham test as directed in paragraph (a)(2) of this section, omitting the injection.

(2) *Procedure.* Using equipment and diluents described in § 436.31, as necessary, perform the test in an area where the animals are housed or under similar environmental conditions. On the day of the test: Withhold all food from the animals being used until after completion of the test, except that access to water may be allowed; and determine the ''control temperature'' of each animal by inserting the temperature-measuring device into the rectum of the test animal to a depth of not less than 7.5 centimeters and allowing sufficient time to reach a maximum temperature, as previously determined, before taking the reading. In any one test use only those animals whose control temperatures do not deviate by more than 1° C. from each other and do not use any animal with a temperature exceeding 39.8° C. The control temperature recorded for each rabbit constitutes the temperature from which any subsequent rise following the injection of the material is calculated. If the product is packaged for dispensing and is in a combination package with a container of diluent, dilute the product as directed in the labeling. Warm the product to be tested to approximately 37° C. Dilute the sample with sterile, pyrogen-free saline (prepared as described in § 436.31(b)(2)) to the appropriate concentration specified in the individual section for each antibiotic to be tested. Inject a test dose of 1 milliliter of the diluted sample per kilogram of rabbit weight into an ear vein of each of three rabbits within 30 minutes subsequent to the control temperature reading. Record the temperature at 1, 2, and 3 hours subsequent to the injection.

(3) *Evaluation.* If no rabbit shows an individual rise in temperature of 0.6° C. or more above its respective control temperature, and if the sum of the three temperature rises does not exceed 1.4° C., the sample meets the requirements for the absence of pyrogens. If one or two rabbits show a temperature rise of 0.6° C. or more, or if the sum of the temperature rises exceeds 1.4° C., repeat the test using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.6° C. or more, and if the sum of the eight temperature rises does not exceed 3.7° C., the sample meets the requirements for the absence of pyrogens.

(b) *Method 2.* Proceed as directed in paragraph (a) of this section, except dilute the sample with pyrogen-free water (diluent 1).

(c) *Method 3.* Proceed as directed in paragraph (a) of this section, except dilute the sample with pyrogen-free water (diluent 1) and inject a test dose of 2.0 milliliters of the diluted sample per kilogram of rabbit weight.

(d) *Method 4.* Proceed as directed in paragraph (a) of this section, except inject a test dose of 0.5 milliliter of the diluted sample per kilogram of rabbit weight.

(e) *Method 5.* Proceed as directed in paragraph (a) of this section, except dilute the sample with pyrogen-free water (diluent 1) and inject a test dose of 0.5 milliliter of the diluted sample per kilogram of rabbit weight.

(f) *Method 6.* Proceed as directed in paragraph (a) of this section, except dilute sample with 0.05*N* sodium hydroxide (diluent 9).

(g) *Method 7.* Proceed as directed in paragraph (a) of this section, except dilute sample with sodium carbonate solution (diluent 13).

(h) *Method 8.* Proceed as directed in paragraph (a) of this section, except inject a test dose of 2.0 milliliters of the diluted sample per kilogram of rabbit weight.

(i) *Method 9.* Proceed as directed in paragraph (a) of this section, except dilute sample with pyrogen-free sodium carbonate solution (diluent 15).

(j) *Method 10.* Proceed as directed in paragraph (a) of this section, except dilute the sample with sodium carbonate solution (diluent 16).

[39 FR 18944, May 30, 1974, as amended at 40 FR 51625, Nov. 6, 1975; 45 FR 22921, Apr. 4, 1980; 50 FR 48397, Nov. 25, 1985; 53 FR 13401, Apr. 25, 1988]

§ 436.35 Depressor substances test.

Proceed as directed in the USP XX depressor substances test. Prepare the sample test solution as follows: For each antibiotic listed in the table below, select the appropriate diluent and test dose (concentration and volume). If the product is packaged for dispensing and is in a combination package with a container of diluent, dilute the product as directed in the labeling.

Antibiotic	Diluent[1]	Concentration of test solution[2]	Volume of test solution to be injected[3]
Bleomycin sulfate	4	[4]0.5	1.0
Capreomycin sulfate	4	3.0	1.0
Chlortetracycline hydrochloride	3	5.0	.6
Clindamycin phosphate	4	5.0	1.0
Daunorubicin hydrochloride	4	1.5	1.0
Dihydrostreptomycin sulfate	4	3.0	1.0
Doxorubicin hydrochloride	4	1.5	1.0
Doxycycline hyclate	4	5.0	1.0
Lincomycin hydrochloride monohydrate	4	3.0	1.0
Minocycline hydrochloride	4	5.0	.6
Plicamycin	3	0.050	1.0
Mitomycin	4	0.050	1.0
Oxytetracycline[5]	3	5.0	.6
Oxytetracycline hydrochloride	4	5.0	.6
Rolitetracycline	4	5.0	.6
Rolitetracycline nitrate	4	5.0	.6
Sodium colistimethate	4	3.0	1.6
Spectinomycin hydrochloride	4	15.0	1.0
Streptomycin sulfate	4	3.0	1.0
Tetracycline hydrochloride	4	5.0	.6
Tetracycline phosphate[5]	3	5.0	.6
Vidarabine monohydrate[5]	4	1.0	1.0

[1] Diluent number as listed in sec. 436.31(b).
[2] Milligrams of activity per milliliter.
[3] Milliliters per kilogram of body weight.
[4] The concentration of the test solution is expressed in units per milliliter in lieu of milligrams of activity per milliliter.
[5] To prepare the test solution, proceed as directed in the individual section of the antibiotic drug regulation in this chapter for the antibiotic to be tested.

[46 FR 60568, Dec. 11, 1981, as amended at 46 FR 61071, Dec. 15, 1981; 49 FR 5096, Feb. 10, 1984]

Subpart D—Microbiological Assay Methods

§ 436.100 Laboratory equipment.

Equipment should be selected which is adequate for its intended use and should be thoroughly cleansed after each use to remove any antibiotic residues. The equipment should be kept covered when not in use. Clean glassware intended for holding and transferring the test organisms should be sterilized in a hot air oven at 200–220° C. for 2 hours. Volumetric flasks, pipettes, or accurately calibrated diluting devices should be used when diluting standard and sample solutions.

(a) *Microbiological agar diffusion assay*—(1) *Cylinders.* Use stainless steel cylinders with an outside diameter of 8 millimeters (±0.1 millimeter), an inside diameter of 6 millimeters (±0.1 millimeter), and a length of 10 millimeters (±0.1 millimeter).

(2) *Plates.* Plastic or glass Petri dishes may be used, having dimensions of 20 by 100 millimeters. Covers should be of suitable material.

(b) *Microbiological turbidimetric assay*—(1) *Tubes.* Tubes which give satisfactory results and have uniform length and diameter should be used. If reusable tubes are employed, care must be taken to remove not only all antibiotic residues from the previous test but also all traces of cleaning solution.

(2) *Colorimeter.* Use a suitable photoelectric colorimeter at a wavelength of 530 millimicrons. Set the instrument at zero absorbance with clear,

uninoculated broth prepared as described in the applicable method for the antibiotic being assayed.

[39 FR 18944, May 30, 1974, as amended at 41 FR 34743, Aug. 17, 1976]

§ 436.101 Solutions.

(a) Antibiotic assay solutions are prepared as follows (solution numbers 1, 2, 3, 4, and 6 correspond to those used in ''Assay Methods of Antibiotics,'' D. C. Grove and W. A. Randall, Medical Encyclopedia, Inc., New York, N.Y. (1955), p. 222), which is incorporated by reference. Copies are available from the Medical Encyclopedia Inc., 30 East 60th St., New York, NY 11220, or available for inspection at the Office of the Federal Register, 800 North Capitol Street, NW., suite 700, Washington, DC.

(1) *Solution 1* (*1 percent potassium phosphate buffer, pH 6.0*).

Dibasic potassium phosphate: 2.0 gm.
Monobasic potassium phosphate: 8.0 gm.
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 5.95 to 6.05 after sterilization.

(2) *Solution 2* (*citrate buffer solution pH 6.3*).

Citric acid: 13.2 gm.
Sodium hydroxide: 7.06 gm.
Sodium citrate: 97.0 gm.
Distilled water, q.s: 1,000.0 ml.

Adjust with 10 percent citric acid solution or 10*N* sodium hydroxide to yield pH 6.2 to 6.4 after sterilization.

(3) *Solution 3* (*0.1M potassium phosphate buffer, pH 8.0*).

Dibasic potassium phosphate: 16.73 gm.
Monobasic potassium phosphate: 0.523 gm.
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 7.9 to 8.1 after sterilization.

(4) *Solution 4*(*0.1M potassium phosphate buffer, pH 4.5*).

Monobasic potassium phosphate: 13.6 gm.
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 4.45 to 4.55 after sterilization.

(5) [Reserved]

(6) *Solution 6* (*10 percent potassium phosphate buffer, pH 6.0*).

Dibasic potassium phosphate: 20.0 gm.
Monobasic potassium phosphate: 80.0 gm.
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 5.95 to 6.05 after sterilization.

(7)–(9) [Reserved]

(10) *Solution 10* (*0.2M potassium phosphate buffer, pH 10.5*).

Dibasic potassium phosphate: 35.0 gm.
10 N potassium hydroxide: 2.0 ml.
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 10.4 to 10.6 after sterilization.

(11) *Solution 11* (*10 percent potassium phosphate buffer, pH 2.5*).

Monobasic potassium phosphate: 100.0 gm.
Concentrated hydrochloric acid: 0.2 ml. (approximately).
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 2.0 to 2.8 after sterilization.

(12) *Solution 12* (*10 percent potassium phosphate buffer, pH 7.0*).

Monobasic potassium phosphate: 100.0 gm.
Distilled water, q.s: 1,000.0 ml.

Adjust with 18*N* phosphoric acid or 10*N* potassium hydroxide to yield a pH 6.95 to 7.05 after sterilization.

(13) *Solution 13* (*0.01N methanolic hydrochloric acid*).

1.0N hydrochloric acid: 10.0 ml.
Methyl alcohol, q.s: 1,000.0 ml.

(14) *Solution 14* (*2 percent sodium bicarbonate solution*).

Sodium bicarbonate: 20.0 gm.
Distilled water, q.s: 1,000.0 ml.

Prepare daily.

(15) *Solution 15* (*80 percent isopropyl alcohol solution*).

Isopropyl alcohol: 800.0 ml.
Distilled water, q.s: 1,000.0 ml.

(16) *Solution 16* (*0.1 M potassium phosphate buffer, pH 7.0*).

Dibasic potassium phosphate: 13.6 gm.
Monobasic potassium phosphate: 4.0 gm.
Distilled water, q.s.: 1,000.0 ml.

Adjust with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to yield a pH 6.8 to 7.2 after sterilization.

(17) *Solution 17* (*5 percent methyl alcohol in 1 percent potassium phosphate buffer, pH 6.0*).

Methyl alcohol: 50.0 ml.
1 percent potassium phosphate buffer, pH 6.0, q.s.: 1,000.0 ml.

(18) *Solution 18 (0.054M sodium phosphate buffer, pH 6.9).*

Sodium dihydrogen phosphate monohydrate: 3.97 gm.
Disodium hydrogen phosphate anhydrous: 3.55 gm.
Distilled water, q.s.: 1,000.0 mL.

[39 FR 18944, May 30, 1974, as amended at 40 FR 52004, Nov. 7, 1975; 45 FR 75194, Nov. 14, 1980; 47 FR 9396, Mar. 5, 1982]

§ 436.102 Culture media.

(a) *Ingredients.* Use ingredients that conform to the standards, if any, prescribed by the U.S.P. or N.F. In lieu of preparing the media from the individual ingredients specified, they may be made from dehydrated mixtures that, when reconstituted with distilled water, have the same composition as such media. Minor modifications of the individual ingredients specified in this section are permissible if the resulting media possess growth-promoting properties at least equal to the media described.

(b) *Description of media.* Medium numbers 1, 2, 3, 4, 5, 8, 9, 10, 11, and 13 correspond to those used in ''Assay Methods of Antibiotics,'' D. C. Grove and W. A. Randall, Medical Encyclopedia, Inc., New York, N.Y. (1955) p. 220, which is incorporated by reference. Copies are available from Medical Encyclopedia Inc., 30 East 60th St., New York, NY, or available for inspection at the Office of the Federal Register, 800 North Capitol Street, NW., suite 700, Washington, DC. Medium numbers 18 through 21 correspond to those used in ''Outline of Details for Official Microbiological Assays of Antibiotics,'' A. Kirshbaum and B. Arret, ''Journal of Pharmaceutical Sciences,'' vol. 56, No. 4, April 1967, p. 512, which is incorporated by reference. Copies are available from the American Pharmaceutical Association, 2215 Constitution Ave. NW., Washington, DC 20037, or available for inspection at the Office of the Federal Register (see address in this paragraph).

(1) *Medium 1.*

Peptone: 6.0 gm.
Pancreatic digest of casein: 4.0 gm.
Yeast extract: 3.0 gm.
Beef extract: 1.5 gm.
Dextrose: 1.0 gm.
Agar: 15.0 gm.
Distilled water, q.s: 1,000.0 ml.
pH 6.5 to 6.6 after sterilization.

(2) *Medium 2.*

Peptone: 6.0 gm.
Yeast extract: 3.0 gm.
Beef extract: 1.5 gm.
Agar: 15.0 gm.
Distilled water, q.s: 1,000.0 ml.
pH 6.5 to 6.6 after sterilization.

(3) *Medium 3.*

Peptone: 5.0 gm.
Yeast extract: 1.5 gm.
Beef extract: 1.5 gm.
Sodium chloride: 3.5 gm.
Dextrose: 1.0 gm.
Dipotassium phosphate: 3.68 gm.
Potassium dihydrogen phosphate: 1.32 gm.
Distilled water, q.s: 1,000.0 ml.
pH 6.95 to 7.05 after sterilization.

(4) *Medium 4.*

Peptone: 6.0 gm.
Yeast extract: 3.0 gm.
Beef extract: 1.5 gm.
Dextrose: 1.0 gm.
Agar: 15.0 gm.
Distilled water, q.s: 1,000.0 ml.
pH 6.5 to 6.6 after sterilization.

(5) *Medium 5.* Medium 5 is the same as medium 2, except adjust the final pH to 7.8 to 8.0 after sterilization.

(6)–(7) [Reserved]

(8) *Medium 8.* Medium 8 is the same as medium 2, except adjust the final pH to 5.8 to 6.0 after sterilization.

(9) *Medium 9.*

Pancreatic digest of casein: 17.0 gm.
Papaic digest of soybean: 3.0 gm.
Sodium chloride: 5.0 gm.
Dipotassium phosphate: 2.5 gm.
Dextrose: 2.5 gm.
Agar: 20.0 gm.
Distilled water, q.s: 1,000.0 ml.
pH 7.2 to 7.3 after sterilization.

(10) *Medium 10.* Medium 10 is the same as medium 9, except:

Agar: 12.0 gm.
Polysorbate 80 (add polysorbate 80 after boiling the medium to dissolve the agar): 10.0 ml.
pH 7.2 to 7.3 after sterilization.

(11) *Medium 11.* Medium 11 is the same as medium 1, except adjust the final pH to 7.8 to 8.0 after sterilization.

(12) [Reserved]

(13) *Medium 13.*

Peptone: 10.0 gm.

Dextrose: 20.0 gm.
Distilled water, q.s: 1,000.0 ml.
pH 5.6 to 5.7 after sterilization.

(14)–(18) [Reserved]

(19) *Medium 19.*

Peptone: 9.4 gm.
Yeast extract: 4.7 gm.
Beef extract: 2.4 gm.
Sodium chloride: 10.0 gm.
Dextrose: 10.0 gm.
Agar: 23.5 gm.
Distilled water, q.s: 1,000.0 ml.
pH 6.0 to 6.2 after sterilization.

(20)–(31) [Reserved]

(32) *Medium 32.* Prepare as medium 1, except add 300 milligrams of hydrated manganese sulfate ($MnSO_4 \cdot H_2O$) to each liter of medium.

(33) *Medium 33.* Use medium 1, sterilized and cooled to 50° C. Aseptically add sufficient sterile sodium novobiocin solution to give a final concentration of 10 micrograms of novobiocin activity per milliliter of medium. Sterile sodium novobiocin solution is prepared by filtering a solution containing 2.5 milligrams of novobiocin per milliliter of distilled water through a membrane filter of 0.22–micron porosity.

(34) *Medium 34.*

Glycerol: 10.0 gm.
Peptone: 10.0 gm.
Beef extract: 10.0 gm.
Sodium chloride: 3.0 gm.
Distilled water, q.s.: 1,000.0 ml.
pH 7.0 after sterilization.

(35) *Medium 35.* Same as medium 34, except add 17.0 grams of agar to each liter of medium.

(36) *Medium 36.*

Pancreatic digest of casein	15.0 gm.
Papaic digest of soybean	5.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.3 after sterilization	

(37) *Medium 37.*

Pancreatic digest of casein: 17.0 gm.
Soybean peptone: 3.0 gm.
Dextrose: 2.5 gm.
Sodium chloride: 5.0 gm.
Dipotassium phosphate: 2.5 gm.
Distilled water, q.s.: 1,000.0 ml.
pH 7.3 after sterilization.

(38) *Medium 38.*

Peptone: 15.0 gm.
Papaic digest of soybean meal: 5.0 gm.
Sodium chloride: 4.0 gm.
Sodium sulfite: 0.2 gm.
L-cystine: 0.7 gm.
Dextrose: 5.5 gm.
Agar: 15.0 gm.
Distilled water, q.s.: 1,000.0 ml.
pH 7.0 after sterilization.

[39 FR 18944, May 30, 1974, as amended at 40 FR 52004, Nov. 7, 1975; 42 FR 14092, Mar. 15, 1977; 47 FR 9396, Mar. 5, 1982; 47 FR 22514, May 25, 1982]

§ 436.103 Test organisms.

(a) *Preparation of test organism suspensions.* For each test organism listed in the following table, select the media (as listed by medium number in § 436.102(b)), incubation period of the Roux bottle, suggested dilution factor, and suggested storage period for the particular test organism and proceed by the appropriate method described in paragraph (b) of this section. Test organism letters A through K, M, and N correspond to those used in ''Outline of Details for Official Microbiological Assays of Antibiotics,'' A. Kirshbaum and B. Arret, ''Journal of Pharmaceutical Sciences,'' Vol. 56, No. 4, p. 512 (April 1967), which is incorporated by reference. Copies are available from the American Pharmaceutical Association, 2215 Constitution Ave. NW., Washington, DC 20037, or available for inspection at the Office of the Federal Register, 800 North Capitol Street, NW., suite 700, Washington, DC.

Test organisms	Method used	Medium used for the—		Incubation period of Roux bottle	Suggested dilution factor	Suggested storage period of suspensions under refrigeration
		Slants	Roux bottles			
Test organism A—*Staphylococcus aureus* (ATCC 6538P)[2].	1	1	1	24 hours	1:20	1 week.
Test organism B—*Micrococcus luteus* (ATCC 7468)[2].	1	1	1	24 hours	1:30	2 weeks.
Test organism C—*Micrococcus luteus* (ATCC 9341)[2].	1	1	1	24 hours	1:40	2 weeks.
Test organism D—*Staphyloccocus epidermidis* (ATCC 12228)[2].	1	1	1	24 hours	[1] 1:14	1 week.

Test organisms	Method used	Medium used for the—		Incubation period of Roux bottle	Suggested dilution factor	Suggested storage period of suspensions under refrigeration
		Slants	Roux bottles			
Test organism E—*Saccharomyces cerevisiae* (ATCC 9763)[2].	6	19	.....	.....	1:30	4 weeks.
	or					
	7	19	19	48 hours	1:30	4 weeks.
Test organism F—*Bordetella bronchiseptica* (ATCC 4617)[2].	1	1	1	24 hours	1:20	2 weeks.
Test organism G—*Bacillus cereus* var. *mycoides* (ATCC 11778)[2].	3	1	1	1 week	.....	6 months.
Test organism H—*Bacillus subtilis* (ATCC 6633)[2].	1	1	1	24 hours	.....	6 months.
	or					
	2	1	32	5 days	.....	6 months.
Test organism I—*Klebsiella pneumoniae* (ATCC 10031)[2].	1	1	1	24 hours	1:25	1 week.
Test organism J—*Escherichia coli* (ATCC 10536).	1	1	1	24 hours	1:20	2 weeks.
Test organism K—*Streptococcus faecium* (ATCC 10541)[2].	5	.....	.....	.....	.....	24 hours.
Test organism L—*Micrococcus luteus* (ATCC 10240)[2].	1	1	1	24 hours	1:35	4 weeks.
Test organism O—*Staphylococcus aureus,* resistant to novobiocin (ATCC 12692)[2].	1	33	33	24 hours	1:10	4 weeks.
Test organism T—*Saccharomyces cerevisiae* (ATCC 2601)[2].	7	19	19	48 hours	1:30	4 weeks.
Test organism V—*Micrococcusluteus,* resistant to dihydrostreptomycin (ATCC 10240A)[2].	1	1	1	48 hours	1:35	4 weeks.
Test organism W—*Pseudomonas aeruginosa* (ATCC 25619)[2].	1	1	1	24 hours	1:25	2 weeks.
Test organism X—*Mycobacterium smegmatis* (ATCC 607)..	8	36	.....	.....	.....	2 weeks.
Test organism Y—*Pseudomonas aeruginosa* (ATCC 29336)[2].	9	36	36	24 hours	1:50	1 week.

[1] If the antibiotic to be tested is paromomycin, the dilution factor is 1:25.
[2] Available from American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD. 20852.

(b) *Methods for preparation of test organism suspensions*—(1) *Method 1*—(i) *Preparation of suspension.* Maintain organisms on agar slants containing 10 milliliters of the appropriate medium. Incubate the slants at 32° C.–35° C. for 24 hours. Using 3 milliliters of sterile U.S.P. saline T.S., wash the growth from the agar slant onto a large agar surface, such as a Roux bottle, containing 250 milliliters of the appropriate medium. Spread the suspension of organisms over the entire surface of the Roux bottle with the aid of sterile glass beads. Incubate the Roux bottle at 32° C.–35° C. Wash the resulting growth from the agar surface with 50 milliliters of sterile U.S.P. saline T.S.

(ii) *Standardization of suspension.* Determine the dilution factor that will give a 25–percent light transmission at a wavelength of 580 millimicrons using a suitable photoelectric colorimeter and a 13–millimeter diameter test tube as an absorption cell. It may be necessary to adjust the suspension. Determine the amount of suspension to be added to each 100 milliliters of agar or nutrient broth by the use of test plates or test broth. Store the test organism suspension under refrigeration.

(2) *Method 2.* Proceed as directed in paragraph (b)(1) of this section, except in lieu of paragraph (b)(1)(ii) thereof, heat-shock and standardize the suspension as follows: Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50 to 70 milliliters of sterile U.S.P. saline T.S. and heat the suspension for 30 minutes at 70° C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 milliliters of agar. Maintain the spore suspension under refrigeration.

(3) *Method 3.* Proceed as directed in paragraph (b)(1) of this section, except in lieu of paragraph (b)(1)(ii) thereof, heat-shock and standardize the suspension as follows: Heat the suspension for

30 minutes at 70° C. Wash the spore suspension three times with 25 to 50 milliliters of sterile distilled water. Resuspend the organisms in 50 to 70 milliliters of sterile distilled water and heat-shock again for 30 minutes at 70° C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 milliliters of agar. Maintain the spore suspension under refrigeration.

(4) [Reserved]

(5) *Method 5.* Maintain the test organisms in 100–milliliter quantities of nutrient broth—Medium 3 as described in § 436.102(b)(3). For the test prepare a fresh subculture by transferring a loopful of the stock culture to 100 milliliters of the same nutrient broth and incubate for 16 to 18 hours at 37° C. Store this broth culture under refrigeration.

(6) *Method 6.* Maintain the test organisms on agar slants containing 10 milliliters of the medium specified in paragraph (a) of this section. Incubate the slants at 32° C.–35° C. for 24 hours. Inoculate 100 milliliters of nutrient broth—Medium 13 as described in § 436.102(b)(13). Incubate for 16 to 18 hours at 37° C. Proceed as directed in paragraph (b)(1)(ii) of this section.

(7) *Method 7.* Proceed as directed in paragraph (b)(1) of this section, except incubate the slants at 30° C. for 24 hours and incubate the Roux bottle at 30° C. for 48 hours.

(8) *Method 8.* Maintain organisms on agar slants containing 10 milliliters of the appropriate medium and transfer to a fresh slant about once a week. Incubate the slants at 37° C for 48 hours. Using 3 milliliters of sterile U.S.P. saline T.S., wash the growth from the agar slant into a 500–milliliter Erlenmeyer flask containing 100 milliliters of medium 34, as described in § 436.102(b) (34), and 50 grams of glass beads. Agitate the culture by rotation at a speed of 130 cycles per minute and a radius of 3.5 centimeters at 27° C for 5 days. Determine the amount of suspension to be added to each 100 milliliters of agar by the use of test plates. Store the test organism suspension under refrigeration.

(9) *Method 9.* Proceed as directed in paragraph (b)(1) of this section, except incubate the slant and Roux bottle at 37° C and wash the resulting growth from the agar surface with 50 milliliters of Medium 37 as described in § 436.102(b)(37).

[39 FR 18944, May 30, 1974, as amended at 40 FR 52004, Nov. 7, 1975; 42 FR 14092, Mar. 15, 1977; 42 FR 18058, Apr. 5, 1977; 44 FR 10378, Feb. 20, 1979; 47 FR 22514, May 25, 1982; 47 FR 27552, June 25, 1982]

§ 436.104 Penicillin activity.

Use penicillin-free equipment and glassware.

(a) *Preparation of inoculated plates.* Proceed as directed in § 436.105(a), using 10 milliliters of medium 1 for the base layer. For the seed layer, use 4 milliliters of medium 4, inoculated with the amount of test organism C which gave the clearest, sharpest zones of inhibition measuring 17 to 21 millimeters in diameter when standardized as described in § 436.103(b)(1)(ii). Use the plates the same day they are prepared.

(b) *Preparation of working standard stock solutions and standard response lines solutions.* Proceed as directed for penicillin G in § 436.105(b), except dilute the working standard stock solution to a final concentration of 100 units of penicillin G per milliliter and use the following final concentrations for the standard response line: 0.005, 0.0125, 0.025, 0.050, 0.100, and 0.200 unit of penicillin G per milliliter. The 0.050 unit of penicillin G–per–milliliter solution is the reference concentration of the assay.

(c) *Sample preparation.* Dissolve 1.0 gram of the sample in sufficient distilled water to make 18 milliliters. Filter if not clear. Transfer 9.0 milliliters to a separatory funnel, and add 20 milliliters of amyl acetate. Add 1 milliliter of 10 percent potassium phosphate buffer, pH 2.5 (solution 11 as described in § 436.101), shake, allow to separate, and draw off the aqueous layer into a second separatory funnel. Check the pH of the aqueous solution with pH paper, and readjust with concentrated hydrochloric acid if the pH is three or above. Extract again with 20 milliliters or amyl acetate, discard the aqueous phase, and combine the amyl acetate extracts. Wash the extracts with 10 milliliters of 1 percent potassium phosphate buffer, pH 2.5, and discard the

buffer wash. Extract the penicillin from the amyl acetate with a 10–milliliter aliquot of 1 percent potassium phosphate buffer, pH 6.0 (solution 1 as described in § 436.101). This is the assay solution.

(d) *Procedure for assay.* For the standard response line, use a total of 15 plates (three plates for each response line solution, except the reference concentration solution, which is included on each plate). On each set of three plates, fill three alternate cylinders with the reference concentration solution and the other three cylinders with the concentration of the response line under test. Thus, there will be 45 reference concentration zones of inhibition and nine zones of inhibition for each of the other concentrations of the response line. Treat a portion of the sample solution (2 to 5 milliliters) with 0.1 milliliter of penicillinase solution and incubate at 37° C. for 1 hour. For each sample tested, use three plates. On each plate fill two cylinders with the 0.050 unit of penicillin G per milliliter standard, two cylinders with the untreated sample, and two cylinders with the penicillinase-treated sample. Incubate all plates, including those of the standard response line, overnight at 30° C. A zone of inhibition with the untreated sample and no zone with the penicillinase-treated sample are a positive test for penicillin. If a positive test is obtained, measure the diameters of the zones of inhibition using an appropriate measuring device such as a millimeter rule, calipers, or an optical projector.

(e) *Estimation of penicillin G activity.* To prepare the standard response line, average the diameters of the standard reference concentration and average the diameters of the standard response line concentration tested for each set of three plates. Average also all 45 diameters of the reference concentration. The average of the 45 diameters of the reference concentration is the correction point of the response line. Correct the average diameter obtained for each concentration to the figure it would be if the average reference concentration diameter for that set of three plates were the same as the correction point. Thus, if in correcting the 0.025 penicillin G concentration, the average of the 45 readings of the 0.050 unit of penicillin G–per–milliliter concentration is 18.5 millimeters and the average of the 0.050 unit of pencillin G–per milliliter concentration of this set of three plates is 18.3 millimeters, the correction is +0.2 millimeters. If the average reading of the 0.025 unit of pencillin G–per–milliliter concentration of these same three plates is 15.5 millimeters, the corrected value is 15.7 millimeters. Plot these corrected values, including the average of the 0.050 unit of penicillin G-per-milliliter concentration, on semilogarithmic graph paper using the pencillin concentration in units per milliliter on the logarithmic scale and the diameter of the zone of inhibition on the arithmetic scale. Draw the line of best fit through these points. To estimate the sample potency, average the zone diameters of the standard and the zone diameters of the sample on the three plates used. If the average zone diameter of the sample is lower than that of the standard, subtract the difference between them from the reference concentration diameter of the standard response line. From the response line, read the concentrations corresponding to these corrected values of zone diameters. Multiply the concentration by the dilution factor to obtain the units of penicillin G per sample size tested.

[39 FR 18944, May 30, 1974, as amended at 41 FR 34743, Apr. 17, 1976]

§ 436.105 Microbiological agar diffusion assay.

Using the sample solution prepared as described in the section for the particular antibiotic to be tested, proceed as described in paragraphs (a), (b), (c), and (d) of this section.

(a) *Preparation of inoculated plates.* For each antibiotic listed in the table in this paragraph, select the media (as listed by medium number in § 436.102(b)), the amount of media to be used in the base and seed layers, the test organism (as listed in § 436.103(a)), and the suggested inoculum and prepare the inoculated plates as follows: Prepare the base layer by adding the appropriate amount of melted agar to each Petri dish (nominal dimensions 20 by 100 millimeters). Distribute the agar

evenly in each dish on a flat, level surface, placing a cover on each plate in turn; if a nonporous cover is used, leave it slightly ajar to prevent accumulation of condensed moisture from the hot agar base layer. After the agar hardens, seat the nonporous cover on each plate. To prepare the seed layer, add the suggested inoculum of the test organism suspension to a sufficient amount of agar, which has been melted and cooled to 48° C–50° C. Swirl the flask to obtain a homogeneous suspension, and add the appropriate amount of the inoculated media to each of the plates containing the uninoculated base agar. Spread evenly over the agar surface, cover, and allow to harden on a flat, level surface. After the agar has hardened, place 6 cylinders described in § 436.100(a)(1) on the inoculated agar surface so that they are at approximately 60° intervals on a 2.8—centimeter radius.

Antibiotic	Media to be used (as listed by medium number in § 436.102(b))		Milliliters of media to be used in the base and seed layers		Test organism	Suggested volume of standardized inoculum to be added to each 100 milliliters of seed agar	Incubation Temperature for the plates
	Base layer	Seed layer	Base layer	Seed layer			
						Milliliters	*Degrees C.*
Amoxicillin	11	11	21	4	C	0.5	32–35
Amphotericin B	None	19	None	8	E	1.0	29–31
Ampicillin	11	11	21	4	C	0.5	32–35
Bacitracin	2	1	21	4	B	0.3	32–35
Bacitracin	2	1	21	4	L	0.3	32–35
Bleomycin	35	35	10	6	X	1.0	32–35
Carbenicillin	9	10	21	4	W	[2] 0.5	36–37.5
Cefactor	2	1	21	5	A	0.05	36–37.5
Cefadroxil	2	1	21	4	A	0.05	36–37.5
Cefamandole	2	1	21	5	A	0.06	36–37.5
Cefazolin	2	1	21	4	A	0.05	32–35
Cefotaxime	2	1	21	5	A	0.1	36–37.5
Cefoxitin	2	1	21	5	A	0.1	36–37.5
Cephalexin	2	1	21	4	A	0.05	32–35
Cephaloglycin	2	1	21	4	A	0.2	32–35
Cephaloridine	2	1	21	4	A	0.1	32–35
Cephalothin	2	1	21	4	A	0.1	32–35
Cephapirin	2	1	21	4	A	0.08	32–35
Cephradine	2	1	21	4	A	0.05	32–35
Clindamycin	11	11	21	4	C	1.5	36–37.5
Cloxacillin	2	1	21	4	A	0.1	32–35
Colistimethate, sodium	9	10	21	4	F	0.1	36–37.5
Colistin	9	10	21	4	F	0.1	36–37.5
Cyclacillin	11	11	21	4	C	0.5	36–37.5
Dactinomycin	5	5	10	4	H	[1]	36–37.5
Dicloxacillin	2	1	21	4	A	0.1	32–35
Dihydrostreptomycin	5	5	21	4	H	[1]	36–37.5
Erythromycin	11	11	21	4	C	1.5	32–35
Gentamicin	11	11	21	4	D	0.03	36–37.5
Kanamycin B	5	5	21	4	H	[1]	36–37.5
Methicillin	2	1	21	4	A	0.3	32–35
Mitomycin	8	8	10	4	H	0.5	36–37.5
Nafcillin	2	1	21	4	A	0.3	32–35
Natamycin	None	19	None	8	E	0.8	29–31
Neomycin	11	11	21	4	A	0.4	32–35
Neomycin	11	11	21	4	D	1.0	36–37.5
Netilmicin	11	11	20	5	D	0.25	36–37.5
Novobiocin	2	1	21	4	D	4.0	34–36
Nystatin	None	19	None	8	T	1.0	29–31
Oleandomycin	11	11	21	4	D	1.0	36–37.5
Oxacillin	2	1	21	4	A	0.3	32–35
Paromomycin	11	11	21	4	D	2.0	36–37.5
Penicillin G	2	1	21	4	A	1.0	32–35
Penicillin V	2	1	21	4	A	1.0	32–35
Plicamycin	8	8	10	4	A	0.1	32–35
Polymyxin B	9	10	21	4	F	0.1	36–37.5
Rifampin	2	2	21	4	H	0.1	29–31
Sisomicin	11	11	21	4	D	0.03	36–37.5
Streptomycin	5	5	21	4	H	[1]	36–37.5
Ticarcillin	38	38	21	4	Y	1.5	36–37.5

Antibiotic	Media to be used (as listed by medium number in § 436.102(b))		Milliliters of media to be used in the base and seed layers		Test organism	Suggested volume of standardized inoculum to be added to each 100 milliliters of seed agar	Incubation Temperature for the plates
	Base layer	Seed layer	Base layer	Seed layer			
						Milliliters	*Degrees C.*
Vancomycin	8	8	10	4	H	(1)	36–37.5

[1] Determine the amount of the inoculum by the use of test plates.
[2] Use dilution of the suspension that gives 25 percent light transmission in lieu of the stock suspension.

(b) *Preparation of working standard stock solutions and standard response line solutions.* For each antibiotic listed in the table in this paragraph, select the working standard drying conditions, solvent(s), concentrations, and storage time for the standard solutions and proceed as follows: If necessary, dry the working standard as described in § 436.200; dissolve and dilute an accurately weighed portion to the proper concentration to prepare the working standard stock solution. Store the working standard stock solution under refrigeration and do not use longer than the recommended storage time. Further dilute an aliquot of the working standard stock solution to the proper concentrations to prepare the standard response line solutions. The reference concentration of the assay is the mid concentration of the response line.

Antibiotic	Working standard stock solutions					Standard response line concentrations	
	Drying conditions (method number as listed in §436.200)	Initial solvent	Diluent (solution number as listed in §436.101(a))	Final concentration units or milligrams per milliliter	Storage time under refrigeration	Diluent	Final concentrations, units or micrograms of antibiotic activity per milliliter
Amoxicillin	Not dried		Distilled water	1.0 mg	7 days	3	0.064, 0.080, 0.100, 0.125 and 0.156, µg. (Prepare the standard response line simultaneously with the sample solution.)
Amphotericin B	1		Dimethylsulfoxide	1 mg.[1]	Use same day	10	0.64 0.80, 1.00, 1.25, 1.56 µg. (Prepare the standard response line simultaneously with the sample solution.)
Ampicillin	Not dried		Distilled water	0.1	1 week	3	0.064, 0.080, 0.100, 0.125 0.156 µg. (Prepare the standard response line simultaneously with the sample solution.)
Bacitracin zinc	1		0.01*N* HCl	100 units	Use same day	1	0.64, 0.80, 1.0, 1.25, 1.56 units.
Bleomycin	7		16	2 units	2 weeks	16	0.01, 0.02, 0.04, 0.08, 0.16 unit.
Carbenicillin	Not dried		1	1 mg	2 weeks	1	12.8, 16.0, 20.0, 25.0, 31.2 µg.
Cefaclor	Not dried		1	1 mg	1 day	1	3.2, 4.0, 5.0, 6.25, 7.81 µg.
Cefadroxil	Not dried		1	1mg	Use same day	1	12.8,16.0, 20.0, 25.0, and 31.2µg.
Cefamandole	Not dried		3	1mg[5]	1 day	1	1.28, 1.60, 2.00, 2.50, 3.12 µg.
Cefazolin	Not dried	10,000 µg per ml. in solution 6.	1	1 mg	5 days	1	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cefotaxime	Not dried	1		1 mg	Use same day	1	6.4, 8.0, 10.0, 12.5, 15.6 µg.
Cefoxitin	Not dried		1	1 mg	Use same day	1	12.8, 16.0, 20.0, 25, 31.2 µg.
Cephalexin	Not dried		1	1 mg	7 days	1	12.8, 16.0, 20.0, 25.0, 31.2 µg.
Cephaloglycin	Not dried		Distilled water	100 µg	1 week	4	6.4, 8.0, 10.0, 12.5, 15.6 µg.
Cephaloridine	1		1	1 mg	5 days	1	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cephalothin	1		1	1 mg	5 days	1	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cephapirin	Not dried		1	1 mg	3 days	1	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cephradine	Not dried		1	1 mg	5 days	1	6.4, 8.0, 10.0, 12.5, 15.6 µg.
Clindamycin	Not dried		Distilled water	1 mg	1 month	3	0.64, 0.80, 1.00, 1.25 1.56 µg.
Cloxacillin	Not dried		1	1 mg	7 days	1	3.20, 4.00, 5.00, 6.25, 7.81 µg.
Colistimethate, sodium	1	10,000 µg. per ml. in distilled water.	6	1 mg	Use same day	6	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Colistin	1	10,000 µg. per ml. in distilled water.	6	1 mg	2 weeks	6	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Cyclacillin	Not dried		Distilled water	1 mg	1 day	3	0.64, 0.80, 1.0, 1.25, 1.56 µg. (Prepare the standard response line simultaneously with the sample solution.)
Dactinomycin	1	10,000 µg. per ml. in methyl alcohol.	3	1 mg	3 months	3	0.50, 0.71, 1.00, 1.41, 2.00 µg.
Dicloxacillin	Not dried		1	1 mg	7 days	1	3.20, 4.00, 5.00, 6.25, 7.81 µg.
Dihydrostreptomycin	5		3	1 mg	30 days	3	0.64, 0.80, 1.00, 1.25, 1.56 µg.

Antibiotic	Working standard stock solutions					Standard response line concentrations	
	Drying conditions (method number as listed in § 436.200)	Initial solvent	Diluent (solution number as listed in § 436.101(a))	Final concentration units or milligrams per milliliter	Storage time under refrigeration	Diluent	Final concentrations, units or micrograms of antibiotic activity per milliliter
Erythromycin	1	10,000 µg. per ml. in methyl alcohol.	3	1 mg	14 days	3	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Gentamicin	3		3	1 mg	1 month	3	0.064, 0.080, 0.100, 0.125, 0.156 µg.
Kanamycin B (use the kanamycin sulfate working standard).	Not dried		3	1 mg	1 month	3	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Methicillin	Not dried		1	1 mg	4 days	1	6.4, 8.0, 10.0, 12.5, 15.6 µg.
Mitomycin	Not dried		1	1 mg	14 days	1	0.50, 0.71, 1.0, 1.41, 2.0 µg.
Nafcillin	Not dried		1	1 mg	2 days	1	1.28, 1.60, 2.00, 2.50, 3.12 µg.
Natamycin	Not dried		Dimethylsulfoxide	1 mg [7]	Use same day	10	3.20, 4.00, 5.00, 6.25, 7.81 µg. (Prepare the standard response line solutions simultaneously with the sample solution to be tested using red low actinic glassware. Use solutions within 2 hours after preparation.)
Neomycin	1		3	1 mg	2 weeks	3	0.64, 0.80, 1.00, 1.25, 1.56 µg. (if test organism D is used); 6.4, 8.0, 10.0, 12.5, 15.6 µg. (if test organism A is used).
Netilmicin	Not dried [9]		3	1 mg	7 days	3	0.064, 0.080, 0.100, 0.125, 0.156 µ g.
Novobiocin	5	10,000 µg. per ml. in absolute ethyl alcohol.	3	1 mg	5 days	6	0.320, 0.400, 0.500, 0.625, 0.781 µg.
Nystatin [10]	4		Dimethylformamide	1,000 units [2]	Use same day	6	12.8, 16.0, 20.0, 25.0, 31.2 units. (Prepare the standard response line solutions simultaneously with the sample solution to be tested using red low actinic glassware.)
Oleandomycin	Not dried	10,000 µg. per ml. in ethyl alcohol.	3	1 mg	30 days	3	3.20, 4.00, 5.00, 6.25, 7.81 µg.
Oxacillin	Not dried		1	1 mg	3 days	1	3.20, 4.00, 5.00, 6.25, 7.81 µg.
Paromomycin	1		3	1 mg	3 weeks	3	0.64, 0.80, 1.00, 1.25, 1.56 µg.
Penicillin G	Not dried		1	1,000 units	4 days	1	0.64, 0.80, 1.00, 1.25, 1.56 units.
Penicillin V Potassium	Not dried		1	100 units	4 days	1	0.64, 0.80, 1.00, 1.25, 1.56 units.
Plicamycin	7		Distilled water	0.1 mg	1 day	1	0.50, 0.7, 1.00, 1.41, 2.00 µg.
Polymyxin B	1	Distilled water [3]	6	10,000 units	2 weeks	6	6.4, 8.0, 10.0, 12.5, 15.6 units.
Rifampin	Not dried		Methyl alcohol	1 mg	1 day	1	3.20, 4.00, 5.00, 6.25, 7.81, µg.
Streptomycin	1		3	1 mg	30 days	3	0.64, 0.80, 1.00, 1.25, 1.56 µg.

Sisomicin[6]	Not dried[8]		3	1 mg	14 days	3	0.064, 0.080, 0.100, 0.125, 0.156 µg.
Ticarcillin	Not dried		1	1 mg	1 day	1	3.20, 4.00, 5.00, 6.25, 7.81 µg.
Vancomycin	1	Distilled water	1 mg	1 week	4	6.4, 8.0, 10.0, 12.5, 15.6 µg.	

[1] Further dilute aliquots of the working standard stock solution with dimethylsulfoxide to give concentrations of 12.8, 16, 20.0, 25, and 31.2 micrograms per milliliter.
[2] Further dilute aliquots of the working standard stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 units per milliliter.
[3] Add 2 milliliters of distilled water for each 5 milligrams of weighed working standard material.
[4] Further dilute aliquots of the working standard stock solution with dimethylformamide to give concentrations of 64, 80, 100, 125, and 156 micrograms per milliliter.
[5] The final concentration of the working standard stock solution is allowed to hydrolyze in a 37° C. constant temperature water bath for 60 minutes.
[6] Working standard should be stored below minus 20° C under an atmosphere of nitrogen. Sisomicin is hygroscopic and care should be exercised during weighing.
[7] Further dilute aliquots of the working standard stock solution with dimethylsulfoxide to give concentrations 64.0, 80.0, 100, 125, and 156 micrograms per milliliter.
[8] Weigh a separate portion of the working standard and determine the loss on drying by the method described in § 436.200(c) of this chapter. Use this value to determine the anhydrous content of the working standard.
[9] Working standard should be stored below minus 10° C under an atmosphere of nitrogen. Netilmicin sulfate is hygroscopic and care should be exercised during weighing.
[10] For assay of nystatin pastilles, use 80 percent aqueous dimethylformamide as the initial solvent and as diluent for all dilutions where dimethylformamide is required.

(c) *Procedure for assay.* For the standard response line, use a total of 12 plates—three plates for each response line solution, except the reference concentration solution which is included on each plate. On each set of three plates, fill three alternate cylinders with the reference concentration solution and the other three cylinders with the concentration of the response line under test. Thus, there will be 36 reference concentration zones of inhibition and nine zones of inhibition for each of the four other concentrations of the response line. For each sample tested use three plates. Fill three alternate cylinders on each plate with the standard reference concentration solution and the other three cylinders with the sample reference concentration solution. After all the plates have incubated for 16 to 18 hours at the appropriate incubation temperature for each antibiotic listed in the table in paragraph (b) of this section, measure the diameters of the zones of inhibition using an appropriate measuring device such as a millimeter rule, calipers, or an optical projector.

(d) *Estimation of potency.* To prepare the standard response line, average the diameters of the standard reference concentration and average the diameters of the standard response line concentration tested for each set of three plates. Average also all 36 diameters of the reference concentration for all four sets of plates. The average of the 36 diameters of the reference concentration is the correction point of the response line. Correct the average diameter obtained for each concentration to the figure it would be if the average reference concentration diameter for that set of three plates were the same as the correction point. Thus, if in correcting the highest concentration of the response line, the average of the 36 diameters of the reference concentration is 16.5 millimeters and the average of the reference concentration of the set of three plates (the set containing the highest concentration of the response line) is 16.3 millimeters, the correction is +0.2 millimeter. If the average reading of the highest concentration of the response line of these same three plates is 16.9 millimeters, the corrected diameter is then 17.1 millimeters. Plot these corrected diameters, including the average of the 36 diameters of the reference concentration on 2-cycle semilog paper, using the concentration of the antibotic in micrograms or units per milliliter as the ordinate (the logarithmic scale), and the diameter of the zone of inhibition as the abscissa. The response line is drawn either through these points by inspection or through points plotted for highest and lowest zone diameters obtained by means of the following equation:

$$L = \frac{3a + 2b + c - e}{5}$$

$$H = \frac{3e + 2d + c - a}{5}$$

where:

L=Calculated zone diameter for the lowest concentration of the standard response line;

H=Calculated zone diameter for the highest concentration of the standard response line;

c=Average zone diameter of 36 readings of the reference point standard solution;

a, *b*, *d*, *e*=Corrected average values for the other standard solutions, lowest to highest concentration, respectively.

To estimate the potency of the sample, average the zone diameters of the standard and the zone diameters of the sample on the three plates used. If the average zone diameter of the sample is larger than that of the standard, add the difference between them to the reference concentration diameter of the standard response line. If the average zone diameter of the sample is lower than that of the standard, subtract the difference between them from the reference concentration diameter of the standard response line. From the response line, read the concentrations corresponding to these corrected values of zone diameters. Multiply the concentration by the appropriate dilution factor to obtain the antibiotic content of the sample.

[39 FR 18944, May 30, 1974]

EDITORIAL NOTE: For FEDERAL REGISTER citations affecting § 436.105, see the List of CFR Sections Affected appearing in the Finding Aids section of this volume.

§ 436.106 Microbiological turbidimetric assay.

Using the sample solution prepared as described in the section for the particular antibiotic to be tested, proceed as described in paragraphs (a), (b), and (c) of this section.

(a) *Preparation of working standard stock solutions and standard response line solutions.* For each antibiotic listed in the table in this paragraph, select the working standard, drying conditions, solvent(s), concentrations, and storage time for the standard solutions and proceed as follows: If necessary, dry the working standard as described in § 436.200; dissolve and dilute an accurately weighed portion to the proper concentration for the working standard stock solution. Store the working standard stock solution under refrigeration and do not use longer than the recommended storage time. Prepare the proper concentrations for the standard response line solutions by further diluting an aliquot of the working standard stock solution. The reference concentration of the assay is the mid concentration of the standard response line.

Antibiotic	Working standard stock solutions					Standard response line concentrations	
	Drying conditions (method number as listed in § 436.200)	Initial solvent	Diluent (solution number as listed in § 436.101(a))	Final concentration units or milligrams per milliliter	Storage time under refrigeration	Diluent (solution number as listed in § 436.101(a))	Final concentrations—units or micrograms of antibiotic activity per milliliter
Amikacin	Not dried		Distilled water	1 mg	2 weeks	Distilled water	8.0, 8.9, 10.0, 11.2, 12.5 µg.
Candicidin [1]	6		Dimethyl sulfoxide	1 mg	Use same day	Distilled water	0.030, 0.043, 0.060, 0.085, 0.120 µg. (Prepare standard response line simultaneously with the sample solution.)
Capreomycin	5	Distilled water		1 mg	7 days	Distilled water	80.0, 89.0, 100.0, 112,0, 125.0 µg.
Chloramphenicol	Not dried	Ethyl alcohol (10,000 µg. per ml.).	Distilled water	1 mg	1 month	Distilled water	2.00, 2.24, 2.50, 2.80, 3.12 µg.
Chlortetracycline	Not dried		0.01*N* HCl	1 mg	4 days	Distilled water	0.048, 0.054, 0.060, 0.067, 0.075 µg.
Cycloserine	1		Distilled water	1 mg	1 month	Distilled water	40.0, 44.5, 50.0, 56.0, 62.5 µg.
Demeclocycline	1		0.1*N* HCl	1 mg	4 days	Distilled water	0.080, 0.089, 0.100, 0.112, 0.125 µg.
Dihydrostreptomycin	5		Distilled water	1 mg	30 days	Distilled water	24.0, 26.8, 30.0, 33.5, 37.5 µg.
Doxycycline	Not dried		0.1*N* HCl	1 mg	5 days	Distilled water	0.080, 0.089, 0.100, 0.112, 0.125 µg.
Gramicidin	1		alcohol U.S.P. XX	1 mg	30 days	alcohol U.S.P. XX.	0.032, 0.0356, 0.040, 0.0448, 0.050 µg.
Kanamycin	Not dried		Distilled water	1 mg	1 month	Distilled water	8.0, 8.9, 10.0, 11.2, 12.5 µg.
Lincomycin	Not dried		Distilled water	1 mg	1 month	Distilled water	0.400, 0.447, 0.500, 0.559, 0.625 µg.
Meclocycline	Not dried		13	1 mg	Use same day	Distilled water	0.048, 0.054, 0.06, 0.067, 0.075 µg.
Methacycline	1		Distilled water	1 mg	7 days	Distilled water	0.048, 0.054, 0.060, 0.067, 0.075 µg.
Oxytetracycline	Not dried		0.1*N* HCl	1 mg	4 days	Distilled water	0.192, 0.215, 0.240, 0.268, 0.300 µg.
Rolitetracycline	1		Distilled water	1 mg	1 day	Distilled water	0.192, 0.215, 0.240, 0.268, 0.300 µg.
Spectinomycin	Not dried		Distilled water	1 mg	1 month	Distilled water	24.0, 26.8, 30.0, 33.5, 37.5 µg.
Streptomycin	1		Distilled water	1 mg	30 days	Distilled water	24.0, 26.8, 30.0, 33.5, 37.5 µg.
Tetracycline	Not dried		0.1*N* HCl	1 mg	1 day	Distilled water	0.192, 0.215, 0.240, 0.268, 0.300 µg.
Tobramycin	Not dried		Distilled water	1 mg	2 weeks	Distilled water	2.00, 2.236, 2.5, 2.795, 3.125 µg.
Troleandomycin	1		15	1 mg	Use same day	Distilled water	20.0, 22.25, 25.0, 28.0, 31.25 µg.
Tyrothricin [2]							

[1] Use sterile equipment for all stages of this assay.
[2] The gramicidin working standard and the gramicidin standard response line concentrations are used for the assay of tyrothricin.

(b) *Procedure for assay.* For each antibiotic listed in the table in this paragraph, select the test organism (as listed in § 436.103(a)), nutrient broth (as listed by medium number in § 436.102(b)), and suggested inoculum and proceed as follows: Place 1.0 milliliter (or 0.1 milliliter in the case of gramicidin and tyrothricin) of each concentration of the standard response line (prepare as described in paragraph (a) of this section) and of the sample solution in each set of three replicate tubes (as described in § 436.100(b)(1)). Fifteen tubes are used for the five-point standard response line and three for each sample. To each tube add 9 milliliters of the inoculated broth and place immediately in a water bath at the appropriate temperature for 2 to 4 hours. The exact length of the incubation period should be determined by observation of growth in the reference concentration tube of the standard. Remove the tubes from the water bath and add 0.5 milliliter of a 12-percent formaldehyde solution to each tube. Determine the absorbance value of each tube in a suitable photoelectric colorimeter, at a wavelength of 530 millimicrons. Set the instrument at zero absorbance with an uninoculated blank composed of the same amounts of nutrient broth and formaldehyde used in the assay.

NOTE: The amount of working standard and sample solutions may be reduced as long as all other solutions used are reduced proportionately.

Antibiotic	Test organism	Medium (nutrient broth)	Suggested volume of standardized inoculum to be added to each 100 milliliters of medium (nutrient broth)	Incubation temperature
Amikacin	A	3	0.1	36–37.5
Candicidin [1]	E	13	0.2	27–29
Capreomycin	I	3	0.05	36–37.5
Chloramphenicol	J	3	0.7	36–37.5
Chlortetracycline	A	3	0.1	36–37.5
Cycloserine	A	3	0.4	36–37.5
Demeclocycline	A	3	0.1	36–37.5
Dihydrostreptomycin	I	3	0.1	36–37.5
Doxycycline	A	3	0.1	36–37.5
Gramicidin	K	3	1.0	36–37.5
Kanamycin	A	3	0.2	36–37.5
Lincomycin	A	3	0.1	36–37.5
Meclocycline	A	3	0.2	36–37.5
Methacycline	A	3	0.1	36–37.5
Oxytetracycline	A	3	0.1	36–37.5
Rolitetracycline	A	3	0.1	36–37.5
Spectinomycin	J	3	0.1	36–37.5
Streptomycin	I	3	0.1	36–37.5
Tetracycline	A	3	0.1	36–37.5
Tobramycin	A	3	0.15	36–37.5
Troleandomycin	I	3	0.1	36–37.5
Tyrothricin	K	3	1.0	36–37.5

[1] Use sterile equipment for all stages of this assay.

$$L = \frac{3a + 2b + c - e}{5}$$

$$H = \frac{3e + 2d + c - a}{5}$$

where:

L=Calculated absorbance value for the lowest concentration of the standard response line.

H=Calculated absorbance value for the highest concentration of the standard response line.

a, b, c, d, e=Average absorbance values for each concentration of the standard response line, lowest to the highest, respectively.

(c) *Estimation of potency.* To prepare the standard response line, plot the average absorbance values for each concentration of the standard response line on one-cycle semilogarithmic graph paper with the absorbance values on the arithmetric scale and concentrations on the logarithmic scale. The response line is drawn either through these points by inspection or through points plotted for highest and lowest absorbance values obtained by means of the following equations.

To estimate the potency of the sample, average the absorbance values for the sample and determine the antibiotic concentration from the standard response line. Multiply the concentration by the appropriate dilution factor to obtain the antibiotic content of the sample.

[39 FR 18944, May 30, 1974, as amended at 40 FR 57797, Dec. 12, 1975; 41 FR 49483, Nov. 9, 1976; 44 FR 22057, Apr. 13, 1979; 46 FR 3835, 3839, Jan. 16, 1981; 46 FR 16682, Mar. 13, 1981; 46 FR 33512, June 30, 1981; 46 FR 61072, Dec. 15, 1981; 47 FR 22515, May 25, 1982; 47 FR 23708, June 1, 1982; 48 FR 3960, Jan. 28, 1983; 53 FR 32607, Aug. 26, 1988]

Subpart E—General Chemical Tests for Antibiotics

§ 436.200 Loss on drying.

Use the method specified in the individual section for each antibiotic.

(a) *Method 1.* In an atmosphere of about 10 percent relative humidity, grind the sample, if necessary, to obtain a fine powder. When tablets, troches, or capsules are to be tested, use four tablets, troches, or capsules in preparing the sample. Transfer about 100 milligrams of the sample to a tared weighing bottle equipped with a ground-glass stopper. Weigh the bottle and place it in a vacuum oven, tilting the stopper on its side so that there is no closure during the drying period. Dry at a temperature of 60° C. and a pressure of 5 millimeters of mercury or less for 3 hours. At the end of the drying period, fill the vacuum oven with air dried by passing it through a drying agent such as sulfuric acid or silica gel. Replace the stopper and place the weighing bottle in a desiccator over a desiccating agent, such as phosphorous pentoxide or silica gel, allow to cool to room temperature, and reweigh. Calculate the percent of loss.

(b) *Method 2.* Proceed as directed in paragraph (a) of this section, except use a tared weighing bottle or weighing tube equipped with a capillary-tube stopper, the capillary having an inside diameter of 0.20–0.25 millimeter, and place it in a vacuum oven without removing the stopper.

(c) *Method 3.* Proceed as directed in paragraph (a) of this section, except dry the sample at a temperature of 110° C. and a pressure of 5 millimeters of mercury or less for 3 hours.

(d) *Method 4.* Proceed as directed in paragraph (a) of this section, except dry the sample at a temperature of 40° C. and a pressure of 5 millimeters of mercury or less for 2 hours.

(e) *Method 5.* Proceed as directed in paragraph (a) of this section, except dry the sample at a temperature of 100° C. and a pressure of 5 millimeters of mercury or less for 4 hours.

(f) *Method 6.* Proceed as directed in paragraph (a) of this section, except dry the sample at a temperature of 40° C. and a pressure of 5 millimeters of mercury or less for 3 hours.

(g) *Method 7.* Proceed as directed in paragraph (a) of this section, except dry the sample at a temperature of 25° C. and a pressure of 5 millimeters of mercury or less for 4 hours.

(h) *Method 8.* Proceed as directed in paragraph (a) of this section, except transfer approximately 300 milligrams of the sample to a tared weighing bottle equipped with a ground-glass stopper; dry the sample at a temperature of 25 ° C and a pressure of 5 millimeters of mercury or less for 4 hours, and then dry the sample at a temperature of 100 °C and a pressure of 5 millimeters of mercury or less for 3 additional hours.

(i) *Method 9.* Use a suitable thermogravimetric apparatus prepared for vacuum operation. Rapidly and thoroughly grind a portion of the sample and promptly transfer 5 to 10 milligrams to the sample pan. Place the system under vacuum and allow it to come to equilibrium before heating. Obtain an accurate sample weight and

continuously record the weight loss as the sample is heated at a rate of 20° per minute from room temperature to about 200 ° C. The weight loss plateau, or inflection, at about 150 ° C is taken as the total volatile weight loss. Calculate the percent weight loss on drying.

[39 FR 18944, May 30, 1974, as amended at 50 FR 48397, Nov. 25, 1985; 51 FR 11572, Apr. 4, 1986]

§ 436.201 Moisture determination.

(a) *Equipment*—(1) *Apparatus.* Use a closed system consisting of all glass automatic burettes, platinum electrodes, and a magnetic stirrer connected to a suitable electrometric apparatus. This apparatus embodies a simple electrical circuit which serves to pass 5 to 10 microamperes of direct current between a pair of platinum electrodes immersed in the solution to be titrated. At the endpoint of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 seconds or longer, depending upon the solution being titrated.

(2) *Titrating vessel.* Use a suitable titrating vessel which has been previously dried at 105° C. and cooled in a desiccator.

(b) *Reagents*—(1) *Karl Fischer reagent.* Dissolve 125 grams of iodine in 170 milliliters of pyridine, add 670 milliliters of methanol and cool. To 100 milliliters of pyridine kept in an ice bath, add sulfur dioxide until the volume reaches 200 milliliters. Slowly add this solution to the cooled iodine-methanol-pyridine mixture and shake well. (A commercially prepared Karl Fischer reagent, pyridine containing or pyridine-free, may be used.) Preserve the reagent in glass-stoppered bottles protected from light and from moisture in the air.

(2) *Methanol solution.* Add sufficient water (usually 2 milligrams per milliliter) to methanol so that each milliliter of the resulting methanol solution is equivalent to about 0.5 milliliter of Karl Fischer reagent.

(3) *Solvents*—(i) *Solvent A.* Methanol:chloroform:carbon tetrachloride (1:2:2 by volume).

(ii) *Solvent B.* Chloroform:carbon tetrachloride (1:1 by volume).

(iii) *Solvent C.* Anhydrous methanol.

(c) *Standardization of reagents*—(1) *Water equivalence of Karl Fischer reagent.* Standardize the Karl Fischer reagent no longer than 1 hour before use by one of the following methods.

(i) Accurately weigh 25–35 milligrams of water into a dry titration vessel and add 20 milliliters of solvent A. Start the stirrer and titrate to the endpoint by adding measured quantities of Karl Fischer reagent. Calculate the water equivalence of the Karl Fischer reagent as follows:

$$e = \frac{W}{V_T - V_A}$$

where:

e=Water equivalence of the Karl Fischer reagent in terms of milligrams of water per milliliter;

W=Milligrams of water;

V_T=Milliliters of Karl Fischer reagent used;

V_A=Milliliters of Karl Fischer reagent equivalent to the 20 milliliters of solvent A, determined as directed in paragraph (c)(3) of this section.

(ii) Accurately weigh about 25–35 milligrams of water into a dry titration vessel, add an excess of Karl Fischer reagent, start the stirrer, and titrate to the endpoint with methanol solution. Calculate the water equivalence of the Karl Fischer reagent as follows:

$$e = \frac{W}{V_T - V_A}$$

where:

e=Water equivalence of the Karl Fischer reagent in terms of milligrams of water per milliliter;

W=Milligrams of water;

V_T=Milliliters of Karl Fischer reagent used;

V_m=Milliliters of methanol solution used;

f=Milliliters of Karl Fischer reagent equivalent to each milliliter of methanol solution determined as directed in paragraph (c)(2) of this section.

(2) *Karl Fischer reagent equivalence of methanol solution.* Titrate a known volume of Karl Fischer reagent with methanol solution until the endpoint is reached. Calculate the milliliters of Karl Fischer reagent equivalent to each milliliter of methanol solution as follows:

$$e = \frac{W}{(V_T - V_m) \times f}$$

where:

f=Milliliters of Karl Fischer reagent equivalent to each milliliter of methanol solution;
V_T=Milliliters of Karl Fischer reagent used;
V_m=Milliliters of methanol solution used.

(3) *Karl Fischer reagent equivalence of solvents.* (i) Solvent A: Use 20 milliliters of solvent A as the sample. Start the stirrer and titrate to the endpoint by adding measured quantities of Karl Fischer reagent.

(ii) Solvent B: Use 10 milliliters of solvent B as the sample. Add an excess of Karl Fischer reagent to the sample and start the stirrer. Titrate to the endpoint with methanol solution.

(iii) *Solvent C.* Use 20 milliliters of solvent C as the sample. Start the stirrer and titrate to the endpoint by adding measured quantities of Karl Fischer reagent.

(iv) Calculate the Karl Fischer reagent equivalence of the solvents as follows:

$V_A = V_C = V_T$,

$V_B = (V_T - V_m)$ X f

where:

V_A, V_B, and V_C=Milliliters of Karl Fischer reagent equivalent to the aliquots used of solvents A, B, and C, respectively;
V_T=Milliliters of Karl Fischer reagent used;
V_m=Milliliters of methanol solution used;
f=Milliliters of Karl Fischer reagent equivalent to each milliliter of methanol solution determined as directed in paragraph (c)(2) of this section.

(d) *Sample preparation*—(1) *Powders.* In the case of tablets, grind 4 tablets to a fine powder. In the case of capsules containing enteric-coated pellets, grind the pellets to a fine power. If the maximum moisture limit is greater than 1 percent, accurately weigh about 300 milligrams of the sample into a dry titrating vessel. If the maximum moisture limit is less than 1 percent, accurately weigh 1 to 2 grams of the sample. Proceed as directed in paragraph (e)(1) or (2) of this section.

(2) *Ointments and oils.* (i) Transfer about 1 to 2 grams, accurately weighed, into a dry titrating vessel. Proceed as directed in paragraph (e)(1) of this section; or

(ii) Transfer about 1 to 2 grams, accurately weighed, into a dry titrating vessel. Add 10 milliliters of solvent B and proceed as directed in paragraph (e)(2) of this section.

(3) *Aerosols with propellant.* Place the immediate container to be tested in a suitable freezing unit having a temperature of not higher than 0° C. for at least 2 hours. Remove the container from the freezing unit, puncture it, mix the entire contents by swirling. Proceed as directed in paragraph (e)(3) of this section, using an accurately measured 10-milliliter aliquot from the container as the sample and allowing the solution to warm to at least 10° C. before determining the endpoint.

(4) *Hygroscopic powders.* Weigh the immediate container. Using a suitable dry hypodermic needle and syringe, inject 3 milliliters of anhydrous methanol into the container and shake to dissolve the contents. Using the same syringe, remove the withdrawable contents and transfer into the titration vessel. Rinse the syringe and needle by drawing in an additional 3 milliliters of anhydrous methanol. Add the rinsings to the titration vessel. Titrate the solution immediately, proceeding as directed in paragraph (e)(3) of this section. Determine the Karl Fischer equivalent (in milliliters), if any, of the anhydrous methanol by titrating a blank of the same total volume used in preparing the sample and rinsing the syringe and needle. Dry the immediate container and its closure for three hours at 100° C., cool to room temperature in a desiccator, and weigh. Determine the weight of sample tested by subtracting the weight obtained from the original weight of the immediate container.

(5) *Solutions.* Proceed as directed in paragraph (e)(3) of this section, using about 1 to 2 grams of the sample, accurately weighted.

(e) *Titration procedures and calculations*—(1) *Procedure 1.* Add 20 milliliters of solvent A to the sample. Start the stirrer and titrate to the endpoint by adding measured quantities of Karl Fischer reagent. Determine the percent moisture in the sample as follows:

$$\text{Percent moisture} = \frac{(V_T - V_A) \times e \times 100}{W_s}$$

where:

e=Water equivalence of the Karl Fischer reagent determined as directed in paragraph (c)(1) of this section;

V_T=Milliliters of Karl Fischer reagent used;

V_A=Milliliters of Karl Fischer reagent equivalent to the 20 milliliters of solvent A, determined as directed in paragarph (c)(3) of this section;

W_s=Weight of the sample in milligrams.

(2) *Procedure 2.* Add an excess of Karl Fischer reagent to the sample, start the stirrer, and titrate to the endpoint with methanol solution. Calculate the percent moisture in the sample as follows:

(i) For powders:

$$\text{Percent moisture} = \frac{(V_T - V_m f) \times e \times 100}{W_s}$$

(ii) For oils and ointments:

$$\text{Percent moisture} = \frac{(V_T - V_m f - V_B) \times e \times 100}{W_s}$$

where:

V_T=Milliliters of Karl Fischer reagent used;

V_m=Milliliters of methanol solution used;

f=Milliliters of Karl Fischer reagent equivalent to each milliliter of methanol solution determined as directed in paragraph (c)(2) of this section.

V_B=Milliliters of Karl Fischer reagent equivalent to the 10 milliliters of solvent B determined as directed in paragraph (c)(3) of this section;

e=Water equivalence of the Karl Fischer reagent determined as directed in paragraph (c)(1) of this section;

W_s=Weight of the sample in milligrams.

(3) *Procedure 3.* Add about 20 milliliters of solvent A to a dry titrating vessel and proceed as directed in titration procedure 1 or 2. Disregard the volume of reagents used to determine the endpoint. Promptly introduce an accurately weighed or measured quantity of sample into the titrating vessel and titrate to the endpoint using either titration procedure 1 or 2 without additional solvents. Calculate the percent moisture in the sample as follows:

(i) If titration procedure 1 is used:

$$\text{Percent moisture in weighed samples} = \frac{V_T \times e \times 100}{W_s}$$

$$\text{Percent moisture in aerosols} = \frac{V_T \times e}{\text{Milliliters of sample} \times 10}$$

Percent moisture in hygroscopic powders=

$$\text{Percent moisture in hygroscopic powders} = \frac{(V_T - V_b) \times e \times 100}{W_s}$$

(ii) If titration procedure 2 is used:

$$\text{Percent moisture in weighed samples} = \frac{(V_T - V_m f) \times e \times 100}{W_s}$$

$$\text{Percent moisture in aerosols} = \frac{(V_T - V_m f) \times e}{\text{Milliliters of sample} \times 10}$$

$$\text{Percent moisture in hygroscopic powders} = \frac{(V_T - V_m f - V_b) \times e \times 100}{W_s}$$

where:

V_T=Milliliters of Karl Fischer reagent used;

V_m=Milliliters of methanol solution used;

f=Milliliters of Karl Fischer reagent equivalent to each milliliter of methanol solution determined as directed in paragraph (c)(2) of this section;

V_b=Karl Fischer equivalent (in milliliters) of the methanol used as a sample solvent;

e=Water equivalence of the Karl Fischer reagent determined as directed in paragraph (c)(1) of this section.

[39 FR 18944, May 30, 1974, as amended at 48 FR 51292, Nov. 8, 1983; 50 FR 41679, Oct. 15, 1985; 51 FR 22071, June 18, 1986; 51 FR 27532, Aug. 1, 1986]

§ 436.202 pH.

(a) *Apparatus.* A suitable potentiometer fitted with two electrodes, one being constructed of glass and sensitive to hydrogen ion activity and the other being a calomel or a silver/silver chloride reference electrode. A combination electrode with glass electrode and reference electrode contained in the same system may be used.

(b) *Standardization.* Select two standard buffer solutions such that the expected pH value of the sample is within their pH range and is also within 2 pH

unit of one of the standard buffer solutions. Standardize the pH meter with the two buffer solutions. Make any necessary adjustment of the meter if the observed pH value of either standard solution differs by more than 0.05 pH units of its known value.

(c) *Sample preparation.* If necessary, dilute the sample with carbon dioxide-free distilled water to the concentration specified in the individual section for each antibiotic.

(d) *Test procedure.* Determine the pH of the sample at 25°±2° C. Rinse the electrode(s) between determinations first with distilled water and then with a portion of the next sample to be tested. Store electrode(s) with tips immersed in water.

[39 FR 18944, May 30, 1974, as amended at 42 FR 29857, June 10, 1977; 42 FR 31449, June 21, 1977]

§ 436.203 Crystallinity.

Use the method specified in the individual section for each antibiotic.

(a) *Method 1.* To prepare the sample for examination, mount a few particles in mineral oil on a clean glass slide. Examine the sample by means of a polarizing microscope. The particles reveal the phenomena of birefringence and extinction positions on revolving the microscope stage.

(b) *Method 2.* Proceed as directed in paragraph (a) of this section, except to prepare the sample for examination, mount a few particles in mineral oil, add 1 drop of ethyl alcohol, and allow to react for about 30 seconds.

§ 436.204 Iodometric assay.

(a) *Reagents.* (1) 0.01*N* Sodium thiosulfate (2.482 grams $Na_2S_2O_35H_2O$ and 125 milligrams Na_2CO_3 per liter).

(2) 1.0*N* Sodium hydroxide.

(3) 1.2*N* Hydrochloric acid.

(4) 0.01*N* Iodine solution (prepared from 0.1*N* iodine U.S.P.).

(5) Starch iodide paste, T.S. (U.S.P.).

(b) *Preparation of sample and working standard solutions*—(1) *Workingstandard solutions.* From the following table, select the initial solvent, diluent, and final concentration as listed for each antibiotic working standard. Dissolve and dilute an accurately weighed portion to the specified final concentration and proceed as directed in paragraphs (c) and (d) of this section.

Antibiotic	Initial solvent	Diluent (solution number as listed in § 436.101(a))	Final concentration in units or milligrams of activity per milliliter of standard solution
Amoxicillin	None	Distilled water	1.0 mg.
Ampicillin	do	do	1.25 mg.
Cephalexin	do	do	2 mg.
Cephaloridine	do	do	2 mg.
Cephalothin	do	do	2 mg.
Cephapirin	do	do	2 mg.
Cloxacillin	do	do	1.25 mg.
Cyclacillin	do	do	1.0 mg.
Dicloxacillin	do	1	1.25 mg.
Methicillin	do	1	1.25 mg.
Nafcillin	do	1	1.25 mg.
Oxacillin	do	1	1.25 mg.
Penicillin G	do	1	2,000 units.
Penicillin V potassium	do	1	2,000 units.

(2) *Bulk antibiotic solutions.* From the following table, select the initial solvent, diluent, and final concentration as listed for each antibiotic. Dissolve an accurately weighed aliquot (approximately 30 to 60 milligrams) of the sample, dilute to the appropriate final concentration, and proceed as directed in paragraphs (c) and (d) of this section.

Antibiotic	Initial solvent	Diluent (solution number as listed in § 436.101(a))	Final concentration in units or milligrams of activity per milliliter of sample
Amoxicillin trihydrate	None	Distilled water	1.0 mg.
Ampicillin	do	do	1.25 mg.
Ampicillin sodium	do	1	1.25 mg.
Ampicillin trihydrate	do	Distilled water	125 mg.

Antibiotic	Initial solvent	Diluent (solution number as listed in § 436.101(a))	Final concentration in units or milligrams of activity per milliliter of sample
Bacampicillin hydrochloride	None	.....do	1.25 mg.[2]
Cephalexin	.....do	.....do	2 mg.
Cephaloridine	.....do	.....do	2 mg.
Cephalothin sodium	.....do	.....do	2 mg.
Cephapirin sodium	.....do	.....do	2 mg.
Cloxacillin sodium monohydrate	.....do	.....do	1.25 mg.
Cloxacillin sodium monohydrate	.....do	.....do	1.25 mg.
Dicloxacillin sodium monohydrate	.....do	1	1.25 mg.
Cyclacillin	.....do	Distilled water	1.0 mg.
Methicillin sodium monohydrate	.....do	1	1.25 mg.
Mezlocillin	.....do	Distilled water	2.0 mg.
Nafcillin sodium monohydrate	.....do	1	1.25 mg.
Oxacillin sodium monohydrate	.....do	1	1.25 mg.
Penicillin G benzathine blank solution	.....do	Distilled water	2,000 units.
Penicillin G benzathine inactivated solution	.....do	1*N* NaOH	2,000 units.[1]
Penicillin G potassium	.....do	1	2,000 units.
Penicillin G procaine	2 ml methyl alcohol	1	2,000 units.
Penicillin G sodium	None	1	2,000 units.
Penicillin V	2 ml methyl alcohol	1	2,000 units.
Penicillin V potassium	.....do	1	2,000 units.

[1] Allow to stand in 1*N* NaOH for 15 minutes before assaying.
[2] The final concentration of bacampicillin hydrochloride is calculated in milligrams of ampicillin activity per milliliter of sample. The ampicillin working standard is used for the assay of bacampicillin hydrochloride.

(3) *Finished product solutions.* Prepare the sample for assay as directed in the individual section for each antibiotic product to be tested by diluting to the concentration prescribed in the table in paragraph (b)(2) of this section and proceed as described in paragraphs (c) and (d) of this section.

(c) *Inactivated sample and standard solutions.* (1) Transfer 2.0 milliliters each of the sample and the appropriate working standard solutions to glass-stoppered Erlenmeyer flasks.

(2) Add 2.0 milliliters of 1*N* sodium hydroxide, except if the sample has been diluted in 1*N* sodium hydroxide, and allow to stand at room temperature for 15 minutes.

(3) Add 2.0 milliliters of 1.2*N* hydrochloric acid.

(4) Add 10.0 milliliters of 0.01*N* iodine solution, stopper, allow to stand at room temperature for 15 minutes, and proceed as directed in paragraph (e) of this section.

(d) *Blank determination.* Transfer 2.0 milliliters each of the sample and the appropriate working standard solutions to glass-stoppered Erlenmeyer flasks. Add 10.0 milliliters of 0.01*N* iodine solution and immediately proceed as directed in paragraph (e) of this section.

(e) *Titration procedure.* Titrate the excess iodine using 0.01*N* sodium thiosulfate. Toward the end of the titration, add 1 drop of the starch iodide paste. Continue the titration by the addition of 0.01- to 0.02-milliliter portions of 0.01*N* sodium thiosulfate, shaking vigorously after each addition. The endpoint is reached when the blue color of the starch-iodine complex is discharged. Calculate the antibiotic content as described in paragraph (f) of this section.

(f) *Calculations*—(1) *F factor determination.* Using the appropriate working standard for the particular antibiotic to be tested, assay the standard as directed in this section. Calculate the F factor (the units of micrograms of activity equivalent of each milliliter of 0.01*N* sodium thiosulfate consumed) by means of the following formula:

$$F = \frac{W_s \times P}{V_s}$$

where:

W_s=Actual weight in milligrams of standard in the 2.0 milliliters titrated;

P=Potency of the working standard in units or micrograms per milligram;

V_s=Milliliters of sodium thiosulfate used in the working standard blank determination minus the milliliters of sodium thiosulfate used in the titration of the inactivated working standard solution (the difference is the equivalent of the number of milliliters of 0.01*N* iodine absorbed by the inactivated standard).

(2) *Bulk antibiotic.* Calculate the potency of the sample in units or micrograms per milligram by means of the following formula:

$$\frac{V_u \times F}{W_u}$$

where:

V_u=Milliliters of sodium thiosulfate used in the sample blank determination minus the milliliters of sodium thiosulfate used in the titration of the inactivated sample solution (the difference is the equivalent of the number of milliliters of 0.01*N iodine absorbed by the inactivated sample);*

W_u=Actual weight in miligrams of sample in the 2.0 milliliters titrated.

(3) *Finished products.* Calculate the potency of the sample in units or milligrams by means of the appropriate one of the following formulas:

$$\text{Units of antibiotic per dose or item} = \frac{V_u \times F \times d}{2n}$$

$$\text{Milligrams of antibiotic per dose or item} = \frac{V_u \times F \times d}{n \times 2{,}000}$$

where:

d=Dilution factor for the sample;

n=Number of doses or items in the sample assayed.

[39 FR 18944, May 30, 1974, as amended at 39 FR 34032, Sept. 23, 1974; 42 FR 59856, Nov. 22, 1977; 44 FR 10378, Feb. 20, 1979; 46 FR 2980, Jan. 13, 1981; 46 FR 25602, May 8, 1981; 46 FR 46312, Sept. 18, 1981; 46 FR 58298, Dec. 1, 1981; 46 FR 61072, Dec. 15, 1981; 49 FR 6091, Feb. 17, 1984]

§ 436.205 Hydroxylamine colorimetric assay.

(a) *Reagents*—(1) *Hydroxylamine hydrochloride solution.* Dissolve 350 grams of hydroxylamine hydrochloride in sufficient distilled water to make 1 liter.

(2) *Buffer.* Dissolve 173 grams of sodium hydroxide and 20.6 grams of sodium acetate in sufficient distilled water to make 1 liter.

(3) *Neutral hydroxylamine.* Mix 1 volume each of hydroxylamine hydrochloride solution described in paragraph (a)(1) of this section and the buffer described in paragraph (a)(2) of this section. Check the pH and if necessary adjust to pH 7.0±0.1 by adding an additional amount of one of the components. To 1 volume of this neutralized solution add 8 volumes of distilled water and 2 volumes of 95 percent ethanol. This solution should be used for 1 day only.

(4) *Ferric ammonium sulfate.* Dissolve 272 grams of ferric ammonium sulfate in a mixture of 26 milliliters of concentrated sulfuric acid and sufficient distilled water to make 1 liter. This reagent may be used for 1 week when stored in a brown bottle at room temperature.

(b) *Preparation of working standard solutions.* From the following table, select the diluent and final concentration as listed for each antibiotic working standard. Dissolve and dilute an accurately weighed portion to the specified final concentration and proceed as directed in paragraph (d) of this section.

Antibiotic	Diluent (solution number as listed in § 436.101(a))	Final concentration in milligrams per milliliter of standard solution
Amoxicillin	Distilled water	1.0
Ampicillin	.....do	1.25
Cefazolin [1]		1.0
Cephaloridine	Distilled water	1.0
Cephalothin	.....do	2.0
Cephapirin	.....do	1.0
Cloxacillin	1	1.25
Cyclacillin	Distilled water	1.25
Dicloxacillin	1	1.25
Methicillin	1	1.25
Nafcillin	1	1.25
Oxacillin	1	1.25
Penicillin G	1	1.25
Penicillin G procaine	17	2.0
Penicillin V Potassium	1	1.25

[1] To prepare the working standard solution, proceed as directed in the individual section of the antibiotic drug regulation in this chapter for the antibiotic to be tested.

(c) *Preparation of sample solutions.* From the following table, select the diluent and final concentration as listed for each antibiotic. Dissolve an accurately weighed portion of the sample, dilute to the appropriate final concentration, and proceed as directed in paragraph (d) of this section; if the product is packaged for dispensing, dilute an aliquot of the stock solution (prepared as described in the individual monograph) to the appropriate concentration and then proceed as directed in paragraph (d) of this section.

Antibiotic	Diluent (solution number as listed in § 436.101(a))	Final concentration in milligrams per milliliter of sample
Amoxicillin trihydrate	Distilled water	1.0
Ampicillin	do	1.25
Ampicillin sodium	1	1.25
Ampicillin trihydrate	Distilled water	1.25
Bacampicillin hydrochloride	do	[1]1.2
Cefazolin sodium	1	1.0
Cephaloridine	Distilled water	1.0
Cephalothin sodium	do	2.0
Cephapirin sodium	do	1.0
Cloxacillin sodium monohydrate.	1	1.25
Cyclacillin	Distilled water	1.25
Dicloxacillin sodium monohydrate.	do	1.25
Methicillin sodium monohydrate.	1	1.25
Nafcillin sodium monohydrate	1	1.25
Oxacillin sodium monohydrate	1	1.25
Penicillin G potassium	1	1.25
Penicillin G procaine	17	2.0
Penicillin G sodium	1	1.25
Penicillin V	17	1.25
Penicillin V potassium	1	1.25

[1] The final concentration of bacampicillin hydrochloride is calculated in milligrams of ampicillin per milliliter of sample. The ampicillin working standard is used for the assay of bacampicillin hydrochloride.

(d) *Procedure.* Using a volume of from 1 to 2 milliliters of standard or sample solution, add an equal volume of water and mix. Add the following reagents in the specified volumetric proportions with respect to the sample or standard solutions: Add 1.25 volumes of neutral hydroxylamine reagent and allow to react for 5 minutes. Add 1.25 volumes of ferric ammonium sulfate reagent, mix, and after 3 minutes determine the absorbance of the resulting solution at the wavelength of 480 millimicrons, using a suitable spectrophotometer and a reagent blank prepared by treating a volume of water in the same manner as the standard or sample solution. The time elapsed after the addition of the ferric ammonium sulfate reagent and the reading of the absorbance must be precisely the same (within 10 seconds) for each solution. Calculate the potency of the sample in units or micrograms per milligram as follows:

$$\text{Units or micrograms per milligram of sample} = \frac{A_1 \times \text{Potency (in units or micrograms per milliliter of standard solution)}}{A_2 \times \text{Milligrams of sample per milliliter of sample solution}}$$

A_1=Absorbance of sample solution.
A_2=Absorbance of standard solution.

[39 FR 18944, May 30, 1974, as amended at 39 FR 34032, Sept. 23, 1974; 39 FR 44012, Dec. 20, 1974; 42 FR 59856, Nov. 22, 1977; 44 FR 10378, Feb. 20, 1979; 45 FR 16474, Mar. 14, 1980; 46 FR 2981, Jan. 13, 1981; 46 FR 25602, May 8, 1981; 46 FR 61072, Dec. 15, 1981; 49 FR 34350, Aug. 30, 1984]

§ 436.206 Test for metal particles in ophthalmic ointments.

(a) *Procedure.* Extrude the contents of each of 10 tubes as completely as practicable into separate, clear, glass Petri dishes (60 millimeters in diameter), cover the dishes, and heat to 80° C. to 85° C. for at least 2 hours or until the ointment has melted completely and evenly in the dishes. A higher temperature of 100° C.±2° C. may be used if necessary to allow adequate settling of metal particles. Allow the ointment to cool to room temperature without agitation. Invert each Petri dish on the stage of a suitable microscope adjusted to furnish 30 times magnification and equipped with an eye-piece micrometer disc which has been calibrated at the magnification being used. In addition to the usual source of light, direct an illuminator from above the ointment at a 45° angle. Examine the entire bottom of the Petri dish for metal particles. By varying the intensity of the illuminator from above, such metal particles are recognized by their characteristic reflection of light. Count the total number of metal particles exceeding 50 microns in any single dimension.

(b) *Evaluation.* The batch is acceptable if (1) a total of not more than 50 such particles is found in 10 tubes; and (2) not more than one tube is found to contain more than eight such particles. If the batch fails the above test, repeat

the test on 20 additional tubes of ointment. The total number of metal particles exceeding 50 microns in any single dimension from the 30 tubes tested shall not exceed 150, with not more than three tubes containing more than eight such particles.

[39 FR 18944, May 30, 1974; 40 FR 11869, Mar. 14, 1975]

§ 436.207 Residue on ignition.

Use the method specified in the individual section for each antibiotic.

(a) *Method 1.* Place approximately 1 gram of the sample, accurately weighed, in a tared porcelain crucible and carefully ignite at a low temperature until thoroughly charred. The crucible may be loosely covered with a porcelain lid during the charring. Add 2 milliliters of nitric acid and 5 drops of sulfuric acid to the contents of the crucible and cautiously heat until white fumes are evolved, then ignite, preferably in a muffle furnace, at 500° C. to 600° C. until the carbon is all burned off. Cool the crucible in a desiccator and weigh. From the weight of residue obtained, calculate the sulfated ash content.

(b) *Method 2.* Proceed as directed in paragraph (a) of this section, except use 2 milliliters of sulfuric acid and do not use the nitric acid.

§ 436.208 Heavy metals determination.

(a) *Reagents*—(1) *Ammonia solution.* Prepare an aqueous solution containing not less than 9 grams and not more than 10 grams of ammonia (NH_3) per 100 milliliters.

(2) *6 percent acetic acid.* Dilute 60 milliliters of glacial acetic acid with sufficient water to give a solution of 1,000 milliliters.

(3) *Hydrogen sulfide solution.* Prepare a saturated solution of hydrogen sulfide by passing hydrogen sulfide into cold water for a sufficient time. It is suitable if it produces an immediate copious precipitate when added to an equal volume of 1*N* ferric chloride. Prepare a fresh hydrogen sulfide solution each time a heavy metals test is to be performed.

(4) *Lead nitrate stock solution.* Dissolve 159.8 milligrams of lead nitrate with 100 milliliters of 0.15*N* nitric acid, and dilute with water to a volume of 1,000 milliliters. Prepare and store this solution in glass containers free from soluble lead salts.

(5) *Standard lead solution.* Dilute a 10-milliliter aliquot of the lead nitrate stock solution to 100 milliliters with water. This solution must be freshly prepared each time a heavy metals test is performed. One milliliter of this standard lead solution represents a lead level of 10 parts per million in a 1.0-gram sample or 20 parts per million in a 0.5-gram sample.

(b) *Preparation of the sample.* Use the sulfated ash obtained as described in § 436.207(a). If the heavy metal limit is greater than 30 parts per million, the sulfated ash may be obtained from a 0.5-gram sample. Add 2 milliliters of hydrochloric acid to the sulfated ash and slowly evaporate to dryness on a steam bath. Moisten the residue with 1 drop of hydrochloric acid, add 10 milliliters of hot water, and digest by heating on the steam bath for 2 minutes. After cooling to room temperature, add ammonia solution dropwise until a pH of 7.2 is reached, then add 2 milliliters of 6 percent acetic acid. Filter the solution, if necessary, and wash the crucible and the filter with about 10 milliliters of water. Combine the washings with the filtrate and dilute to exactly 25 milliliters with water.

(c) *Procedure.* Prepare a series of five standard lead solutions, in increments of 10 parts per million, in which the solution of lowest concentration contains 20 parts of lead per million less than the maximum limit of heavy metals permitted for the sample. Transfer the necessary quantities of standard lead solution described in paragraph (a)(5) of this section directly into metal-free 50-milliliter Nessler tubes of uniform diameter, add 2 milliliters of 6 percent acetic acid to each, and adjust each to a final volume of 25 milliliters with water. Transfer the 25-milliliter solution of the sample described in paragraph (b) of this section to another Nessler tube. Add 10 milliliters of hydrogen sulfide solution to each standard and sample solution, mix well, and allow to stand for 10 minutes. View downward over a white surface; the color of the solution of the sample should be no darker than the standard

that contains the lead equivalent of the heavy metals limit of the test.

§ 436.209 Melting range or temperature.

(a) *Apparatus.* Melting range apparatus consists of a glass container for a bath of colorless fluid, a suitable stirring device, an accurate thermometer, and a controlled source of heat. Any apparatus or method of equal accuracy may be used. The accuracy should be checked periodically by use of melting point standards, preferably those that melt near the expected melting range of the product to be tested. The bath fluid is selected with a view to the temperature required, but light paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still 2 centimeters above the bottom of the bath.

(b) *Sample preparation.* If necessary, reduce the sample to a fine powder and store it in a desiccator over sulfuric acid for 24 hours. If a method for loss on drying is included in the section for the antibiotic to be tested, a sample dried by that method may be used.

(c) *Test procedure.* Use a capillary glass tube about 10 centimeters long and 0.8 to 1.2 millimeters internal diameter with the wall 0.2 to 0.3 millimeter in thickness. Charge the tube with a sufficient amount of the dry power to form a column 2.5 to 3.5 millimeters high from the sealed end when packed down as closely as possible by moderate tapping on a solid surface. Heat the bath until a temperature 10°±1° C. below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3°±0.5° C. per minute until melting is completed. The temperature at which the column of the sample is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the sample becomes liquid throughout is defined as the end of melting or the melting point.

[39 FR 18944, May 30, 1974, as amended at 41 FR 24883, June 21, 1976]

§ 436.210 Specific rotation.

(a) *Test procedure.* The appropriate solvent, test concentration, and polarimeter tube length are specified in the section for each antibiotic to be tested. Accurately weigh the sample to be tested in a glass-stoppered volumetric flask, dissolve in the appropriate solvent, and dilute to the specified test concentration at 25° C. Maintain the solution at 25° C. and transfer to the appropriate polarimeter tube. Determine the angular rotation of both solvent and sample solution in a suitable polarimeter, using a sodium light source or a white light source with a 589.3-millimicron filter. The zero correction is the average of the blank readings and is subtracted from the average observed rotation of the sample solution if the two figures are of the same sign, or is added if they are opposite in sign, to give the corrected angular rotation of the sample solution. The determination must be completed within one-half hour from the time the solution is prepared.

(b) *Calculations.* Determine the specific rotation. $[\alpha]$, by the following formula:

$$[a]t_x = \frac{100a}{lc}$$

where:

a=The corrected angular rotation of the sample solution in degrees at temperature *t* using a light source of a wavelength of *x* millimicrons;

l=The length of the polarimeter tube in decimeters;

c=The concentration of the solution expressed as number of grams of substance in 100 milliliters of solution.

§ 436.211 Identity test by infrared spectrophotometry.

(a) *Apparatus*—(1) *Spectrophotometer.* A suitable spectrophotometer capable of recording the infrared absorption spectrum in the 2 to 15 micron range.

(2) *Hydraulic press.* A 30-ton hydraulic press with 12-inch square platens.

(b) *Sample preparation methods.* Use the sample preparation method specified in the individual section for each antibiotic.

(1) *Potassium bromide discs.* Quantities of materials specified are for a 13-millimeter die. Appropriate adjustments

should be made in the quantities of materials when dies of other sizes are used. To prepare a 1.0 percent mixture weigh approximately 2 milligrams of the sample and mix thoroughly with 200 milligrams of dried potassium bromide (infrared spectrophotometric quality). For a 0.5 percent potassium bromide mixture, use 1 milligram of sample. For a 0.25 percent potassium bromide mixture, use 0.5 milligram of sample. A mortar and pestle, a ball mill, or other suitable mixing device may be used. Transfer the uniformly milled mixture to the die, evacuate gradually while raising the pressure to 3,000 pounds per square inch until evacuation is complete, then raise the pressure to 16,000 pounds per square inch, and hold that pressure for 2 to 3 minutes. Release the pressure, dismantle the die, and recover the potassium bromide disc. Mount the disc in a suitable holder and proceed as directed in paragraph (c) of this section.

(2) *Mineral oil mull.* Weigh approximately 20 milligrams of the sample into an agate mortar and add 2 drops of mineral oil. Triturate thoroughly with a pestle until a uniform consistency is obtained. Use two rock salt plates as an absorption cell. Place a small drop of the mull in the center of one of the plates. Gently put the other plate on the mull and slowly squeeze the plates together to spread the mull uniformly. Clamp the two plates firmly together in a metal holder. Examine the assembled cell by holding it up to the light. It should appear smooth and free of any air bubbles. Proceed as directed in paragraph (c) of this section.

(3) *1 percent solution.* Prepare a 1 percent solution of the sample in chloroform and use 1.0 millimeter matched absorption cells. Proceed as directed in paragraph (c) of this section.

(c) *Procedure.* Place the sample, prepared as directed in paragraph (b) of this section, in the spectrophotometer. Determine the infrared absorbance spectrum between the wavelengths of 2 to 15 microns. To be suitable the spectrum should have a transmittance of between 20 and 70 percent at most of the wavelengths showing significant absorption. Compare the spectrum to that of an authentic sample of the same antibiotic prepared in an identical manner. To pass the infrared identity test, the absorption spectrum of the sample should compare qualitatively with that of the authentic sample.

§ 436.212 Disintegration test.

(a) *Apparatus*—(1) *Basket-rack assembly*. The basket-rack assembly consists of 6 open-ended glass tubes, each 7.75±0.25 centimeters long and having an inside diameter of approximately 21.5 millimeters and a wall approximately 2 millimeters thick; the tubes are held in a vertical position by two plastic plates, each about 9 centimeters in diameter and 6 millimeters in thickness, with six holes, each about 24 millimeters in diameter, equidistant from the center of the plate and equally spaced from one another. Attached by screws to the undersurface of the lower plate is 10-mesh No. 23 (0.025 inch) W. and M. gauge woven stainless steel wire cloth. The glass tubes and the upper plastic plate are secured in position at the top by means of a stainless steel plate, about 9 centimeters in diameter and 1 millimeter in thickness, having six perforations each about 20 millimeters in diameter, which coincide with those of the upper plastic plate and the upper open ends of the glass tubes. A central shaft about 8 centimeters in length, the upper end of which terminates in an eye through which a string or wire may be inserted, is attached to the stainless steel plate. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plastic plates and the steel plate. The design of the basket-rack assembly may be varied somewhat provided the specifications for the glass tubes and the screen mesh size are maintained.

(2) *Disks.* Each tube is provided with a slotted and perforated cylindrical disk 9.5±0.15 millimeters thick and 20.7±0.15 millimeters in diameter. The disk is made of a suitable, transparent plastic material having a specific gravity of between 1.18 and 1.20. Five 2-millimeter holes extend between the ends of the cylinder, one of the holes being through the cylinder axis and the others parallel with it and equally spaced on a 6-millimeter radius from it.

Equally spaced on the sides of the cylinder are four notches that form V-shaped planes perpendicular to the ends of the cylinder. The dimensions of each notch are such that the openings on the bottom of the cylinder are 1.60 millimeters square and those on the top are 9.5 millimeters wide and 2.55 millimeters deep. All surfaces of the disk are smooth.

(3) *Raising and lowering device.* Use a device for raising and lowering the basket in the immersion fluid at a constant rate between 28 and 32 cycles per minute through a distance of not less than 5 centimeters and not more than 6 centimeters.

(b) *Immersion fluids.* During the performance of the tests all immersion fluids are maintained at a temperature of 37°±2° C. by using a thermostatically controlled water bath.

(1) Distilled water.

(2) Simulated gastric fluid: Dissolve 2.0 grams of sodium chloride and 7.0 milliliters of hydrochloric acid in about 500 milliliters of water. Dissolve 3.2 grams of pepsin in this solution and add sufficient water to make 1,000 milliliters. This solution has a pH of about 1.2.

(3) Simulated intestinal fluid: Dissolve 6.8 grams of monobasic potassium phosphate in 250 milliliters of water, mix and add 190 milliliters of 0.2*N* sodium hydroxide and 400 milliliters of water. Add 10.0 grams of pancreatin, mix, and adjust the resulting solution with 0.2*N* sodium hydroxide to a pH of 7.5±0.1. Dilute to 1,000 milliliters.

(c) *Immersion vessel.* Use a suitable vessel, such as a 1-liter beaker.

(d) *Operation.* Add enough immersion fluid to the immersion vessel so that when the basket-rack assembly is placed on the raising and lowering device at the highest point of the upward stroke, the wire mesh remains at least 2.5 centimeters below the surface of the fluid and descends to not less than 2.5 centimeters from the bottom of the immersion vessel.

(e) *Procedure*—(1) *Uncoated or filmcoated tablets.* Place one tablet into each of the six tubes of the basket, add a disk to each tube, and operate the apparatus, using simulated gastric fluid as the immersion fluid. At the end of the time limit specified in the individual section for the particular antibiotic tablet being tested, lift the basket from the fluid and observe the tablets.

(2) *Plain-coated tablets.* Place one tablet in each of the six tubes of the basket, add a disk to each tube, and operate the apparatus, using simulated gastric fluids as the immersion fluid. After 30 minutes, lift the basket from the fluid and observe the tablets. If the tablets have not disintegrated completely, substitute simulated intestinal fluid as the immersion fluid and continue the test for a total period of time (including previous immersion in simulated gastric fluid) equal to the time limit specified in the individual section for the particular antibiotic tablet being tested. Lift the basket and observe the tablets.

(3) *Enteric-coated tablets.* Place one tablet in each of the six tubes of the basket and operate the apparatus, using simulated gastric fluid as the immersion fluid. One hour later, lift the basket from the fluid and observe the tablets. If the tablets show no distinct evidence of dissolution or disintegration, add a disk to each tube and operate the apparatus, using simulated intestinal fluid as the immersion fluid, for a total period of time (including the previous immersion in simulated gastric fluid) equal to the time limit specified in the individual section for the particular antibiotic tablet being tested. Lift the basket and observe the tablets.

(4) *Pastilles.* Place one pastille into each of the six tubes of the basket, add a disk to each tube, and operate the apparatus, using distilled water as the immersion fluid. At the end of the time limit specified in the individual section for the particular antibiotic pastille being tested, lift the basket from the fluid and observe the pastilles.

(5) *Capsules.* Place one capsule into each of the six tubes of the basket, add a disk to each tube, and operate the apparatus, using distilled water as the immersion fluid. At the end of the time limit specified in the individual section for the capsules being tested, lift the basket from the fluid and observe the capsules.

(f) *Evaluation.* Complete disintegration is defined as the state in which any residue of the tablet, pastille, or

capsule (except fragments of the insoluble coating) remaining on the screen is a soft mass having no palpably firm core. The tablets, pastilles, or capsules pass the disintegration test if all of the units tested disintegrate completely under the conditions and time specified in the individual section for the antibiotic tablet, pastille, or capsule being tested. If one or two tablets, pastilles, or capsules fail to disintegrate completely, repeat the test on 12 additional tablets, pastilles, or capsules. The tablets, pastilles, or capsules pass the disintegration test if not less than 16 of the total 18 tested disintegrate completely. Enteric coated tablets fail the disintegration test if they show any distinct evidence of dissolution or disintegration after 1 hour immersion in simulated gastric fluid.

[39 FR 18944, May 30, 1974, as amended at 52 FR 4617, Feb. 13, 1987; 55 FR 19873, May 14, 1990]

§ 436.213 Nonaqueous titrations.

(a) *Equipment*—(1) *Apparatus.* Use a closed system consisting of a suitable titrimeter equipped with a potentiometer, an automatic burette, a chart recorder, and a glass calomel combinatin electrode (with saturated methanolic potassium chloride as the electrolyte).

(2) *Titration vessel.* Use a 100-milliliter tall form beaker without a spout.

(b) *Reagents*—(1) Methyl alcohol, reagent grade, anhydrous.

(2) Dimethylsulfoxide, A.C.S., reagent grade.

(3) Glacial acetic acid, A.C.S., reagent grade.

(4) Lithium methoxide reagent: 0.02*N* lithium methoxide in methyl alcohol, standardized against primary grade benzoic acid.

(5) Perchloric acid reagent: 0.02*N* perchloric acid in glacial acetic acid, standardized against primary grade potassium acid phthalate.

(c) *Preparation of sample solutions.* Select the weight of the sample and the solvent listed for each antibiotic. Transfer the accurately weighed sample to a titration vessel. Add the appropriate solvent, cover, and stir magnetically until the sample is dissolved. Proceed as directed in paragraph (e) of this section, using the procedure or procedures specified in the individual section for each antibiotic.

Antibiotic	Weight in milligrams of sample	Solvent
Amoxicillin-acid titration	100	20 milliliters dimethylsulfoxide and 30 milliliters methyl alcohol.*
Amoxicillin-base titration.	100	50 milliliters glacial acetic acid.
Ampicillin-acid titration	100	20 milliliters dimethylsulfoxide and 30 milliliters methyl alcohol.*
Ampicillin-base titration	100	50 milliliters glacial acetic acid.
Ampicillin sodium-base titration.	50	Do.
Cephaloglycin-base titration.	50	Do.
Cephapirin sodium-base titration.	50	50 milliliters glacial acetic acid.
Cyclacillin-acid titration	100	20 milliliters dimethylsulfoxide and 30 milliliters methyl alcohol.*
Cyclacillin-base titration	100	50 milliliters glacial acetic acid.
Tobramycin-base titration.	30	50 ml glacial acetic acid.

*The methyl alcohol is added after the sample has dissolved in dimethylsulfoxide.

(d) *Blank determination.* Place the same volume of solvent used to prepare the sample solution into a titration vessel and proceed as directed in paragraph (e) of this section, using the procedure or procedures specified in the individual section for each antibiotic.

(e) *Titration procedures*—(1) *Acid titration.* Equilibrate the electrode by soaking it overnight in the solvent used for preparing the sample solution. Start the magnetic stirrer and titrate the sample solution with the lithium methoxide reagent. Record the change in potential of the solution with the addition of the titrant. Determine the number of milliliters of reagent consumed at neutralization (the inflection point of the titration curve). Calculate the antibiotic content as directed in the individual section.

(2) *Base titration.* Proceed as directed in paragraph (e)(1) of this section, except use the perchloric acid reagent as the titrant and calculate the antibiotic

content as directed in the individual section.

[39 FR 18944, May 30, 1974, as amended at 40 FR 22251, Apr. 22, 1975; 40 FR 23725, June 2, 1975; 40 FR 57797, Dec. 12, 1975; 46 FR 2981, Jan. 13, 1981]

§ 436.214 Heat stability.

Store an accurately weighed portion of the sample of approximately 30 milligrams in an unstoppered 50-milliliter Erlenmeyer flask for 4 days in an electric oven at 100° C±1° C. At the end of this period, remove the flask from the oven and allow to cool in a desiccator. Accurately weigh an unheated portion of the original sample of approximately 30 milligrams. Assay both the heated and unheated samples for potency as directed in § 436.204 or § 436.205 of this chapter. Determine the percent loss from the difference in potency between the unheated original sample and the heat-treated sample.

[42 FR 59856, Nov. 22, 1977]

§ 436.215 Dissolution test.

(a) *Equipment.* Use either Apparatus 1 or 2 as described in the United States Pharmacopeia XXI dissolution test.

(b) *Procedure.* For each dosage form listed in the table in this paragraph select the appropriate dissolution medium, rotation rate, sampling time, and apparatus, and proceed as set forth in either Apparatus 1 or 2 methodology of the United States Pharmacopeia XXI dissolution test. Determine the amount of drug substance dissolved by performing the assay described in paragraph (c) of this section. The amount of dissolution medium removed for sampling purposes may be disregarded if the amount removed is not more than 15 milliliters. If more than 15 milliliters is removed, then correct for the volume removed.

Dosage form	Dissolution medium	Rotation rate [1]	Sampling time(s)	Apparatus
Amoxicillin trihydrate and clavulanate potassium chewable tablets..	900 mL distilled water	75	30 min	2
Amoxicillin trihydrate and clavulanate potassium tablets.	.....do	75	.....do	2
Azithromycin capsules.	900 mL 0.10 *M* sodium phosphate buffer, pH 6.0, 0.1 mg/mL trypsin.	100	45 min	2
Bacampicillin hydrochloride tablets.	.....do	75	.....do	2
Cefadroxil hemihydrate capsules.	900 mL distilled water	100	45 min	1
Cefadroxil hemihydrate tablets.	900 mL distilled water	50	30 min	2
Cefixime tablets	900 mL 0.05 *M* potassium phosphate buffer, pH 7.2.	100	45 min	1
Cefpodoxime proxetil tablets	900 mL pH 3.0 glycine buffer	75	30 min	2
Cefprozil tablets.	900 mL purified water	100	45 min	1
Cefuroxime axetil for oral suspension	900 mL Sorenson's Modified Phosphate Buffer, pH 7.0.	50	30 min	2
Cefuroxime axetil tablets.	900 mL 0.07*N* hydrochloric acid	55	15 min. and 45 min	2
Cephalexin hydrochloride monohydrate tablets..	900 mL distilled water	150	45 min	1
Cephradine dihydrate capsules.	900 mL 0.12*N* hydrochloric acid	75	60 min.	2
Clarithromycin tablets.	900 mL 0.10 *M* sodium acetate buffer, pH 5.0.	50	30 min.	2
Doxycycline hyclate tablets.	900 mL distilled water	75	60 min and 90 min	2
Doxycycline monohydrate hydrochloric acid capsules..	900 mL 0.1*N* hydrochloric acid.	75	60 min	2
Erythromycin particles in tablets	900 mL 0.05*M* potassium phosphate buffer, pH 6.8.	75	45 min.	2
Loracarbef capsules.	900 mL distilled water.	50	30 min	2
Oxytetracycline hydrochloride capsules..	900 mL distilled water	75	30 min and 60 min	2
Rifabutin capsules	900 mL 0.01 *N* hydrochloric acid	100	45 min	1
Tetracycline hydrochloride capsules (except 500-mg)..	.....do	75	.....do	2
Tetracycline hydrochloride capsules (500-mg)..	.....do	75	30 min, 60 min, and 90 min	2
Tetracycline hydrochloride tablets.	.....do	75	30 min and 60 min	2
Vancomycin hydrochloride capsules.	900 mL distilled water.	100	45 min	1

[1] Rotation rate of basket or paddle stirring element (revolutions per minute).

(c) *Antibiotic drug content*—(1) *Tetracycline hydrochloride*—(i) *Preparation of working standard solution.* Accurately weigh 20 to 30 milligrams of tetracycline hydrochloride working standard into a suitable-sized volumetric flask. Dissolve and dilute to volume with water. Further dilute an accurately measured portion with distilled water to obtain a known concentration of 0.01 to 0.02 milligram of tetracycline hydrochloride per milliliter.

(ii) *Preparation of sample solutions.* Dilute an accurately measured portion of the sample with sufficient distilled water to obtain a concentration of 0.01 to 0.02 milligram of tetracycline hydrochloride per milliliter (estimated).

(iii) *Procedure.* Using a suitable spectrophotometer and water as the blank, determine the absorbance of each standard and sample solution at the absorbance peak at approximately 276 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(iv) *Calculation.* Determine the total amount of tetracycline hydrochloride dissolved as follows:

$$T = \frac{A_u \times c \times d \times 900^*}{A_s}$$

where:

T=Total milligrams of drug dissolved;
A_u=Absorbance of sample;
c=Concentration of standard in milligrams;
d=Dilution factor of sample filtrate;
A_s=Absorbance of standard.

*If more than 15 mL of dissolution medium is removed, correct for the volume removed.

(2) *Oxytetracycline hydrochloride; preparation of working standard-solution.* (i) Accurately weigh 30 milligrams of oxytetracycline-base working standard into a suitable-sized volumetric flask. Add 5 milliliters of 0.1*N* hydrochloric acid and swirl the flask to dissolve oxytetracycline base. Dilute an accurately measured portion with distilled water to obtain a known concentration of 0.01 to 0.02 milligram of oxytetracycline per milliliter.

(ii) Proceed as directed in paragraphs (c)(1) (ii), (iii), and (iv) of this section except measure the absorbance at the absorption peak at approximately 273 nanometers.

(3) *Doxycycline hyclate.* Proceed as directed in paragraph (c)(1) of this section, except use the doxycycline working standard.

(4) *Bacampicillin hydrochloride.* Use the ampicillin working standard as the standard of comparison and assay for ampicillin content by either of the following methods.

(i) *Iodometric assay.* Proceed as directed in §436.204 of this chapter, except dilute the working standard to a final concentration of 0.3 milligram of ampicillin per milliliter and use the sample solution as it is removed from the dissolution vessel without further dilution.

(ii) *Hydroxylamine colorimetric assay.* Proceed as directed in §442.40(b)(1)(ii) of this chapter, except:

(*a*) *Buffer.* In lieu of the buffer described in §442.40(b)(1)(ii)(*b*)(*2*) of this chapter, use the buffer prepared as follows: Dissolve 200 grams of primary standard tris (hydroxymethyl) aminomethane in sufficient distilled water to make 1 liter. Filter before use.

(*b*) *Preparation of the working standard solution.* Dissolve and dilute an accurately weighed portion of the ampicillin working standard with sufficient distilled water to obtain a final concentration of 0.3 milligram of ampicillin per milliliter;

(*c*) *Sample solution.* Use the sample solution as it is removed from the dissolution vessel without further dilution; and

(*d*) *Calculations.* Determine the total amount of ampicillin dissolved as follows:

$$T = \frac{(A_u)(c)(d)(900^*)}{A_s}$$

where:

T=Total milligrams of ampicillin equivalent dissolved;
A_u=Absorbance of sample;
c=Concentration of working standard solution in milligrams per milliliter;
d=Dilution factor of sample filtrate;
A_s=Absorbance of standard.

*If more than 15 mL of dissolution medium is removed, correct for the volume removed.

(5) *Cephradine dihydrate*—(i) *Preparation of working standard solution.* Accurately weigh approximately 40 milligrams of cephradine working standard

into a suitable-sized volumetric flask. Dissolve and dilute to volume with 0.12*N* hydrochloric acid. Further dilute with a buffer solution (prepared by dissolving 27.2 grams of sodium acetate trihydrate in a mixture of 12 milliliters of glacial acetic acid and sufficient distilled water to make 2 liters) to obtain a known concentration of 0.01 to 0.03 milligram of cephradine per milliliter.

(ii) *Preparation of sample solution.* Filter the sample and dilute an accurately measured portion of the filtrate with sufficient buffer solution, described in paragraph (c)(5)(i) of this section, to obtain a concentration of 0.01 to 0.03 milligram of cephradine per milliliter (estimated).

(iii) Proceed as directed in paragraphs (c)(1) (iii) and (iv) of this section, except measure the absorbance at the absorption peak at approximately 262 nanometers.

(6) *Amoxicillin trihydrate.* Assay for the amoxicillin content as described in § 440.103d of this chapter, except use the sample as it is removed from the dissolution vessel.

(7) *Vancomycin hydrochloride.* Assay for the vancomycin content as described in § 436.105 of this chapter, except use the sample as it is removed from the dissolution test.

(8) *Erythromycin*—(i) *Preparation of working standard solution.* Accurately weigh approximately 140 milligrams of erythromycin working standard into a 250-milliliter volumetric flask and dissolve in 10 milliliters of methyl alcohol. Add water nearly to volume, mix, and allow the solution to cool. Dilute to volume with water and mix. On the day of use, dilute an accurately measured aliquot with water to obtain a known concentration of 0.28 milligram of erythromycin per milliliter (before adjusting for standard potency).

(ii) *Preparation of sample solution.* Dilute an accurately measured portion of the filtered sample with sufficient 0.05*M* potassium phosphate buffer, pH 6.8, to obtain a concentration of about 0.28 milligram of erythromycin per milliliter (estimated).

(iii) *Procedure.* Transfer 5.0-milliliter aliquots of the working standard solution and sample solution to 25-milliliter volumetric flasks and treat as follows: Add 2.0 milliliters of water, allow to stand for 5 minutes with intermittent swirling. Add 15.0 milliliters of 0.25*N* sodium hydroxide, dilute to volume with sufficient 0.05*M* potassium phosphate buffer, pH 6.8, and mix. Heat to 60 °C for 5 minutes and allow to cool. Using a suitable spectrophotometer and a blank (prepared as per the procedure above except that 2.0 milliliters of 0.5N sulfuric acid is substituted for the 2.0 milliliters of water) for each solution, determine the absorbance of each working standard and sample solution at the absorbance peak at approximately 236 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(iv) *Calculation.* Proceed as directed in paragraph (c)(1)(iv) of this section.

(9) *Cefuroxime axetil tablets and powder for oral suspension*—(i) *Preparation of working standard solution*—(*a*) *Cefuroxime axetil tablets.* Accurately weigh approximately 60 milligrams of cefuroxime axetil working standard into a suitable-sized volumetric flask. Dissolve in 5 milliliters of methanol and dilute to volume with 0.07*N* hydrochloric acid to obtain a known concentration equivalent to 0.01 to 0.02 milligram of cefuroxime activity per milliliter.

(*b*) *Cefuroxime axetil for oral suspension.* Accurately weigh approximately 15 milligrams of cefuroxime axetil working standard into a 100-milliliter volumetric flask. Dissolve in 5 milliliters of methanol and dilute to volume with Sorenson's Modified Phosphate Buffer, pH 7.0 (4.2 grams of sodium dihydrogen orthophosphate dihydrate and 14.3 grams of hydrogen disodium orthophosphate dodecahydrate per liter of water).

(ii) *Preparation of sample solution*—(*a*) *Cefuroxime axetil tablets.* Filter through a 0.45-micron filter and dilute an accurately measured portion of the filtrate with sufficient 0.07*N* hydrochloric acid to obtain a concentration equivalent to 0.01 to 0.02 milligram of cefuroxime activity per milliliter (estimated).

(*b*) *Cefuroxime axetil for oral suspension.* Filter the sample through an 8-micron filter. A coarse prefilter may be used to prevent clogging. Use the filtrate solution without further dilution.

(iii) *Procedure*—(*a*) *Cefuroxime axetil tablets.* Using a suitable spectrophotometer and 0.07*N* hydrochloric acid as the blank, determine the absorbance of each standard and sample solution at the absorbance peak at approximately 280 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(*b*) *Cefuroxime axetil for oral suspension.* Using a suitable spectrophotometer and Sorenson's Modified Phosphate Buffer, pH 7.0 (4.2 grams of sodium dihydrogen orthophosphate dihydrate and 14.3 grams of hydrogen disodium orthophosphate dodecahydrate per liter of water) as the blank, determine the absorbance of each standard and sample solution at the absorbance peak at approximately 280 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(iv) *Calculations.* Determine the total amount of cefuroxime activity dissolved as follows:

$$T = \frac{A_u \times c \times d \times 900}{A_s}$$

where:

T = Total milligrams of cefuroxime activity dissolved;

A_U = Absorbance of sample;

c = Cefuroxime activity of working standard solution in milligrams per milliliter;

d = Dilution factor of sample filtrate; and

A_s = Absorbance of standard.

(10) *Cefixime*—(i) *Preparation of working standard solution.* Accurately weigh approximately 25 milligrams of cefixime working standard into a 500-milliliter volumetic flask. Wet the powder with 0.5 milliliters of methanol, and dilute to volume with 0.05 *M* potassium phosphate buffer, pH 7.2 (prepared by dissolving 6.8 grams of monobasic potassium phosphate in distilled water to a volume of one liter. The pH is adjusted to 7.2 with 1.0*N* NaOH). Sonicate to assure dissolution and mix.

(ii) *Preparation of sample solution.* Forty-five minutes after the beginning of the rotation, withdraw and filter a portion of the solution. For the 400-milligram tablets, pipet 10.0 milliliters of the filtered sample solution into a 100-milliliter volumetric flask. For the 200-milligram tablets, pipet 10.0 milliliters of the filtered sample into a 50-milliliter volumetric flask. Dilute to volume with 0.05 *M* postassium phosphate buffer, pH 7.2.

(iii) *Procedure.* Proceed as directed in paragraphs (c)(1) (iii) and (iv) of this section, except measure the absorbance of the peak at approximately 320 nanometers using 0.05 *M* potassium phosphate buffer, pH 7.2 as the blank.

(11) *Cephalexin hydrochloride monohydrate.* Assay for cephalexin activity of the cephalexin hydrochloride monohydrate as directed in § 442.28 of this chapter, and use U.S.P. dissolution apparatus 1 (10 mesh basket). Use the sample as it is removed from the dissolution vessel.

(12) *Doxycycline monohydrate.* Proceed as directed in paragraph (c)(1) of this section, except use the doxycycline standard.

(13) *Clarithromycin.* Proceed as directed in § 452.50(b)(1) of this chapter except:

(i) *Dissolution medium.* Instead of the mobile phase described in § 452.50(b)(1)(i) of this chapter, use 0.10 *M* sodium acetate buffer prepared as follows: Weigh 13.6 grams of sodium acetate trihyrate into a container sufficient to hold 1 liter of solution. Dissolve the salt in 750 milliliters of distilled water. Adjust the pH of the solution to 5.0±0.05 with glacial acetic acid. Dilute to 1,000 milliliters with distilled water.

(ii) *Preparation of the standard and sample solutions*—(*a*) *Standard solution.* Dissolve (with shaking or sonication) an accurately weighed portion of the clarithromycin working standard, in sufficient methanol to obtain a solution having a known concentration of approximately 625 micrograms per milliliter of clarithromycin. Quantitatively transfer and dilute an aliquot of this solution with mobile phase (described in § 452.50(b)(1)(i) of this chapter) and mix to obtain a solution of known concentration of approximately 125 micrograms per milliliter of clarithromycin.

(*b*) *Sample solution.* Use the sample solution as it is removed from the dissolution vessel after diluting and mixing with mobile phase (described in § 452.50(b)(1)(i) of this chapter) 1:2 for

the 250-milligram tablet and 1:4 for the 500-milligram tablet.

(*c*) *Calculations.* Determine the total amount of clarithromycin activity dissolved as follows:

$$T = \frac{A_U \times c \times d \times 900}{A_s}$$

where:

T = Total milligrams of clarithromycin activity dissolved;

A_U = Area of the clarithromycin peak (at a retention time equal to that observed for the standard) in the chromatogram of the sample;

A_S = Area of the clarithromycin peak in the chromatogram of the clarithromycin standard;

c = Clarithromycin activity in the clarithromycin working standard solution in milligrams per milliliter; and

d = Dilution factor of sample filtrate.

(14) *Azithromycin.* Proceed as directed in § 452.60(b)(1) of this chapter, except:

(i) *Dissolution medium.* Dissolve 85.2 grams of sodium phosphate dibasic and dilute to volume with ultrapure deionized or high-performance liquid chromatographic-grade water in a stoppered 2-liter graduated cylinder. Dilute this entire solution in an appropriate, suitably sized container with 4 liters of ultrapure deionized or high-performance liquid chromatographic-grade water. Adjust the pH to 6.0±0.05 with concentrated hydrochloric acid (about 40.5 milliliters). Add 600 milligrams of trypsin and mix well.

(ii) *Preparation of the standard and sample solutions*—(*a*) *Standard solution.* Accurately weigh approximately 15 milligrams of the azithromycin working standard into a 50-milliliter volumetric flask. Add 25 milliliters of the dissolution medium and sonicate briefly. Dilute to volume with dissolution medium and mix well. Pipet 2.0 milliliters of this solution into a 25-milliliter volumetric flask and dilute to volume with the mobile phase described in § 452.60(b)(1)(i) of this chapter. Pipet 4.0 milliliters of this solution into a 25-milliliter volumetric flask and bring to volume with the mobile phase.

(*b*) *Sample solution.* Filter the sample solutions through a 0.45-micron filter before use. Pipet 2.0 milliliters of the filtered aliquot into a 25-milliliter volumetric flask and dilute to volume with the mobile phase described in § 452.60(b)(1)(i) of this chapter. Pipet 4.0 milliliters of this solution into another 25-milliliter volumetric flask and bring to volume with the mobile phase. The solution is stable at room temperature for 24 hours.

(*c*) *Calculations.* Determine the percent of azithromycin dissolved as follows:

$$\text{Percent azithromycin dissolved} = \frac{A_U \times P_s \times D_F \times 100}{A_s \times W_u}$$

where:

A_U = Area of the azithromycin peak (at a retention time equal to that observed for the standard) in the chromatogram of the sample;

A_S = Area of the azithromycin peak in the chromatogram of the azithromycin standard;

P_S = Azithromycin activity in the azithromycin working standard solution in micrograms per milliliter;

$$DF = \text{Dilution factor} = \frac{900^{1} \times 25 \times 25}{2 \times 4}$$

[1] If more than 15 milliliters of dissolution medium are removed, correct for the volume removed; and

W_U = Theoretical azithromycin content (mg) of capsule.

(15) *Cefprozil.* Proceed as directed in § 442.80(b)(1) of this chapter except:

(i) *Sample solutions.* Filter the sample solutions through a 0.45-micron filter before use. Use the sample solution as it is removed from the dissolution vessel without further dilution for the 250-milligram tablet; prepare the sample solution for the 500-milligram tablet by diluting a 5-milliliter aliquot of the filtered solution to volume in a 10-milliliter volumetric flask with distilled water.

(ii) *Calculations.* Determine the total percent of cefprozil dissolved as follows:

$$\text{Total percentage dissolved} = \frac{(\text{mg cefprozil (Z) dissolved} + \text{mg cefprozil (E) dissolved})}{\text{label claim}}$$

$$\text{Milligrams of cefprozil (Z) or cefprozil (E) dissolved} = \frac{A_U \times c \times d \times 900}{A_s}$$

where:
A_U = Area of the cefprozil (Z) or cefprozil (E) response in the chromatogram of the sample (at a retention time equal to that observed for the standard);
A_S = Area of the cefprozil (Z) or cefprozil (E) response in the chromatogram of the cefprozil (Z) or cefprozil (E) standard;
c = Concentration of the cefprozil (Z) or cefprozil (E) working standard solution in milligrams per milliliter; and
d = Dilution factor of the sample filtrate.

(16) *Loracarbef*—(i) *Preparation of the working standard solution.* Accurately weigh approximately 110 milligrams of the loracarbef working standard into a suitable-sized volumetric flask. Dissolve and dilute to volume with water. Further dilute an accurately measured portion with distilled water to obtain a known concentration of 0.02 milligram of loracarbef activity per milliliter.

(ii) *Preparation of sample solutions.* Dilute an accurately measured portion of the sample with sufficient distilled water to obtain a concentration of 0.02 milligram of loracarbef activity per milliliter (estimated).

(iii) *Procedure.* Using a suitable spectrophotometer and water as the blank, determine the absorbance of each standard and sample solution at the absorbance maximum at approximately 260 nanometers. Determine the exact position of the absorbance maximum for the particular instrument used.

(iv) *Calculations.* Determine the total amount of loracarbef dissolved as follows:

$$T = \frac{A_U \times c \times d \times 900}{A_s}$$

where:
T = Total milligrams of loracarbef activity dissolved;
A_U = Absorbance of sample;
A_S = Absorbance of the standard;
c = Concentration of the working standard solution in milligrams per milliliter; and
d = Dilution factor of the sample filtrate.

(17) *Cefadroxil hemihydrate.* Proceed as directed in paragraph (c)(1) of this section, except use the cefadroxil working standard and measure the absorbance at the absorption peak of approximately 264 nanometers.

(18) *Rifabutin*—(i) *Preparation of the working standard solution.* Accurately weigh approximately 45 milligrams of the rifabutin working standard into a suitable-sized volumetric flask. Dissolve and dilute to volume with 0.01*N* hydrochloric acid (prepared by diluting 5.0 milliliters of hydrochloric acid (37 percent) to 6 liters with distilled water) to obtain a concentration of approximately 13 micrograms rifabutin activity per milliliter.

(ii) *Preparation of sample solutions.* Forty-five minutes after the beginning of the rotation, withdraw a 10-milliliter aliquot from the vessel. Dilute a 2-milliliter portion of the sample to 25 milliliters with 0.01*N* hydrochloric acid.

(iii) *Procedure.* Using a suitable spectrophotometer and 0.01*N* hydrochloric acid as the blank, determine the absorbance of each standard and sample solution at the absorbance maximum at approximately 280 nanometers. Determine the exact position of the absorbance maximum for the particular instrument used.

(iv) *Calculations.* Determine the total amount of rifabutin dissolved as follows:

$$T = \frac{A_U \times c \times d \times 900}{A_s \times 1{,}000}$$

where:
T = Total milligrams of rifabutin activity dissolved;
A_U = Absorbance of sample;
A_S = Absorbance of the standard;
c = Rifabutin activity of the working standard solution in micrograms per milliliter; and
d = Dilution factor of the sample filtrate.

(19) *Cefpodoxime proxetil*—(i) *Dissolution fluid: 0.04 molar glycine buffer, pH 3.0*—(A) *Stock solution.* Dissolve 54.5 grams of glycine (aminoacetic acid) and 42.6 grams of sodium chloride in about 500 milliliters of deionized water in a 1-liter volumetric flask. Add cautiously, and with swirling, 14.2 milliliters of concentrated hydrochloric acid. Cool to room temperature. Dilute to volume with deionized water and mix. Check the pH of the solution obtained by diluting 50 milliliters of the stock solution to 900 milliliters with deionized water. The pH should be 3.0±0.1. If necessary, adjust the pH of the stock solution with 50 percent sodium hydroxide or concentrated hydrochloric acid. Recheck that the pH of the working solution is 3.0±0.1.

(B) *Working solution.* Dilute 50 milliliters of stock solution to 900 milliliters with deionized water.

(ii) *Preparation of the working standard solutions.* Accurately weigh approximately 28 milligrams for the 100-milligram tablets and 56 milligrams for the 200-milligram tablets of the cefpodoxime proxetil working standard and dissolve in 10 milliliters of methanol. Dilute to 200 milliliters with dissolution fluid. Prepare fresh daily.

(iii) *Sample solutions.* Filter the sample solutions through a 0.45-micron filter before use. Use the sample solution as it is removed from the dissolution vessel without further dilution.

(iv) *Procedure.* Using a suitable spectrophotometer and water as the blank, determine the absorbance of each standard and sample solution at the absorbance peak at approximately 259 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(v) *Calculations.* Determine the percent of label dissolved as follows:

$$\text{Percent dissolved} = (A_{sam}/A_{std}) \times (C_s/L) \times V \times P \times F1$$

where:

A_{sam} = Absorbance of the sample at 259 nanometers;

A_{std} = Absorbance of the working standard solution at 259 nanometers;

C_s = Concentration of the working standard preparation in milligrams per milliliter;

L = Tablet strength, in milligrams per tablet;

P = Purity of the reference standard in percent;

V = Volume of dissolution fluid used in milliliters (900); and

$F1$ = 0.7666 (conversion factor to free acid equivalents).

(d) *Evaluation.* Use the dissolution acceptance table and interpretation in the United States Pharmacopeia XXI.

[44 FR 48188, Aug. 17, 1979]

EDITORIAL NOTE: For FEDERAL REGISTER citations affecting § 430.215, see the List of CFR Sections Affected appearing in the Finding Aids section of this volume.

§ 436.216 High-performance liquid chromatographic assay.

(a) *Equipment.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, flow rate, and calculations specified in the monograph for the drug being tested. Use a detector sensitivity setting that gives a peak height for the working standard solution that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the equipment described in paragraph (a) of this section. Use the reagents, working standard solution, and sample solution described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed

system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Select the system suitability requirements specified in the monograph for the drug being tested. Then, using the equipment and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Trailing factor or asymmetry factor.* Calculate either the trailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline or the asymmetry factor (A_s) measured at a point 10 percent of the peak height from the baseline; whichever is required in the appropriate monograph, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$=Width of peak at 5 percent height; and
f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

$$A_s = \frac{a+b}{2_a}$$

where:
a=Horizontal distance from point of ascent to point of maximum peak height; and
b=Horizontal distance from the point of maximum peak height to point of descent.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left[\frac{t_R}{W_h}\right]$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of solute; and
W_h=Peak width at half-height.
Calculate the absolute efficiency of the column, (reduced plate height) (h_r

$$h_r = \frac{(L)(10{,}000)}{(n)(d_p)}$$

where:
L=Length of column in centimeters;
n=Number of theoretical plates; and
d_p

(3) *Resolution.* Calculate the resolution (R) as follows:

$$R = \frac{2\left(t_{Rj} - t_{Ri}\right)}{w_i + w_j}$$

where:
t_{Rj}=Retention time of a solute eluting after i (t_{Rj} is larger than $t_{Ri\geq}$)
t_{Ri}=Retention time for any solute;
w_i=Width of peak at baseline for any solute; and
w_j=Width of peak at baseline for any solute eluting after i.

(4) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left(\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right)^{1/2}$$

where:
$\overline{X}$ is the mean of N of individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, except alternate injections of the working standard solution with injections of the sample solution.

(5) *Capacity factor.* Calculate the capacity factor (k), if required in the monograph as follows:

$$k = \frac{t_r - t_m}{t_m}$$

where:
t_r=Retention time of solute; and
t_m=Retention time of solvent or unretained substance, calculated as follows:

$$t_m = \frac{(3.1416)(D^2)(L)(0.75)}{4F}$$

where:
D=Column diameter in centimeters;
L=Column length in centimeters;
0.75=Average total column porosity; and
F=Flow rate in milliliters per minute.

[51 FR 11572, Apr. 4, 1986, as amended at 54 FR 47351, Nov. 14, 1989]

§ 436.217 Film-coat rupture test.

(a) *Immersion fluid.* Dilute 6.0 milliliters of hydrochloric acid to 1,000 milliliters with water. During the performance of the test maintain the immersion fluid at a temperature of 37±0.5 °C by using a thermostatically controlled water bath.

(b) *Immersion vessel.* Use a suitable vessel, such as a 1-liter beaker.

(c) *Operation.* Add 750 milliliters of immersion fluid to the immersion vessel.

(d) *Procedure.* Drop a tablet into the immersion fluid and record the time for the tablet coat to rupture. Repeat the test with a further 19 tablets, testing not more than 10 tablets with a given volume of immersion fluid.

(e) *Evaluation.* The tablets pass the film-coat rupture test if the mean coat rupture time does not exceed 20 seconds and not more than 2 tablets have a coat rupture time exceeding 40 seconds.

[52 FR 42432, Nov. 5, 1987]

Subpart F—Chemical Tests for Specific Antibiotics

§ 436.300 Polarimetric assay of carbenicillin indanyl sodium.

(a) *Equipment.* Polarimeter capable of measuring optical rotatory activity at 365 nanometers: Perkin-Elmer Model 141 or equivalent, with a suitable 1-decimeter polarimeter tube.

(b) *Reagents*—(1) *4-methyl-2-pentanone.* Meets ACS specifications.

(2) *Phosphate-citrate buffer.* Dissolve 61.0 grams of anhydrous disodium phosphate and 11.0 grams of citric acid in 950 milliliters of distilled water. Adjust the pH to 6.0 with 6*N* hydrochloric acid. Dilute to 1,000 milliliters with distilled water.

(c) *Preparation of carbenicillin indanyl sodium sample and working standard solutions.* Accurately weigh approximately 125 milligrams of the carbenicillin indanyl sodium sample or working standard into a 25-milliliter volumetric flask. Dissolve and dilute to volume with distilled water. Transfer a 5-milliliter aliquot to a 50-milliliter glass-stoppered centrifuge tube. Add 15 milliliters of the phosphate-citrate buffer and 20 milliliters of 4-methyl-2-pentanone; stopper and shake the tube for 10 seconds. Centrifuge at 2,000 revolutions per minute for 10 minutes to separate the phases. Remove about 15 milliliters of the upper (4-methyl-2-pentanone solvent) phase and proceed as directed in paragraph (e) of this section.

(d) *Preparation of the blank.* Place a 5-milliliter aliquot of distilled water into a 50-milliliter glass-stoppered centrifuge tube, add 15 milliliters of phosphate-citrate buffer and 20 milliliters of 4-methyl-2-pentanone; stopper and shake the tube for 10 seconds. Centrifuge at 2,000 revolutions per minute for 10 minutes to separate the phases. Remove about 15 milliliters of the upper phase and proceed as directed in paragraph (e) of this section.

(e) *Procedure.* Fill the polarimeter tube with the blank solution prepared as described in paragraph (d) of this section. Place the tube in the polarimeter. Adjust the polarimeter to zero rotation using a light source with a wavelength of 365 nanometers. Use the same procedure to determine the optical rotation of both the sample solution and the working standard solution prepared as directed in paragraph (c) of this section.

(f) *Calculations.* Calculate the carbenicillin content (potency) of the sample on an anhydrous basis as follows:

$$\text{Micrograms of carbenicillin per milligram of sample} = \frac{\text{Degrees of rotation of sample solution} \times \text{weight of working standard} \times 100 \times \text{micrograms of carbenicillin in each milligram of the working standard}}{\text{Degrees of rotation of working standard solution} \times \text{weight of sample} \times (100 - m)}$$

where: m=moisture content of the sample.

§ 436.301 Thin layer chromatography identity test for carbenicillin indanyl.

Using the sample solution prepared as described in the section for the antibiotic drug to be tested, proceed as described in paragraphs (a), (b), (c), and (d) of this section.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 9 × 9 × 3.5 inches lined with Whatman's 3MM chromatographic paper (0.3 millimeters) or equivalent.

(2) *Iodine vapor chamber.* A rectangular tank approximately 9 × 9 × 3.5 inches, with a suitable cover, containing iodine crystals.

(3) *Plates.* Use 20 × 20 centimeters thin layer chromatography plates coated with silica gel G or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Extraction solvent.* Mix ethyl acetate, acetone, pyridine, water, and acetic acid in volumetric proportions of 100:200:25:75:1.5 respectively.

(2) *Developing solvent.* Mix ethyl acetate, acetone, pyridine, water, and acetic acid in volumetric proportions of 300:400:25:75:2 respectively.

(3) *Ferric chloride-potassium ferricyanide reagent.* Immediately before use, mix 100 milliliters of a 1 percent ferric chloride solution in 1 percent hydrochloric acid with 100 milliliters of a 1 percent potassium ferricyanide solution and 75 milliliters of methanol.

(c) *Preparation of working standard solution.* Weigh an amount of the carbenicillin indanyl working standard equivalent to approximately 10 milligrams of carbenicillin into a 50-milliliter Erlenmeyer flask. Dissolve the material in sufficient extraction solvent to make a solution containing 1 milligram carbenicillin per milliliter.

(d) *Procedure.* Pour developing solvent into the bottom of the chromatography tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Prepare a plate as follows: On a line 2 centimeters from the base of the silica gel plate, and at intervals of 2 centimeters, spot 10 microliters of the standard solution and the sample solution. The plate should be air dried for 30 minutes. Place the plate into the chromatography tank. Allow the solvent front to travel about 15 centimeters from the starting line and then remove the plate from the tank. Heat the plate for 30 minutes at 80° C. in a circulating air oven and then allow the plate to cool to room temperature. Place the plate in the iodine vapor chamber for about 30 seconds, remove the plate and spray it with the ferric chloride-potassium ferricyanide reagent. Carbenicillin indanyl appears as a blue spot on a yellow-green background at an R_f of about 0.5. The test is satisfactory if the sample compares qualitatively with the standard.

[39 FR 18944, May 30, 1974, as amended at 41 FR 18509, May 5, 1976]

§ 436.302 Clindamycin vapor phase chromatography.

(a) *Equipment.* Gas chromatograph equipped with a flame ionization detector: Barber-Colman 5,000 or equivalent.

(b) *Reagents.* (1) Pyridine, reagent grade, dried over sodium sulfate.

(2) Chloroform, reagent grade.

(3) Acetic anhydride, reagent grade, used as acteylating agent.

(4) Internal standard: Prepare a solution containing 3 milligrams of cholestane per milliliter in pyridine.

(c) *Typical conditions.* (1) Column: 4 feet × 4 millimeters ID, glass, with 1 percent SE–30 on Diatoport S (60/80 mesh), or equivalent.

(2) Temperatures: Column 200° C.; detector 215° C.; injection port, ambient temperature.

(3) Carrier gas: Helium approximately 120 milliliters per minute.

(4) Detector: Hydrogen flame—hydrogen at 120 pounds per square inch, air at 40 pounds per square inch.

(5) Sensitivity: 1,000; attenuation, 2 for clindamycin, 1 for internal standard: 2×10^{-8} amperes.

(d) *Preparation of clindamycin sample and working standard solutions.* Accurately weigh approximately 15 milligrams of sample or working standard into a glass-stoppered conical 15-milliliter centrifuge tube. Add 1.0 milliliter of chloroform, 1.0 milliliter of internal standard solution, and 0.6 milliliter of acetic anhydride. Agitate the tubes to insure dissolution of the sample and

complete mixing of the liquids. Proceed as directed in paragraph (e) of this section.

(e) *Procedure.* Cover the top of each centrifuge tube with a plastic cap. Punch a small hole in the top of each cap to allow vapor to escape. Place the tubes in a 100° C. drying oven for 2.5 hours. Remove the tubes from the oven and allow to cool. Take the plastic cap from each tube and replace with the glass stopper. Centrifuge 10–15 minutes at 2,000–2,500 r.p.m. to separate the white solid from the liquid in the tube. Inject 0.5 microliter of the clear liquid into the gas chromatograph. Use the conditions and materials listed in paragraphs (a), (b), and (c) of this section. The conditions should be adequate to maintain a stable baseline and provide at least 60 percent deflection of the recorder scale by the clindamycin peak. The resolution of the peaks should be complete. The elution order is: Internal standard, clindamycin, and epiclindamycin (if present). Calculate the clindamycin content as directed in paragraph (f) of this section.

(f) *Calculations.* Calculate the clindamycin content of the sample as follows:

$$\text{Micrograms of clindamycin per milligram} = \frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of the clindamycin sample peak (at a retention time equal to that observed for the clindamycin standard)/ Area of internal standard peak;

R_s=Area of the clindamycin standard peak/ Area of internal standard peak;

W_s=Weight of the clindamycin working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Potency of the clindamycin working standard in micrograms per milligram.

§ 436.303 Clindamycin content of clindamycin palmitate hydrochloride by vapor phase chromatography.

(a) *Equipment.* Gas chromatograph equipped with a flame ionization detector: Hewlett-Packard 7606[4] or equivalent.

(b) *Reagents.* (1) Acetic anhydride, reagent grade.

(2) Pyridine, reagent grade.

(3) Chloroform, reagent grade.

(4) Internal standard: Prepare a solution containing 5 milligrams of cholesteryl benzoate per milliliter in chloroform.

(c) *Typical conditions.* (1) Column: 6 feet × 2 millimeters ID, glass, with 1 percent UC–W98 on Chromosorb WHP (80/100 mesh) or equivalent.

(2) Temperatures: Column 275° C.; detector 290° C.; injection port 280° C.

(3) Carrier gas: Helium approximately 60 milliliters per minute.

(4) Detector: Hydrogen flame ionization—hydrogen at 12 pounds per square inch, air at 32 pounds per square inch.

(5) Sensitivity: 1,000; attenuation, 16; 1×10^{-9} amperes.

(d) *Preparation of clindamycin palmitate hydrochloride sample and working standard solutions.* Accurately weigh approximately 15 milligrams of both the sample and the working standard into separate glass-stoppered, conical 15-milliliter centrifuge tubes. Add 1.0 milliliter of internal standard solution, 1.0 milliliter of pyridine, and 0.5 milliliter of acetic anhydride to each tube. Agitate the tubes to insure dissolution and complete mixing of the liquids. Proceed as directed in paragraph (e) of this section.

(e) *Procedure.* Cover the top of each centrifuge tube with a plastic cap. Punch a small hole in the top of each cap to allow vapor to escape. Place the tubes in a 100° C. drying oven for 2.5 hours. Remove the tubes from the oven and allow to cool. Take the plastic cap from each tube and replace with the glass stopper. Centrifuge 10–15 minutes at 2,000–2,500 r.p.m. to separate the white solid from the liquid in the tube. Inject 1 microliter of the clear liquid into the gas chromatograph. Use the conditions and materials listed in paragraphs (a), (b), and (c) of this section. The conditions should be adequate to maintain a stable baseline and provide at least 40 percent deflection of the recorder scale by the clindamycin palmitate peak. The resolution of the peaks should be complete. The internal standard will be eluted before the clindamycin palmitate. Calculate the clindamycin content as directed in paragraph (f) of this section.

[4] Available from: Hewlett Packard Co., P.O. Box 301, Loveland, CO 80537.

(f) *Calculations.* Calculate the clindamycin content of the sample as follows:

$$\text{Micrograms of clindamycin per milligram} = \frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of the sample peak (at a retention time equal to that observed for the clindamycin palmitate hydrochloride standard)/Area of internal standard peak;

R_s=Area of the clindamycin palmitate hydrochloride standard peak/Area of internal standard peak;

W_s=Weight of the clindamycin palmitate hydrochloride working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Micrograms of clindamycin activity per milligram of clindamycin palmitate hydrochloride working standard.

§ 436.304 Clindamycin phosphate vapor phase chromatography.

(a) *Equipment.* Gas chromatograph equipped with an electronic integrator and with a flame ionization detector that has a sensitivity of at least 1×10^{-10} amperes: Hewlett-Packard 7600[4] or equivalent.

(b) *Reagents.* (1) Trifluoroacetic anhydride.

(2) Intestinal alkaline phosphatase.

(3) pH 9.0 borate buffer: Transfer 3.1 grams of boric acid into a 1-liter volumetric flask containing 500 milliliters of water, mix, and add 21 milliliters of 1.0*N* sodium hydroxide and 10 milliliters of 0.1*M* magnesium chloride. Dilute to volume with water and mix well.

(4) Internal standard: Prepare a chloroform solution containing approximately 0.45 milligram hexacosane per milliliter.

(5) Anhydrous sodium carbonate.

(c) *Typical conditions.* (1) Column: 2 feet × 3 millimeters ID, glass, with 1 percent SE–30 on Diatoport S (80/100 mesh), or equivalent.

(2) Temperatures: Column, 180° C., detector, 215° C., injection port, ambient temperature.

(3) Carrier gas: Helium approximately 60 milliliters per minute.

(4) Detector: Hydrogen flame—hydrogen flow at 40 milliliters per minute. Air flow at 400 milliliters per minute.

(5) Sensitivity: 1×10^{-9} amperes.

(d) *Preparation of clindamycin phosphate sample solution.* Accurately weigh approximately 12 milligrams of the clindamycin phosphate sample into a 50-milliliter glass-stoppered centrifuge tube. Pipet 25 milliliters of the pH 9.0 borate buffer into the centrifuge tube. Add 10 milliliters chloroform and shake vigorously for 15 minutes. Centrifuge the resulting mixture and pipet a 20-milliliter aliquot of the aqueous phase into a 35-milliliter centrifuge tube. Add a weighed amount of intestinal alkaline phosphatase equivalent to 50 units of activity[5] and allow the solution to stand until the enzyme has completely dissolved. Place the tube into a water bath at 37° C.±2° C. for 2.5 hours. After the 2.5-hour hydrolysis, allow the solution to cool and proceed as directed in paragraph (f) of this section.

(e) *Preparation of the clindamycin hydrochloride standard solution.* Accurately weigh approximately 9 milligrams of the clindamycin hydrochloride working standard into a 35-milliliter glass-stoppered centrifuge tube and dissolve in 20 milliliters of pH 9.0 borate buffer. Proceed as directed in paragraph (f) of this section.

(f) *Procedure.* Add 10 milliliters of the internal standard solution to each sample and standard solution. Shake the centrifuge tubes vigorously for 30 minutes and centrifuge. Remove the aqueous layer and discard. Shake the tubes again; mix in an ultrasonic mixer for 2 minutes, then centrifuge. No emulsion should be present at this stage. Remove the remaining aqueous layer by suction and transfer a 3-milliliter aliquot of the chloroform layer to a 1-dram tablet vial containing approximately 1 gram of anhydrous sodium sulfate. Swirl the vial to dry the chloroform and transfer a 1-milliliter aliquot to another 1-dram tablet vial. Using a 0.25-milliliter pipet, add 0.25 milliliter of trifluoracetic anhydride to

[4] See footnote 4 to § 436.303(a).

[5] Defined such that 50 units hydrolyzes at least 20 micromoles of a clindamycin phosphate authentic sample under the assay conditions described in this section.

each of the vials and place into a water bath at 45° C.±2° C. for 30 minutes. Remove the vials from the bath, add about 10 granules of anhydrous sodium carbonate to each vial, and allow to stand for approximately 30 minutes. Centrifuge the vials for approximately 10 minutes at 5,000 r.p.m. Inject 2 microliters of each of the resulting solutions into the gas chromatograph. Use the conditions and materials listed in paragraphs (a), (b), and (c) of this section. The elution order is: Epiclindamycin (if present), clindamycin B (if present), clindamycin, and internal standard. Calculate the clindamycin content as directed in paragraph (g) of this section.

(g) *Calculations.* Calculate the clindamycin content of the sample as follows:

Micrograms of clindamycin per milligram=

$$\frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of the clindamycin sample peak (at a retention time equal to that observed for the clindamycin standard)/ Area of internal standard peak;

R_u=Area of the clindamycin standard peak/ Area of internal standard peak;

W_s=Weight of the clindamycin working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Potency of the clindamycin working standard in micrograms per milligram.

[39 FR 18944, May 30, 1974, as amended at 41 FR 24704, June 18, 1976]

§ 436.305 Thin layer chromatographic identity test for hetacillin.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 9 × 9 × 3.5 inches with a glass solvent trough on the bottom.

(2) *Plates.* Use 20 × 20 centimeter thin layer chromatography plates coated with Silica Gel G or equivalent to a thickness of 250 microns.

(b) *Developing solvent.* Mix 650 milliliters acetone with 100 milliliters distilled water, 100 milliliters benzene, and 25 milliliters acetic acid.

(c) *Spray solution.* Dissolve 300 milligrams of ninhydrin in 100 milliliters of ethanol.

(d) *Preparation of spotting solutions*—(1) *Sample solution.* Use the sample solution prepared as described in the section for the particular product to be tested.

(2) *Reference solutions.* Prepare a solution containing 10 milligrams of an authentic hetacillin sample per milliliter in a 4:1 solution of acetone and 0.1*N* hydrochloric acid, and a solution of ampicillin standard at 1 mg/ml in the same solvent.

(e) *Procedure.* Spot a plate as follows: Apply approximately 10 microliters of the sample solution, 1 µl, of the reference hetacillin solution, and 1 µl, of the ampicillin reference solution on a line 1.5 centimeters from the base of the silica gel plate and at intervals of not less than 2.0 centimeters. Pour developing solvent into the glass trough in the bottom of the chromatography tank. After all spots are thoroughly dry, place the silica gel plate directly into the glass trough of the chromatography tank. Cover and seal the tank. Allow the solvent front to travel about 11.5 centimeters from the bottom of the plate, remove the plate from the tank, and allow to air dry. Apply the spray solution (do not saturate) and place immediately into an oven maintained at 90° C. Heat 15 minutes.

(f) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values.

[39 FR 18944, May 30, 1974, as amended at 45 FR 16472, Mar. 14, 1980]

§ 436.306 Lincomycin gas liquid chromatography.

(a) *Equipment.* Gas chromatograph equipped with a flame ionization detector; Barber-Colman 5000 or equivalent.

(b) *Reagents.* (1) Pyridine, reagent grade, kept over potassium hydroxide.

(2) Methanol, reagent grade, anhydrous.

(3) Ethanol, absolute, reagent grade.

(4) Internal standard: Prepare a solution containing 2 milligrams of tetraphenylcyclopentadienone per milliliter in pyridine.

(5) Silylating reagent: Mix (9+1) of hexamethyldisilazane and trimethyl chlorosilane.

(c) *Typical conditions.* (1) Column: 4 feet × 3 millimeters ID, glass, with 3 percent SE–30 on Gas-Chrom Q (100/120 mesh), or equivalent.

(2) Temperatures: Column 225° C.; detector 280° C., injector 270° C.

(3) Carrier gas: Helium at 15 pounds per square inch.

(4) Detector: Hydrogen flame ionization—hydrogen at 20 pounds per square inch, air at 40 pounds per square inch.

(5) Sensitivity: 100; attenuation 2; current 2×10^{-8} amperes.

(d) *Preparation of lincomycin sample and working standard solutions.* Prepare the sample and working standard as follows: Weigh accurately an aliquot of about 40 milligrams into a 10-milliliter volumetric flask, add sufficient pyridine to dissolve, and make to mark. Transfer a 1-milliliter aliquot to a glass-stoppered conical centrifuge tube and proceed as directed in paragraph (e) of this section.

(e) *Procedure.* Add 0.2 milliliter of the silylating reagent to each centrifuge tube and allow to stand at least 30 minutes. Then add exactly 1 milliliter of the internal standard, shake well, and centrifuge. Inject 5 microliters of the supernatant into the gas chromatograph. Use the typical conditions and materials listed in paragraphs (a), (b), and (c) of this section. The conditions should be adequate to provide at least 60 percent scale deflection with the lincomycin peak and to maintain a stable base line. The resolution of the peaks should be complete. The elution order is lincomycin B, lincomycin, and the internal standard. If necessary, adjust the current setting for the lincomycin B peak to give a satisfactory response relative to that of the lincomycin peak. Calculate the lincomycin content and lincomycin B content as directed in paragraph (f) of this section.

(f) *Calculations.*

$$\text{Lincomycin content of sample in micrograms per milligram} = \frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of the lincomycin sample peak/Area of internal standard peak;

R_s=Area of the lincomycin standard peak/Area of internal standard peak;

W_s=Weight of the lincomycin working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Potency of lincomycin working standard in micrograms per milligram.

$$\text{Percent lincomycin B content} = \frac{B}{A+B} \times 100$$

where:

A=Area of lincomycin peak of the sample;

B=Area of lincomycin B peak of the sample corrected for the attenuation adjustment.

[39 FR 18944, May 30, 1974, as amended at 46 FR 3839, Jan. 16, 1981]

§ 436.307 Spectinomycin vapor phase chromatography.

(a) *Equipment.* Gas chromatograph equipped with a flame ionization detector; Barber-Colman 5,000 or equivalent.

(b) *Reagents.* (1) Dimethylformamide, reagent grade, kept dry over anhydrous sodium sulfate.

(2) Internal standard: Prepare a solution containing 2 milligrams of triphenylantimony per milliliter in dry dimethylformamide.

(3) Silylating reagent: Hexamethyldisilazane.

(c) *Typical conditions.* (1) Column: 4 feet by 4 millimeters ID, glass, with 5 percent SE–52 on Diatoport S (80/100 mesh), or equivalent.

(2) Temperatures: Column 215° C.; detector 270° C.; injection port 265° C.

(3) Carrier gas: Helium 93 milliliters per minute at 15 pounds per square inch.

(4) Detector: Hydrogen flame—hydrogen at 20 pounds per square inch, air at 40 pounds per square inch.

(5) Sensitivity: 1,000; attenuation, 10 for both spectinomycin and internal standard; 2×10^{-6} amperes.

(d) *Preparation of spectinomycin sample and working standard*—(1) *Working standard and bulk antibiotic solutions.* (i) Accurately weigh approximately 30 milligrams of sample or working standard into separate glass-stoppered 25-milliliter Erlenmeyer flasks.

(ii) Add 10 milliliters of the internal standard solution and 1.0 milliliter of hexamethyldisilazane to each flask. Agitate the flasks to insure dissolution of the sample and working standard

and complete mixing of the liquids. Shake the flasks intermittently for 1 hour. Proceed as directed in paragraph (e) of this section.

(2) *Finished product solutions.* Prepare the sample for assay as directed in the individual section for each antibiotic product to be tested.

(e) *Procedure.* Inject 2.5 microliters of each solution into the gas chromatograph. Use the conditions and materials listed in paragraphs (a), (b), and (c) of this section. The conditions should be adequate to maintain a stable base line and provide at least 60 percent deflection of the recorder scale by the spectinomycin peak. The resolution of the peaks should be complete. The internal standard will be eluted before spectinomycin. Calculate the spectinomycin content as directed in paragraph (f) of this section.

(f) *Calculations.* Calculate the spectinomycin content of the sample as follows:

$$\text{Micrograms of spectinomycin per milligram} = \frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of spectinomycin sample peak (at a retention time equal to that observed for the spectinomycin standard)/Area of internal standard peak;

R_s=Area of the spectinomycin standard peak/Area of internal standard peak;

W_s=Weight of the spectinomycin working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Potency of the spectinomycin working standard in micrograms per milligram.

§ 436.308 Paper chromatography identity test for tetracyclines.

(a) *Equipment*—(1) *Sheet* (*chromatographic*). Whatman No. 1 filter paper for chromatography, 20 × 20 centimeters.

(2) *Chamber* (*chromatographic*). Cylindrical glass chromatographic jar, 25 centimeters high by 12 centimeters in diameter, with a ground-glass lid.

(3) *Preparation of solutions*—(i) *pH3.5* buffer. Mix 13.93 volumes of 0.1*M* citric acid with 6.07 volumes of 0.2*M* of disodium phosphate.

(ii) *Solvent* (*organic phase*). Mix chloroform, nitromethane, and pyridine in volumetric proportions of 10:20:3, respectively.

(b) *Preparation of spotting solutions.* Prepare solutions of the working standard and sample as follows: Accurately weigh a portion of the working standard and sample and dilute with methanol to obtain a concentration of 1 milligram per milliliter of antibiotic to be tested.

(c) *Procedure.* Fill the chamber to a depth of 0.6 centimeter with freshly prepared solvent. Draw a starting line about 2.5 centimeters from and parallel to the bottom of the sheet. Wet the sheet thoroughly with the pH 3.5 buffer and blot it firmly between sheets of absorbent paper. Starting about 5 centimeters from the edge of the sheet and at 1.5-centimeter intervals, apply to the starting line 2 microliters each of standard solution, sample solution, and a 1:1 mixture of the standard and sample solutions. Allow a few minutes for the sheet to dry partially, and while still damp place it in the chamber with the bottom edge touching the solvent. When the solvent front has risen about 10 centimeters, remove the sheet from the chamber. Expose the paper to ammonia vapor. Examine the dried sheet under a strong source of ultraviolet light and record the position of any fluorescent spots. Measure the distance the solvent front traveled from the starting line and the distance that the fluorescent spots are from the starting line. Calculate the R_f value by dividing the latter by the former.

[39 FR 18944, May 20, 1974, as amended at 44 FR 30333, May 5, 1979; 45 FR 16472, 16474, Mar. 14, 1980]

§ 436.309 Anhydrotetracyclines and 4-epianhydrotetracycline.

Determination of 4-epianhydrotetracycline and anhydrotetracyclines in tetracycline, tetracycline hydrochloride, tetracycline phosphate, and in dosage forms thereof is as follows:

(a) *Screening procedure for total anhydrotetracyclines content*—(1) *Sample solution preparation*—(i) *Bulk packaged for repacking or for use in the manufacture of another drug.* Accurately weigh approximately 50 milligrams of the sample into a 50-milliliter volumetric flask and add 10 milliliters of 0.1*N* hydrochloric acid. Shake until sample is

completely dissolved, and then dilute to volume with water.

(ii) *Sterile dispensing containers.* Proceed as directed in paragraph (a)(1)(i) of this section.

(iii) *Capsules.* Transfer a representative quantity of capsule contents equivalent to 250 milligrams of tetracycline hydrochloride to a 250-milliliter volumetric flask. Add 50 milliliters of 0.1*N* hydrochloric acid and shake on a mechanical shaker for 5 minutes. Dilute to volume with water and filter through a fluted filter paper. Discard the first 20 milliliters of filtrate and collect the next 20 milliliters.

(iv) *Tablets.* Grind a representative number of tablets to a fine powder. Transfer an amount of the powder equivalent to 250 milligrams of tetracycline hydrochloride to a 250-milliliter volumetric flask. Add 50 milliliters of 0.1*N* hydrochloric acid and shake on a mechanical shaker for 5 minutes. Dilute to volume with water and filter through a fluted filter paper. Discard the first 20 milliliters of filtrate and collect the next 20 milliliters.

(v) *Oral powders and suspensions.* Proceed as described in paragraph (b) of this section.

(2) *Test procedure.* Using a suitable spectrophotometer, determine the absorbance of the sample solution prepared as directed in paragraph (a)(1) of this section at 430 millimicrons using 0.02*N* hydrochloric acid as a blank. Then accurately dilute 1.0 milliliter of the sample solution to 100 milliliters with 0.02*N* hydrochloric acid and determine the absorbance of this solution at 356 millimicrons, using 0.02*N* hydrochloric acid as a blank.

(3) *Calculations.*

$$\text{Percent anhydrotetracyclines} = \frac{[a430 - (a356 \times 0.0019)] \times 100}{195}$$

where:

*a*430=Absorptivity (1%, 1 cm.) of sample at 430 millimicrons;

$$\text{For bulk, absorptivity} = \frac{\text{Absorbance} \times 50 \times 10}{\text{Milligrams of sample}}$$

For sterile dispensing containers, capsules, and tablets; absorptivity=Absorbance×10; *a*356=Absorptivity (1%, 1 cm.) of sample at 356 millimicrons;

$$\text{For bulk, absorptivity} = \frac{\text{Absorbance} \times 50 \times 1000}{\text{Milligrams of sample}}$$

For sterile dispensing containers, capsules and tablets; absorptivity=Absorbance×1,000; 0.0019×Absorbance ratio (*A*430/*A*356) observed with tetracycline;

195=Absorptivity (1%, 1 cm.) of anhydrotetracycline hydrochloride at 430 millimicrons.

(4) *Evaluation.* If the total anhydrotetracyclines content determined by the screening procedure described in paragraph (a) of this section exceeds 2 percent for bulks and 3 percent for injectables, tablets, and capsules, perform the determination for anhydrotetracyclines and 4-epianhydrotetracycline described in paragraph (b) of this section. If the results of the test described in paragraph (a) of this section for total anhydrotetracyclines content are within the required limits in the case of bulks, injectables, tablets, and capsules, these results may be submitted in lieu of the results of the test for 4-epianhydrotetracycline and that test as described in paragraph (b) of this section need not be performed.

(b) *Determination of anhydrotetracyclines content and 4-epianhydrotetra-cycline content*—(1) *Apparatus and reagents*—(i) Chromatographic tubes (15 millimeters ID × 170 millimeters long having an outlet tube 4 millimeters ID × 50 millimeters long).

(ii) pH meter standardized at pH 7.0 and at pH 10.0.

(iii) Diatomaceous earth, acid-washed (Celite 545 or equivalent).

(iv) EDTA buffer. Dissolve 0.1 mole ethylenediaminetetraacetic acid disodium salt in 800 milliliters of water.

Adjust to pH 7.8 with ammonium hydroxide, reagent grade, and dilute to 1 liter with water.

(v) Chloroform, spectrophotometric grade.

(vi) Diluted ammonium hydroxide: Mix 1 volume of ammonium hydroxide, reagent grade, with 9 volumes of distilled water.

(vii) 0.1*N* hydrochloric acid.

(viii) 1.0*N* hydrochloric acid.

(2) *Preparation of support phase.* Add 5 milliliters of EDTA buffer to 10 grams of diatomaceous earth and mix until the diatomaceous earth is uniformly moistened. It will no longer be free-flowing.

(3) *Preparation of sample solutions.* Prepare the sample solutions as follows:

(i) *Tetracycline, tetracycline phosphate complex, and tetracycline hydrochloride bulk packaged for repacking or for use in the manufacture of another drug.* Place an amount of sample equivalent to 250 milligrams of tetracycline hydrochloride into a 50-milliliter beaker and dissolve in 10 milliliters of 0.1*N* hydrochloric acid. Immediately adjust the pH to 7.8 with the diluted ammonium hydroxide, and if necessary, with 1.0*N* hydrochloric acid and 0.1*N* hydrochloric acid. Quantitatively transfer this solution to a 50-milliliter volumetric flask by rinsing the beaker with EDTA buffer, fill to volume with EDTA buffer and shake well. Use this solution without delay to prepare a column as directed in paragraph (b)(4) of this section.

(ii) *Capsules.* Proceed as directed in paragraph (b)(3)(i) of this section, except pool the contents of a representative number of capsules and use an amount of the pooled capsule contents equivalent to 250 milligrams of tetracycline hydrochloride.

(iii) *Tablets.* Proceed as directed in paragraph (b)(3)(i) of this section, except grind tablets to a powder in a small mortar and use an amount of powder equivalent to 250 milligrams of tetracycline hydrochloride.

(iv) *Oral suspension and pediatric drops.* Place 5 milliliters of oral suspension equivalent to 125 milligrams of tetracycline hydrochloride or 2 milliliters of pediatric drops equivalent to 200 milligrams of tetracycline hydrochloride into a 50-milliliter beaker and add sufficient 0.1*N* hydrochloric acid to make 10 milliliters. Quickly adjust the pH to 7.8 with the diluted ammonium hydroxide, and if necessary, with 1*N* hydrochloric acid and 0.1*N* hydrochloric acid. Quantitatively transfer this solution to a 25-milliliter flask by rinsing the beaker with EDTA buffer, fill to volume with EDTA buffer, and shake well. Use this solution without delay to prepare a column as directed in paragraph (b)(4) of this section.

(v) *Oral powders.* Reconstitute as directed in the labeling and proceed as directed in paragraph (b)(3)(iv) of this section.

(vi) *Sterile dispensing containers.* Proceed as directed in paragraph (b)(3)(i) of this section.

(4) *Column preparation.* Pack support phase into the chromatographic tube by increments and firmly tamp down each increment. Do not use any glass wool in the column outlet. Add enough support phase to the column to reach a height of 9 to 11 centimeters; then add 1 milliliter of sample solution to 1 gram of diatomaceous earth in a small beaker, and mix thoroughly. Pack the sample: diatomaceous earth mixture on top of the column. Dry wash the beaker with support phase and pack an additional 1-centimeter layer of support phase on top of the sample layer.

(5) *Column elution and fraction collection.* Within 30 minutes after preparing the column, elute with chloroform. Collect 5 successive fractions of 5 milliliters, 5 milliliters, 10 milliliters, 10 milliliters, and 5 milliliters. During elution, two clear separate yellow bands will appear on the column. The first band is anhydrotetracyclines and will almost always elute in the first 5-milliliter fraction, but occasionally in the first and second 5-milliliter fractions. The second band is 4-epianhydrotetracycline and will elute in the remaining fractions. Label the fraction or fractions containing the first yellow band anhydrotetracyclines. Label the fractions after the first yellow band 4-epianhydrotetracycline. Determine the absorbance of each fraction at a wavelength of 438 nanometers using a suitable spectrophotometer equipped with a 1.0-centimeter cell and chloroform as the blank. If necessary,

make appropriate dilutions with choloroform to obtain a readable value.

(6) *Calculations*—(i) *Percent anhydrotetracyclines.* Calculate the percent anhydrotetracyclines as follows:

$$\text{Number of milligrams of anhydrotetracyclines in each fraction containing anhydrotetracyclines} = \frac{A \times b \times c}{20.28}$$

where:

A=Absorbance of the sample solution at 438 nanometers;

b=Volume of fraction in milliliters;

c=Dilution factor of the fraction (for example, if 2 milliliters of the fraction are diluted to 10 milliliters for reading, *c* will be 5).

20.28=Absorptivity (1 milligram per milliliter, 1 centimeter) of anhydrotetracyclines in chloroform at 438 nanometers.

Total weight of anhydrotetracyclines in the sample=Sum of weights of anhydrotetracyclines in the fractions labeled anhydrotetracyclines × Number of milliliters in the sample solution

Percent anhydrotetracyclines in tetracycline, tetracycline hydrochloride, tetracycline phosphate complex bulk packaged for repacking or for use in the manufacture of another drug=

$$100 \times \frac{\text{Total weight of anhydrotetracyclines in the sample}}{\text{Weight of the sample}}$$

Percent anhydrotetracyclines in dosage forms=

$$100 \times \frac{\text{Total weight of anhydrotetracyclines in the sample}}{\text{Tetracycline content of the sample}}$$

(ii) *Percent 4-epianhydrotetracycline.* Calculate the percent 4-epianhydrotetracycline as follows:

$$\text{Number of milligrams of 4-epianhydrotetracycline in each fraction labeled 4-epianhydrotetracycline} = \frac{A \times b \times c}{20.08}$$

where:

A=Absorbance of the sample solution at 438 nanometers;

b=Volume of the fraction in milliliters;

c=Dilution factor of the fraction (for example, if 2 milliliters of the fraction are diluted to 10 milliliters for reading, *c* will be 5);

20.08=Absorptivity (1 milligram per milliliter, 1 centimeter) of 4-epianhydrotetracycline in chloroform at 438 nanometers.

Total weight of 4-epianhydrotetracycline in the sample=Sum of weights of 4-epianhydrotetracycline in the fractions labeled 4-epianhydrotetracycline × Number of milliliters in the sample solution

Percent 4-epianhydrotetracycline in tetracycline, tetracycline hydrochloride, tetracycline phosphate complex bulk packaged for repacking or for use in the manufacture of another drug=

$$100 \times \frac{\text{Total weight of 4-epianhydrotetracycline in the sample}}{\text{Weight of the sample}}$$

Percent 4-epianhydrotetracycline in dosage forms=

$$100 \times \frac{\text{Total weight of 4-epianhydrotetracycline in the sample}}{\text{Tetracycline content of the sample}}$$

[39 FR 18944, May 30, 1974, as amended at 40 FR 22251, May 22, 1975; 43 FR 11153, Mar. 17, 1978]

§ 436.310 Thin layer chromatography identity test for mitomycin.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 9 × 9 × 3.5 inches, lined with filter paper and with a solvent trough on the bottom.

(2) *Plates.* Use 20 by 20 centimeter thin layer chromatography plates coated with silica gel G or equivalent, to a thickness of 250 microns.

(b) *Reagents*—(1) *Developing solvent.* Mix *n*-butanol, glacial acetic acid, and water in volumetric proportions of 4:2:1, respectively.

(2) *Spray solution.* Prepare a one-percent solution of ninhydrin in ethanol.

(c) *Preparation of spotting solutions.* Prepare solutions of the sample and working standard, each containing 1 milligram of mitomycin per milliliter, in water.

(d) *Procedure.* Pour the developing solvent into the solvent trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for 30 minutes. Prepare a plate as follows: Apply spotting solutions on a line 2.5 centimeters from the base of the silica gel plate and at points 2.0 centimeters apart. Apply approximately 2 microliters of the working standard solution to points 1 and 3. When these spots are dry, apply approximately 2 microliters of sample solution to points 2 and 3. After all spots are thoroughly dry, place the silica gel plate into the trough in the chromatography tank. Cover and seal the tank tightly. Allow the solvent front to travel about 10 centimeters from the starting line. Remove the plate and allow it to air dry. After the plate is dry, spray lightly with the spray solution. Heat the plate in an oven at 110° C. for 10–15 minutes. Mitomycin appears as a pink spot.

(e) *Evaluation*. The sample and standard should have spots of corresponding R_f value (approximately 0.51), and standard and sample combined should appear as a single spot of corresponding R_f value.

[39 FR 18944, May 30, 1974, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.311 Thin layer chromatography identity test for amoxicillin.

Using the sample solution prepared as described in the section for the antibiotic drug to be tested, proceed as described in paragraphs (a) through (e) of this section.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, equipped with a glass solvent trough in the bottom and a tight-fitting cover for the top. Line the inside walls of the tank with Whatman's 3MM chromatographic paper (0.33 millimeters) or equivalent.

(2) *Plates.* Use 20- by 20-centimeter thin layer chromatography plates coated with Silica Gel G or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Developing solvent.* Mix methyl alcohol, chloroform, pyridine, and distilled water in volumetric proportions of 90:80:10:30, respectively.

(2) *Spray solution.* Dissolve 300 milligrams of ninhydrin in 100 milliliters of ethyl alcohol.

(c) *Preparation of working standard.* Weigh an amount of the amoxicillin working standard equivalent to 200 milligrams of amoxicillin into a 50-milliliter volumetric flask and bring to volume with 0.1*N* hydrochloric acid.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for at least 2 hours. Spot duplicate plates by applying approximately 5 microliters each of standard and sample solutions on a line 1.5 centimeters from the base of the plate and at intervals of not less than 2.0 centimeters. All solutions must be spotted within 10 minutes of preparation. Place spotted plate in a desiccator until solvent has evaporated from spots. Place the plate into the glass trough at the bottom of the chromatography tank. Cover the tank. Allow the solvent to reach the 15-centimeter scored mark, remove the plate from the tank and dry with a current of warm air until there is no detectable solvent odor. Apply the ninhydrin spray solution to the plate—do not saturate—and place immediately into an oven maintained at 110° C for 15 minutes.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. Amoxicillin has an R_f value of about 0.53. The sample and standard

should have spots of corresponding R_f values.

[39 FR 34032, Sept. 23, 1974; 48 FR 11427, Mar. 18, 1983, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.312 Atomic absorption method for determining the zinc content of zinc bacitracin.

(a) *Equipment.* An atomic absorbance spectrophotometer equipped with a zinc hollow-cathode discharge lamp, an air-acetylene flame, a nebulizer-burner system for introducing the sample solution into the flame, an optical dispersing device (such as a monochromator) for isolating a resonance line of zinc from others produced by the emission source, and a suitable radiation detector and recorder.

(b) *Preparation of working standard and sample solutions*—(1) *Workingstandard solutions.* Prepare a standard stock solution containing 10 milligrams of zinc per milliliter as follows: Weigh 3.11 grams of zinc oxide into a 250-milliliter volumetric flask, add 80 milliliters of 1*N* HCl, warm to dissolve, cool to room temperature, and dilute to volume with water. Dilute aliquots of this standard stock solution with 0.001*N* HCl to obtain three working standard solutions containing respectively 0.5, 1.5, and 2.5 micrograms of zinc per milliliter.

(2) *Sample solution.* Accurately weigh approximately 200 milligrams of the sample into a 100-milliliter volumetric flask. Dissolve and dilute to volume with 0.01*N* HCl. Transfer a 2.0-milliliter aliquot of this solution to a 200-milliliter volumetric flask and dilute to volume with 0.001*N* HCl.

(c) *Procedure.* Using 0.001*N* HCl as the blank, adjust the absorbance of the instrument to zero at a detection wavelength of 213.8 nanometers. Determine the absorbance of each standard solution and the sample solution at 213.8 nanometers.

(d) *Calculations.* Plot the absorbance versus the concentration of each of the working standard solutions. Draw a straight response line of best fit through these points. Read the concentration of zinc in micrograms per milliliter corresponding to the absorbance of the sample solution. Calculate the percent zinc in the sample as follows:

$$\text{Percent zinc} = \frac{C \times 100{,}000}{\text{Milligrams of sample} \times (100 - m)}$$

where:

C=Concentration of zinc in the sample solution in micrograms per milliliter;

m=Percent moisture in the sample.

[40 FR 15088, Apr. 4, 1975]

§ 436.316 Determination of penicillin G content.

(a) *Reagents.* The reagents are freshly prepared every three days and are of such quality that when used in this procedure with an authentic sample of penicillin G, not less than 97 percent of penicillin G is recovered.

(1) *Amyl acetate (iso-amyl acetate) solution.* Saturate the amyl acetate (boiling range 138.5° C—141.5° C) with the *N*-ethylpiperidine salt of penicillin G by adding 2 milligrams of the salt for each 1.0 milliliter of the solvent. Cool this solution to 0° C—8° C and filter it through a sintered-glass filter immediately before use.

(2) *Acetone solution.* Saturate reagent grade acetone with the *N*-ethylpiperidine salt of penicillin G using 3 milligrams of salt for each 1 milliliter of acetone. Cool this solution to 0° C—8° C and filter it through a sintered-glass filter immediately before use.

(3) *N-ethylpiperidine solution. N*-ethylpiperidine (boiling range 129.5° C—131.0° C) should be stored in brown bottles in a refrigerator. Dilute 1.0 milliliter of this reagent with 4.0 milliliters of amyl acetate. Saturate this solution with the *N*-ethylpiperidine salt of penicillin G, using about 3 milligrams of the salt for each 1.0 milliliter of solution. Cool this solution to 0° C—8° C and filter it through a sintered-glass filter immediately before use.

(4) *Phosphoric acid solution.* Prepare by dissolving 1.0 milliliter of reagent grade phosphoric acid (85 percent) in 4.0 milliliters of water. Cool to 0° C—8° C and shake before using.

(5) *Silica gel.* Use dry silica gel (mesh size 6–16, Tyler standard). Place about

0.5 gram of the silica gel in a micro filter funnel (approximately 10-millimeter diameter) having a fritted-glass disc of medium porosity.

(b) *Procedure.* Accurately weigh from 60 to 70 milligrams of the sample to be tested, except if penicillin G procaine is to be tested weigh 90 to 100 milligrams of sample, into a glass test tube or glass vial of approximately 10–milliliter capacity. Add 2.0 milliliters of water to dissolve or suspend (procaine) the penicillin and cool to 0° C—5° C. Add 2.0 milliliters of amyl acetate solution and 0.5 milliliter of phosphoric acid solution, stopper and shake the container vigorously for approximately 15 seconds. For penicillin G procaine, add a second 0.5-milliliter portion of phosphoric acid solution and shake vigorously. Centrifuge to obtain a clear separation of the two layers (approximately 20 seconds). If any penicillin procaine remains undissolved, add a third 0.5-milliliter portion of phosphoric acid solution, shake the container vigorously, and centrifuge. After centrifuging, remove as much of the amyl acetate layer as possible, usually about 1.7 milliliters to 1.8 milliliters, with a suitable hypodermic needle and syringe and place the portion removed into the filter funnel containing silica gel, described in paragraph (a)(5) of this section. Allow the amyl acetate to remain in contact with the silica gel for exactly 20 seconds, then apply suction and collect the filtrate in a small test tube placed in a suction flash surrounded by cracked ice. Pipet a 1.0-milliliter aliquot of the amyl acetate filtrate into a tared flat-bottom glass tube (approximately 15 x 50 millimeters) containing 1.0 milliliter of acetone solution and 0.5 milliliter of *N*-ethylpiperidine solution. The time elapsing between acidification and the addition of the filtrate to the above reagents should not be more than 3 minutes. Place the glass tube containing the mixture into a large weighing bottle, stopper the bottle and allow to stand for not less than 2 hours in a refrigerator at 0° C—8° C. Remove the liquid from the precipitate by means of a tared micro filter stick and wash with a total of 1.0 milliliter of acetone solution adding the latter by means of a hypodermic syringe equipped with a fine needle. Place the filter stick inside the glass tube, dry under vacuum at room temperature for not less than 1 hour, and weigh. (The *N*-ethylpiperidine penicillin G residues can be saved for saturating reagents).

(c) *Calculations.* Calculate the percent penicillin G content as follows:

$$\text{Percent penicillin G content} = \frac{\text{Milligrams } N\text{-ethylpiperidine penicillin precipitate} \times 149.4}{\text{Weight of sample in milligrams}}$$

[42 FR 59857, Nov. 22, 1977]

§ 436.317 Solubility characteristic test for griseofulvin (ultramicrosize) tablets.

(a) *Apparatus*—(1) *Vessel.* A cylindrical glass tank. The approximate dimensions are 40 centimeters in diameter and at least 23 centimeters in height.

(2) *Heating system.* A 1,500-watt immersion heating element connected to a partial immersion, contact thermometer and an appropriate control relay.

(3) *Circulating system components.* The circulating system consists of three different circulating devices:

(i) Circulating pump of a centrifugal, immersion type. Tubing approximately 1 centimeter outside diameter and 46 centimeters in length is attached to the pump outlet producing a flow rate of approximately 1,600 milliliters per minute when operated as described.

(ii) A ''4-element stirrer'' consisting of a motor and a shaft approximately 45 centimeters long and 8 millimeters in diameter. The motor rotates the vertical shaft in a clockwise direction at approximately 180 revolutions per minute. There are 4 elements or sets of stirring blades on the shaft. One set, located at the bottom of the shaft, is a 3-bladed element of 2.5 centimeters

overall radius with circular blades, 1.8 centimeters in diameter and 1 to 2 millimeters in thickness, pitched at an angle of approximately 45 degrees from the horizontal plane, so that fluid is propelled downward when the shaft is rotated in a clockwise direction. The three remaining sets of stirring blades have 4 blades each, symmetrically positioned about the shaft. Each set of blades is 3.2 centimeters in overall radius. Each blade is rectangular in shape, 2.4 centimeters in length, 1.2 centimeters in height, and 1 to 2 millimeters in thickness. The four sets of blades are located at 5 centimeter intervals on the shaft, the top three being fixed in a staggered configuration.

(iii) A rotating basket device consisting of a motor capable of constant speed of 100±5 revolutions per minute in a clockwise direction, a shaft, and a cylindrical basket. The shaft and the basket are fabricated from Type 316 stainless steel. The shaft is 6 millimeters in diameter and approximately 30 centimeters in length. It must run true on the motor axis so that the basket rotates smoothly and without perceptible wobble. The basket consists of two parts, one of which, the top, is attached to the shaft. It is of solid metal except for a 2-millimeter round vent, and is fitted with three spring clips that allow the removal of the lower part, or the basket proper, to admit the test sample. The detachable part of the basket is fabricated of welded seam stainless steel, 40 mesh woven wire cloth, formed into a cylinder 3.66 centimeters high and 2.5 centimeters in diameter, with a narrow rim of sheet metal around the top.

(4) *Circulating system configuration.* All three circulating devices are located in one half of the tank. In clockwise order they are the circulating pump, the rotating basket, and the 4-element stirrer. There is a distance of 12 to 13 centimeters between each of the three devices. The rotating basket shaft and the stirring shaft are located 9 to 10 centimeters from the tank wall. The 4-element stirrer is positioned 1 to 1.5 centimeters from the bottom of the tank. The rotating basket is fixed at 7 to 8 centimeters from the bottom. The circulating pump intake is located approximately 3 centimeters from the top of the fluid in the tank and 5 to 6 centimeters from the wall of the tank. The pump's outlet hose is held by a clamp so that hose makes a clockwise arc around the inside wall of the tank, descending to a point near the bottom of the tank and 5 to 6 centimeters from the wall, which is 180 degrees from the pump inlet.

(b) *Dissolution medium.* Distilled water.

(c) *Procedure.* Place 24 liters of dissolution medium into the vessel and maintain the temperature at 37±0.5° C by means of the heater, circulating pump, and the 4-element stirrer. Withdraw a 25-milliliter portion of the dissolution medium as a sample-blank solution. Place one tablet into the basket, and lower it into its proper position in the tank. Rotate the basket at 100±5 revolutions per minute in a clockwise direction. After 60 minutes, withdraw a second 25-milliliter portion as the sample solution. Filter the sample-blank solution and the sample solution through water-washed glass wool, or an equivalent filter, discarding the first 10 to 15 milliliters of each filtrate. Determine the amount of griseofulvin dissolved as directed in paragraph (d)(2) of this section.

(d) *Griseofulvin assay*—(1) *Preparation of standard solution and standard-blank solution.* Accurately weigh approximately 50 milligrams of griseofulvin working standard and place into a 100-milliliter volumetric flask. Dissolve and dilute to volume with methyl alcohol. Transfer 2.0 milliliters of this solution to a 200-milliliter volumetric flask and dilute to volume with distilled water. This is the standard solution. Transfer a 2.0-milliliter portion of methyl alcohol to a 200-milliliter volumetric flask and dilute to volume with distilled water. This is the standard-blank solution. Filter the standard-blank solution and the standard solution through water-washed glass wool, or an equivalent filter, discarding the first 10 to 15 milliliters of each filtrate.

(2) *Procedure.* Using a suitable spectrophotometer and distilled water as the blank, determine the absorbance of the four filtered solutions at the absorbance peak at approximately 295

nanometers, using suitable spectrophotometer cells with a 1-centimeter light path. Determine the exact position of the absorbance peak for the particular instrument used.

(3) *Calculation.* Determine the percentage of griseofulvin dissolved as follows:

$$\text{Percent griseofulvin dissolved} = \frac{A_u \times W_s \times V \times 10}{A_s \times P}$$

where:

A_u=Absorbance of the sample solution minus the absorbance of the sample-blank solution;

W_s=Weight of the working standard in milligrams;

V=Volume of the dissolution medium in liters;

A_s=Absorbance of the standard solution minus the absorbance of the standard-blank solution;

P=Labeled potency of the sample in milligrams of griseofulvin per tablet.

(e) *Evaluation.* The tablet passes the solubility characteristic test if it dissolves to the extent of not less than 50 percent at 60 minutes. If the tablet fails to meet this requirement, repeat the test on five additional tablets. The batch passes the solubility characteristic test if not less than 5 of 6 tablets meet the requirement.

[40 FR 41522, Sept. 8, 1975; 40 FR 45426, Oct. 2, 1975]

§ 436.318 Continuous flow thin layer chromatography identity test.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide equipped with a glass solvent trough in the bottom.

(2) *Plates.* Use a 20 × 20 centimeter thin-layer chromatography plate coated with Silica Gel G or equivalent to a thickness of 250 micrometers.

(3) *Cover.* A stainless steel cover with a slot, measuring 21 × 0.6 centimeters, cut in the front edge.

(4) *Supporting platform.* A platform that can be placed in the bottom of the chromatography tank so that the solvent trough is elevated about 3.75 centimeters.

(b) *Reagents*—(1) *Developing solvent.* Mix chloroform, redistilled methanol and concentrated ammonium hydroxide in volumetric proportions of 25:60:30, respectively.

(2) *Spray solution.* Dissolve 1 gram of ninhydrin in 100 milliliters of *n*- butanol and add 1 milliliter of pyridine.

(c) *Preparation of spotting solutions.* Prepare solutions of the sample and working standard, each containing 6 milligrams of antibiotic to be tested per milliliter in distilled water.

(d) *Procedure.* Prepare a plate as follows: On a line 2 centimeters from the base of the silica gel plate, and at intervals of 1 centimeter, spot 3 microliters each of the standard solution and the sample solution. In addition, prepare one spot composed of 3 microliters of the sample solution and 3 microliters of the standard solution. Place the supporting platform in the bottom of the tank and place the solvent trough on it, near the front of the tank. Place a piece of Whatman #3 MM filter paper or equivalent, measuring 20x3 centimeters and folded in half, lengthwise, over the front edge of the tank to form a cushion and drying wick for the plate. Place the plate in the solvent trough with the coated side toward the front of the tank and leaning against the filter paper at the top. Pour the developing solvent into the trough and bottom of the tank. Cover the tank. The plate should extend approximately 1 centimeter beyond the top of the tank and through the slot in the cover. Seal all the openings in the tank with masking tape, except where the plate leans against the filter paper. Remove the plate from the tank after 5.5 hours. Allow the plate to air dry and then heat it for 15 minutes at 110° C in an oven. Remove the plate from the oven and immediately spray it with the spray solution. The compound appears as a pink spot.

(e) *Evaluation.* The sample and standard should have traveled the same distance from the origin, and the standard and sample combined should appear as a single spot that has traveled the same distance as the sample and standard individually.

[40 FR 57797, Dec. 12, 1975]

§ 436.319 Thin layer chromatography identity test for bacitracin and bacitracin zinc.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, equipped with a glass solvent trough in the bottom and a tight-fitting cover for the top. Line the inside walls of the tank with Whatman 3MM chromatographic paper or equivalent.

(2) *Plates.* Use a 20- by 20-centimeter thin layer chromatography plate coated with silica gel G or equivalent to a thickness of 250 micrometers. Activate the plate by heating for 20 minutes at 110° C. Allow to cool to room temperature and use immediately.

(b) *Reagents*—(1) *Developing solvent.* Mix *n*- butanol, water, pyridine, glacial acetic acid, and ethyl alcohol in volumetric proportions of 60:10:6:15:5, respectively.

(2) *Spray solution.* Dissolve 1 gram of ninhydrin in a mixture of 1 milliliter of pyridine and sufficient *n*- butanol to make 100 milliliters.

(c) *Preparation of spotting solutions.* Prepare solutions of the sample and working standard, each containing 6.0 milligrams of bacitracin per milliliter in 1 percent disodium ethylenediamine tetraacetic acid in water.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for at least 30 minutes. Prepare a plate as follows: On a line 2.0 centimeters from the base of the silica gel plate, and at intervals of 2.0 centimeters, spot approximately 1.0 microliter of the standard solution to points 1 and 3. When these spots are dry, apply approximately 1.0 microliter of sample solution to points 2 and 3. After all spots are thoroughly dry, place the base of the silica gel plate directly into the glass trough in the chromatography tank. Cover and seal the tank. Allow the solvent front to travel approximately 13 centimeters from the starting line. Remove the plate from the tank, and allow it to air dry. After the plate is dry, spray lightly with the spray solution. The plate may take 1 hour or more to develop at room temperature. The development may be speeded up by warming the plate in a 110° C oven.

(e) *Evaluation.* The sample and standard should have spots of corresponding R_f value (approximately 0.26) and standard and sample combined should appear as a single spot of corresponding R_f value.

[42 FR 27228, May 27, 1977]

§ 436.320 Ferric chloride colorimetric assay.

(a) *Reagents.* (1) 1*N* hydrochloric acid.

(2) 0.01*N* hydrochloric acid.

(3) Ferric chloride stock solution. Quickly weigh (very hygroscopic) 5.0 grams of $FeCl_3 \cdot 6H_2O$ into a 100-milliliter beaker. Add approximately 10 milliliters of 1*N* hydrochloric acid and stir to dissolve. Quantitatively transfer to a 50-milliliter glass-stoppered amber volumetric flask and make up to volume with water.

(4) Ferric chloride working reagent. Pipette 10.0 milliliters of ferric chloride stock solution into a 2-liter volumetric flask, add 20 milliliters 1*N* hydrochloric acid, and bring to volume with water. Check the pH; it should be between 2.0 and 2.1.

(b) *Standard solution.* Accurately weigh approximately 50 milligrams of the working standard of the antibiotic to be tested and dissolve with 25 milliliters of 0.1*N* hydrochloric acid. Quantitatively transfer to a 250-milliliter volumetric flask and dilute to volume with distilled water. Keep in a glass-stoppered flask and store under refrigeration. Discard solution after 7 days.

(c) *Sample solution.* Accurately weigh approximately 50 milligrams of the sample and dissolve with 25 milliliters of 0.1*N* hydrochloric acid. Quantitatively transfer to a 250-milliliter volumetric flask and dilute to volume with distilled water.

(d) *Procedure.* Pipette exactly 10.0 milliliters of the standard solution and of the sample solution into separate test tubes. To each tube add exactly 10 milliliters of ferric chloride working reagent, mix, and allow to stand 15 minutes. Determine the absorbance of each solution at 490 nanometers in a suitable spectrophotometer against a blank prepared from 10.0 milliliters of

0.01*N* hydrochloric acid and 10.0 milliliters of ferric chloride working reagent.

(e) *Estimation of potency.* Calculate the potency as follows:

$$\text{Micrograms of antibiotic per milligram} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{\text{Milligrams of standard}}{\text{Milligrams of sample}} \times \text{Potency of standards in micrograms per milligram}$$

[43 FR 11154, Mar. 17, 1978; 43 FR 34456, Aug. 4, 1978]

§ 436.321 Griseofulvin gas liquid chromatography.

(a) *Equipment.* Gas chromatograph equipped with an electronic integrator and with a flame ionization detector: Hewlett Packard 7600 or equivalent.

(b) *Reagents.* (1) Chloroform, reagent grade.

(2) Internal standard solution: Prepare a solution containing 1.0 milligram of tetraphenylcyclopentadienone per milliliter in chloroform.

(c) *Typical conditions*—(1) *Column.* 1.2 meters by 4 millimeters ID, glass, packed with 1 percent OV–17 on Gas Chrom Q (100/120 mesh), or equivalent.

(2) *Temperatures.* Column 245° C; detector 260° C; injection port 260° C.

(3) *Carrier gas.* Helium approximately 60 millimeters per minute and 40 pounds per square inch (1.7 kilograms per square centimeter).

(4) *Detector.* Hydrogen flame ionization-hydrogen at 12 pounds per square inch (0.5 kilogram per square centimeter), air at 34 pounds per square inch (1.43 kilograms per square centimeter).

(5) *Sensitivity.* Adjusted to obtain peak heights greater than 50 percent full scale deflection.

(d) *Preparation of griseofulvin sample and working standard solutions.* Accurately weigh approximately 40 milligrams of both the sample and the working standard into separate 25-milliliter volumetric flasks. Add sufficient internal standard solution to dissolve the contents of each flask with vigorous mixing and then dilute to volume with internal standard solution and mix. Proceed as directed in paragraph (e) of this section.

(e) *Procedure.* Inject 1.0 microliter of this solution into the gas chromatograph. Use the typical conditions and materials listed in paragraphs (a), (b), and (c) of this section. The resolution of the peaks should be complete. The griseofulvin peak will elute before the internal standard peak. Calculate the griseofulvin content as directed in paragraph (f) of this section.

(f) *Calculations.* Calculate the griseofulvin content of the sample as follows:

$$\text{Micrograms of griseofulvin per milligram} = \frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of the griseofulvin sample peak (at a retention time equal to that observed for the griseofulvin standard)/Area of the internal standard peak;

R_s=Area of the griseofulvin working standard peak/Area of the internal standard peak;

W_s=Weight of the griseofulvin working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Potency of the griseofulvin working standard in micrograms per milligram.

[44 FR 20660, Apr. 6, 1979]

§ 436.322 High-pressure liquid chromatographic assay for anthracycline antibiotics.

(a) *Equipment.* A suitable high-pressure liquid chromatograph, such as a Waters Associates Model 244[1] or equivalent equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

[1] Available from Waters Associates, Inc., Maple St., Milford, Mass. 10757.

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A suitable integrator;

(6) A 30-centimeter column having an inside diameter of 4.6 millimeters and packed with a suitable reverse phase packing such as: Waters Associates, Micro-Bondapak C18.[1]

(b) *Reagents.* (1) Solvent mixture: Water: acetonitrile (69:31).

(2) Mobile phase: Water: acetonitrile (69:31) adjusted to pH 2 with phosphoric acid. Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(3) Internal standard solution: Prepare a 2.0-milligram-per-milliliter solution of 2-naphthalenesulfonic acid in the solvent mixture.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.5 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the reference standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the initial baseline.

(d) *Procedure.* Use the standard and sample solutions prepared as directed in the individual monographs for the drug being tested. Use the equipment, reagents, and operating conditions listed in paragraphs (a), (b), and (c) of this section. Inject 5 microliters of the standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components (ordinarily this time is 20 minutes). After separation of the standard solution has been completed, inject 5 microliters of the sample solution into the chromatograph and repeat the procedure described for the standard solution. The elution order is: Void volume, internal standard, doxorubicin, dihydrodaunomycin, daunomycin, adriamycinone, dihydrodaunomycinone, bromodaunomycin, daunomycinone, and bis-anhydrodaunomycinone.

(e) *Calculations.* Calculate the anthracycline content as directed in the individual monograph for the drug being tested.

[43 FR 44836, Dec. 29, 1978]

§ 436.323 Continuous flow thin layer chromatography identity test for cefamandole nafate.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide equipped with a glass solvent trough in the bottom.

(2) *Plates.* Use a 20 x 20 centimeter thin-layer chromatography plate coated with silica gel G or equivalent to a thickness of 250 micrometers.

(3) *Cover.* A stainless steel cover with a slot measuring 21 x 0.6 centimeters, cut in the front edge.

(4) *Supporting platform.* A platform that can be placed in the bottom of the chromatography tank so that the solvent trough is elevated about 3.75 centimeters.

(b) *Reagents*—(1) *Developing solvent.* Mix *n*- butanol, glacial acetic acid, and water in volumetric proportions of 4:1:1, respectively.

(2) *Spray solution.* Mix starch iodide solution, glacial acetic acid, and 0.1 *N* iodine test solution, U.S.P. in volumetric proportions of 50:3:1. Prepare the starch iodide solution by mixing starch iodide paste test solution, U.S.P. and water in volumetric proportions of 1:1.

(c) *Preparation of spotting solutions.* Prepare solutions of the sample and working standard, each containing 1 milligram of cefamandole nafate per milliliter in distilled water.

(d) *Procedure.* Prepare a plate as follows: On a line 2 centimeters from the base of the silica gel plate, and at intervals of 1 centimeter, spot 5 microliters each of the standard solution and the sample solution. In addition, prepare one spot composed of 5 microliters of the sample solution and 5 microliters of the standard solution. Place the supporting platform in the bottom of the tank and place the solvent trough on it, near the front of the tank. Place a piece of Whatman #3 MM filter paper or equivalent, measuring 20 x 3 centimeters and folded in half, lengthwise, over the front edge of the tank to form a cushion and drying

wick for the plate. Place the plate in the solvent trough with the coated side toward the front of the tank and leaning against the filter paper at the top. Pour the developing solvent into the trough and bottom of the tank. Cover the tank. The plate should extend approximately 1 centimeter beyond the top of the tank and through the slot in the cover. Seal all the openings in the tank with masking tape, except where the plate leans against the filter paper. Remove the plate from the tank after 4 hours. Allow the plate to air dry and then heat it in an oven for 15 minutes at 110° C. Remove the plate from the oven and immediately spray it with the spray solution. The compound appears as a white spot on a purple background.

(e) *Evaluation.* The sample and standard should have traveled the same distance from the origin, and the combined standard and sample should appear as a single spot that has traveled the same distance as the sample and standard individually.

[44 FR 20664, Apr. 6, 1979]

§ 436.324 Polarographic analysis of cefamandole.

(a) *Equipment*—(1) *Polarograph.* Use a polarograph equipped with a dropping mercury indicating electrode, a platinum auxilliary electrode, and a saturated calomel reference electrode, such as Princeton Applied Research Model 174[1] or equivalent.

(2) *X–Y plotter.* Use a suitable X–Y plotter, such as Houston Omnigraphic Model 2200–3–3[2] or equivalent.

(3) *Nitrogen.* Use a nitrogen tank equipped with a pressure-reducing regulator and a filter to remove traces of oxygen, such as an oxisorb filter[1] or equivalent.

(b) *Reagent.* pH 2.3 Buffer: Dissolve 3.6 grams of dibasic sodium phosphate, 39.4 grams of citric acid, and 70.8 grams of potassium chloride in sufficient distilled water to make 1 liter.

(c) *Operating conditions*—(1) Operating mode: Differential pulse.

(2) Scan range: −0.3 volt to −1.05 volts.

(3) Scan rate: −2 millivolts per second.

(4) Sensitivity: 10 to 20 microamperes or equivalent to keep peak on scale.

(5) Mercury drop time: 1 second per drop.

(6) Modulation amplitude: 25 millivolts.

(7) Display direction: +

(8) Damping: None.

(d) *Preparation of sample and working standard solutions.* Use the cefamandole lithium working standard. Accurately weigh approximately 12 milligrams of sample or working standard into a 50-milliliter volumetric flask. Dissolve the sample or working standard in 4 milliliters of distilled water. Immediately prior to polarography, add 30 milliliters of pH 2.3 buffer, dilute to volume with distilled water, and mix.

(e) *Procedure.* Transfer a portion of the sample or working standard solution to the polarographic cell. Pass a stream of nitrogen through the solution for 5 minutes to remove the dissolved oxygen. After 5 minutes, disperse the nitrogen above the sample. Start the mercury dropping from the mercury dropping electrode, and, using the operating conditions described in paragraph (c) of this section, record the polarogram. Compare the polarogram of the sample to that of the working standard.

(f) *Calculations.* Calculate the potency of cefamandole as follows:

$$\text{Micrograms of cefamandole per milligram} = \frac{A \times \text{Milligrams of working standard} \times \text{Potency of working standard in micrograms per milligram}}{B \times \text{Milligrams of sample}}$$

where:

A=The peak height of the sample;

[1] Available from Princeton Applied Research Corporation, P.O. Box 2565, Princeton, NJ 08540.

[2] Available from Houston Instrument, 8500 Cameron Road, Austin, TX 78753.

B=The peak height of the working standard.

The peak height is obtained from the polarogram by measuring the vertical distance from the peak to the baseline of the sample or working standard.

[44 FR 20664, Apr. 6, 1979, as amended at 47 FR 20756, May 14, 1982]

§ 436.325 High pressure liquid chromatography assay for vidarabine.

(a) *Equipment.* A suitable high pressure liquid chromatograph, such as a Waters Associates Model 244[1] or equivalent, equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A 30-centimeter column having an inside diameter of 4 millimeters and packed with a suitable octadecyl bonded silica phase packing such as Waters Associates, Micro-Bondapak C18.[1]

(b) *Mobile phase.* (1) Transfer 2.2 grams of sodium dioctyl sulfosuccinate and 10 milliliters of glacial acetic acid to a 1-liter volumetric flask. Dissolve with 500 milliliters of methanol, dilute to volume with distilled water, and mix. Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter.

(2) De-gas the mobile phase just before its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.5 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the reference standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the initial baseline.

(d) *Preparation of sample and working standard solutions.* Accurately weigh approximately 24 milligrams of sample or working standard into a 200-milliliter volumetric flask. Add about 150 milliliters of distilled water and heat on a steam bath for 10 minutes. Shake until all the powder is dissolved. Cool to room temperature and dilute to volume with distilled water.

(e) *Procedure.* Using the equipment, mobile phase, and operating conditions listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the sample or working standard solution prepared as directed in paragraph (d) of this section into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. The elution order is void volume, 9-β-D-arabinofuranosylhypoxanthine (if present), vidarabine, and adenine (if present).

(f) *Calculations.* Calculate the vidarabine content as follows:

$$\text{Micrograms of vidarabine per milligram} = \frac{A \times W_s \times f}{B \times W_u}$$

where:

A=Area of the vidarabine sample peak (at a retention time equal to that observed for the standard);

B= Area of the standard peak;

W_s=Weight of standard in milligrams;

W_u=Weight of sample in milligrams; and

f=Potency of standard in micrograms per milligram.

[44 FR 30334, May 25, 1979, as amended at 47 FR 23708, June 1, 1982]

§ 436.326 Thin layer chromatographic identity test for cefoxitin sodium.

Using the sample solution prepared as described in the section for the antibiotic drug to be tested, proceed as described in paragraphs (a), (b), (c), (d), and (e) of this section.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, equipped with a glass solvent trough in the bottom and a tight-fitting cover for the top. Line the inside walls of the tank with Whatman #3 MM, chromatographic paper or equivalent.

(2) *Plates.* Use a 20×20 centimeter thin layer chromatography plate coated with silica gel G or equivalent to a thickness of 250 micrometers.

(b) *Developing solvent.* Mix ethyl acetate, pyridine, *n*-butanol, acetic acid,

[1] Available from Waters Associates, Inc., Maple St., Milford, MA 10757.

and water in volumetric proportions of 42:21:21:6:10, respectively.

(c) *Spray solution.* Immediately before use, mix 100 milliliters of a 1-percent ferric chloride solution in 1 percent hydrochloric acid with 100 milliliters of a 1-percent potassium ferricyanide solution and 75 milliliters of methanol.

(d) *Preparation of working standard solution.* Prepare a solution containing approximately 2.5 milligrams per milliliter of cefoxitin working standard in distilled water.

(e) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Prepare a plate as follows: On a line 2 centimeters from the base of the silica gel plate, and at intervals of 2 centimeters, spot 10 microliters each of the standard solution and the sample solution. After all spots are thoroughly dry, place the silica gel plate directly into the glass trough. Cover and seal the tank. Allow the solvent front to travel about 15 centimeters from the starting line. Remove the plate from the tank and heat it for 1 hour at 60° C in a circulating air oven. Remove the plate from the oven and allow it to cool at room temperature. Apply the spray solution and allow it to air dry. After approximately 15 minutes, the compound appears as a blue spot on a yellow-green background.

(f) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values.

[44 FR 10373, Feb. 20, 1979, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.327 Thin layer chromatographic identity test for cyclacillin.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23 x 23 x 9 centimeters, with a glass solvent trough on the bottom and a tight-fitting cover.

(2) *Plates.* Use 20 x 20 centimeter thin layer chromatography plates coated with Silica Gel G or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Developing solvent.* One percent ammonium formate aqueous solution.

(2) *Spray solution.* Dilute starch iodide paste TS (U.S.P. XIX) with an equal volume of water. Mix diluted starch iodide paste, glacial acetic acid, and 0.1*N* iodine in volumetric proportions of 50:3:1, respectively.

(c) *Assay solutions*—(1) *Preparation of working standard solution.* Accurately weigh an amount of cyclacillin working standard and dissolve the material with sufficient 0.1*N* sodium hydroxide to obtain a solution containing 1 milligram per milliliter. Allow the solution to stand for 15 minutes before using.

(2) *Preparation of sample solution.* Using the sample solution prepared as described in the section for the antibiotic to be tested, proceed as described in paragraphs (d) and (e) of this section.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank. Cover and seal the tank. Allow it to equilibrate. Prepare a plate as follows: On a line 2 centimeters from the base of the thin layer chromatography plate and at intervals of 2 centimeters, spot 5 microliters each of the working standard solution and sample solution. Dry the spots thoroughly with a stream of dry air. Place the plate in the trough in the chromatography tank. Cover and seal the tank. Allow the solvent front to travel about 15 centimeters from the starting line and then remove the plate from the tank. Dry the plate by heating for 30 minutes at 80° C in a circulating air oven. Visualize the spots by applying the spray solution.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line, and the distance the spots are from the starting line. Divide the latter by the former to calculate the R_f value. The sample and standard should appear as white spots against a blue background at an R_f of approximately 0.6. The test is satisfactory if the R_f value of the sample compares with that of the working standard.

[46 FR 2981, Jan. 13, 1981, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.328 High pressure liquid chromatographic assay for sulfisoxazole acetyl content.

(a) *Equipment.* A suitable high pressure liquid chromatograph, such as a Waters Associates Model 244[1] or equivalent equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with a suitable reverse phase packing such as: Waters Associates, Micro-Bondapak C18;[1] and

(6) A suitable integrator.

(b) *Reagents*—(1) *Mobile phase.* Mix acetonitrile (high pressure liquid chromatography grade): water (40:60). Filter the mobile phase through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter. De-gas the mobile phase just prior to its introduction into the chromatograph pumping system.

(2) *Internal standard solution.* Dissolve 0.33 milligram of benzanilide per milliliter in acetonitrile (high pressure liquid chromatography grade). Filter the solution through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.2 milliliters per minute. Use a detector sensitivity setting that gives a peak height for reference standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the baseline.

(d) *Preparation of the working standard and sample solutions*—(1) *Working standard solution.* Prepare a solution containing 1.0 milligram per milliliter of sulfisoxazole acetyl in the internal standard solution.

(2) *Sample solution.* Reconstitute the sample as directed in the labeling. Allow to stand for 1 hour. Shake gently and transfer 5.0 milliliters of the sample to a separatory funnel. Extract the suspension three times with 75-milliliter portions of chloroform. Collect the chloroform layers in a 250-milliliter volumetric flask. Dilute the flask to volume with chloroform and mix. Filter a portion of the solution through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter. Transfer a 4.0-milliliter aliquot of the filtrate into a 25-milliliter glass-stoppered flask and evaporate to dryness under a stream of dry air. Dissolve the residue in 10.0 milliliters of the internal standard solution, stopper, and mix.

(e) *Procedure.* Using the equipment, reagents, and operating conditions listed in paragraphs (a), (b), and (c) of this section, inject 5 microliters of sample or working standard solution prepared as described in paragraph (d) of this section, into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. The elution order is void volume, sulfisoxazole acetyl and benzanilide.

(f) *Calculations.* Calculate the sulfisoxazole content as follows:

$$\text{Milligrams of sulfisoxazole per milliliter of sample} = \frac{A \times \text{Concentration of the standard solution in milligrams per milliliter} \times 125 \times 0.864}{B}$$

where:

A=Area of sample peak (at a retention time equal to that of the standard) divided by the area of the internal standard peak;

B=Area of the standard peak divided by the area of the internal standard peak;

[1] Available from: Waters Associates, Inc., Maple Street, Milford, MA 10757.

0.864=The molecular weight of sulfisoxazole divided by the molecular weight of sulfisoxazole acetyl.

[46 FR 2990, Jan. 13, 1981]

§ 436.329 High-pressure liquid chromatographic assay for meclocycline.

(a) *Equipment.* A suitable high-pressure liquid chromatograph, such as a Waters Associates Model 244[1] or equivalent equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 340 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A suitable integrator;

(6) A column approximately 25 centimeters in length having an inside diameter of approximately 4 millimeters and packed with a suitable reverse-phase packing such as: 10 micrometer silica gel particles bonded to octadecyl silane, Vydac 201 TP Reverse Phase[2] or equivalent.

(b) *Reagents*—(1) *0.001M Ammonium (ethylenedinitrilo) tetraacetate.* Moisten 293 milligrams of (ethylenedinitrilo) tetraacetic acid with 1 milliliter of methanol and dissolve in 7 milliliters of concentrated ammonium hydroxide. Dilute to 900 milliliters with distilled water, adjust the pH to 6.6 with glacial acetic acid, and dilute to 1,000 milliliters with distilled water.

(2) *Mobile phase.* Mix 150 milliliters of tetrahydrofuran (high-pressure liquid chromatography grade) with 850 milliliters of 0.001*M* ammonium (ethylenedinitrilo) tetraacetate. Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 0.8 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the reference standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the initial baseline.

(d) *Preparation of sample and working standard solutions.* Accurately weigh an amount of sample or working standard equivalent to approximately 25 milligrams of meclocycline into a 50-milliliter volumetric flask. Dissolve and dilute to volume with methanol and mix. Transfer exactly 3.0 milliliters of this solution to a 25-milliliter volumetric flask, dilute to volume with mobile phase, and mix.

(e) *Procedure.* Using the equipment, reagents, and operating conditions listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the sample or working standard solution prepared as described in paragraph (d) of this section into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. The elution order is void volume, oxytetracycline (if present), demeclocycline (if present), methacycline (if present), and meclocycline.

(f) *Calculations.* Calculate the meclocycline content as follows:

$$\text{Micrograms of meclocycline per milligram} = \frac{A \times \text{Milligrams of working standard} \times \text{Potency of the working standard in micrograms per milligram}}{B \times \text{Milligrams of sample}}$$

where:

A= Area or peak height of the sample peak (at a retention time equal to that observed for the standard);

B= Area or peak height of the standard peak.

[46 FR 3836, Jan. 16, 1981]

[1] Available from: Waters Associates, Inc., Maple St., Milford, MA 10757.

[2] Available from: The Separations Group, 16640 Spruce St., Hesperia, CA 92345.

§ 436.330 Thin layer chromatographic identity test for bacampicillin.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23 × 23 × 9 centimeters, with a glass solvent trough on the bottom and a tight-fitting cover, lined with Whatman's 3MM chromatographic paper (0.3 millimeter) or equivalent.

(2) *Plates.* Use 20 × 20 centimeter thin layer chromatography plates coated with Silica Gel 60F 254 or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Developing solvent.* Mix methylene chloride, chloroform, and 95 percent ethyl alcohol in volumetric proportions of 100:10:10, respectively.

(2) *Spray solution.* Dissolve 1 gram of ninhydrin in 100 milliliters of *n*-butanol and add 1 milliliter of pyridine.

(c) *Spotting solutions*—(1) *Preparation of working standard solution.* Dissolve and dilute a weighed amount of the bacampicillin hydrochloride working standard with sufficient 95 percent ethyl alcohol to obtain a solution containing 2 milligrams per milliliter.

(2) *Preparation of sample solution.* Dissolve and dilute a weighed amount of the sample with sufficient 95 percent ethyl alcohol to obtain a solution containing 2 milligrams per milliliter. Proceed as described in paragraphs (d) and (e) of this section.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for one hour. Prepare a plate as follows: On a line 2.5 centimeters from the base of the thin layer chromatography plate and at intervals of 2.0 centimeters, spot 5 microliters of the working standard solution to positions 1 and 3. When these spots are dry, apply 5 microliters of the sample solution to points 2 and 3. After all the spots are thoroughly dry, place the plate into the trough in the bottom of the tank. Cover and tightly seal the tank, allow the solvent front to travel about 15 centimeters from the starting line (about 30 minutes) and then remove the plate from the tank. Air dry the plate. Visualize the spots by spraying with spray solution and heating in an oven at 100° C for approximately 10 minutes.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line, and the distance the spots are from the starting line. Divide the latter by the former to calculate the R_f value. Bacampicillin appears as a purple spot at an R_f value of approximately 0.52. The test is satisfactory if the R_f value of the sample compares with that of the working standard. The combined spot should appear as a single spot of corresponding R_f value.

[46 FR 25602, May 8, 1981, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.331 High-pressure liquid chromatographic assay for dactinomycin.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4-centimeter deflection;

(5) A suitable integrator; and

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 micrometers to 10 micrometers in diameter, U.S.P. XX.

(b) *Mobile phase.* Mix acetonitrile (high-pressure liquid chromatography grade): water (60:40). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 2.5 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the initial baseline.

(d) *Preparation of working standard and sample solutions*—(1) *Preparation of*

working standard solution. Prepare a solution containing 0.25 milligram per milliliter of dactinomycin in mobile phase.

(2) *Preparation of sample solution.* Prepare the sample solution as described in the individual monograph for the drug being tested.

(e) *Procedure.* Use the equipment, mobile phase, operating conditions, and working standard and sample solutions described in paragraphs (a), (b), (c), and (d) of this section, and proceed as directed in paragraph (e)(1) of this section.

(1) *System suitability test.* Equilibrate and condition the column by passage of about 10 to 15 void volumes of mobile phase followed by two or more replicate injections of 10 microliters each of the working standard solution. Allow an elution time sufficient to obtain satisfactory separation of expected components after each injection. Record the peak responses and, calculate the relative standard deviation as described for system suitability tests in the U.S.P. XX General Chapter 621 chromatography. Proceed as directed in paragraph (e)(2) of this section if the minimum performance requirement for the relative standard deviation is not more than 1.0 percent. If the minimum performance requirement is not met, adjustment must be made to the system to obtain satisfactory operation before proceeding as described in paragraph (e)(2) of this section.

(2) *Determination of the chromatogram.* Inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(f) *Calculations.* Calculate the dactinomycin content as described in the individual monograph for the drug being tested.

[49 FR 24017, June 11, 1984, as amended at 50 FR 5749, Feb. 12, 1985]

§ 436.332 High-pressure liquid chromatographic assay for moxalactam.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter, U.S.P. XX;

(5) A suitable recorder of at least 25.4 centimeter deflection;

(6) A suitable integrator.

(b) *Mobile phase.* Mix 0.01*M* ammonium acetate:methanol (19:1). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 0.5 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale.

(d) *Preparation of working standard solution.* Transfer the contents of an ampoule of working standard to a tared weighing bottle. Place the unstoppered weighing bottle in a desiccator containing a saturated aqueous solution of potassium carbonate to provide an atmosphere of 42 percent relative humidity. Allow the moisture content of the working standard to equilibrate for 16 hours. Determine the moisture content as described in § 436.201 of this chapter. Equilibrated standard material must be kept in a closed weighing bottle and used within 36 hours of equilibration. Dissolve approximately 50 milligrams of the working standard, accurately weighed and corrected for moisture, with sufficient distilled water to obtain a solution containing 0.5 milligram of

moxalactam per milliliter. Use the prepared solution immediately.

(e) *Preparation of sample solution.* Mix contents of vial thoroughly. Dissolve an accurately weighed portion of approximately 50 milligrams of sample with distilled water to obtain a concentration of 0.5 milligram per milliliter (estimated); also, reconstitute the sample as directed in the labeling. Then using a suitable hypodermic needle and syringe, remove all of the withdrawable contents if it is represented as a single dose container; or, if the labeling specifies the amount of potency in a given volume of the resultant preparation, remove an accurately measured representative portion from each container. Further dilute an aliquot of this solution with distilled water to obtain a concentration of 0.5 milligram per milliliter (estimated). Use the prepared solution immediately.

(f) *Procedure.* Using the equipment, reagents, and operating conditions as listed in paragraphs (a), (b), and (c) of this section, inject 5 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the working standard solution has been completed, inject 5 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(g) *Calculations.* (1) Calculate the moxalactam content in micrograms per milligram as follows:

$$\text{Micrograms of moxalactam per milligram of sample} = \frac{R_u \times W_s \times P}{R_s \times W_u}$$

where:

R_u=Sum of the areas of the moxalactam sample R-isomer and the S-isomer peaks;

R_s=Sum of the areas of the moxalactam working standard R-isomer and the S-isomer peaks;

W_u=Weight of the sample in milligrams;

W_s=Weight of the moxalactam working standard in milligrams;

P=Potency of the moxalactam working standard in micrograms per milligram, corrected for moisture.

(2) Calculate the moxalactam content of the vial as follows:

$$\text{Milligrams of moxalactam per vial} = \frac{R_u \times W_s \times P \times d}{R_s \times 100{,}000}$$

where:

R_u=Sum of the areas of the moxalactam R-isomer and the S-isomer peaks;

R_s=Sum of the areas of the moxalactam working standard R-isomer and the S-isomer peaks;

W_s=Weight of the moxalactam working standard in milligrams;

P=Potency of the moxalactam working standard in micrograms per milligram, corrected for moisture;

d=Dilution factor.

(3) Calculate the ratio of R-isomer to S-isomer as follows:

$$\text{Ratio of R-isomer to S-isomer} = \frac{\text{Area of the R-isomer peak}}{\text{Area of the S-isomer peak}}$$

[46 FR 61069, Dec. 15, 1981]

§ 436.333 Thin layer chromatographic identity test for moxalactam.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, equipped with a glass solvent trough in the bottom and a tight-fitting cover for the top. Line the inside walls of the tank with Whatman #3MM chromatographic paper or equivalent.

(2) *Plates.* Use a 20x20 centimeter thin layer chromotagraphy plate coated with silca gel G or equivalent to a thickness of 250 micrometers.

(b) *Developing solvent.* Mix ethyl acetate, glacial acetic acid, acetonitrile, and water in volumetric proportions of 42:14:14:18, respectively.

(c) *Preparation of spotting solutions.* Prepare solutions of the sample and working standard, each containing 10 milligrams per milliliter of moxalactam in distilled water.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Prepare a plate as follows: On a line 2 centimeters from the base of the silica gel plate, and at intervals of 2 centimeters, spot 10 microliters each of the standard solution and the sample solution. After all spots are thoroughly dry, place the silica gel plate directly into the glass trough. Cover and seal the tank. Allow the solvent front to travel about 15 centimeters from the starting line. Remove the plate from the tank and air dry. Expose the plate to iodine vapors for 40 minutes. Immediately circumscribe all spots using a suitable marker.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values and intensity.

[46 FR 61070, Dec. 15, 1981, as amended at 49 FR 2242, Jan. 19, 1984]

§ 436.334 High-pressure liquid chromatographic assay for piperacillin.

(a) *Equipment.* A high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4-centimeter deflection;

(5) A suitable integrator;

(6) A 25-centimeter column having an inside diameter of 4.6 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter (United States Pharmacopeia XX).

(b) *Reagents.* (1) 0.2*M* monobasic sodium phosphate: Dissolve 27.60 grams of monobasic sodium phosphate with sufficient water to make 1,000 milliliters.

(2) 10 percent tetrabutylammonium hydroxide in water.

(3) Ampicillin-piperacillin solution: Dissolve and dilute 25 milligrams of ampicillin and 5 milligrams of piperacillin monohydrate with sufficient mobile phase to obtain 100 milliliters, and mix.

(c) *Mobile phase.* Methanol:water:0.2*M* monobasic sodium phosphate:10 percent tetrabutylammonium hydroxide (450:447:100:3) adjusted to pH 5.5±0.02 with phosphoric acid. The concentration of reagents may be varied to obtain acceptable operation of the system. De-gas the mobile phase just prior to its introduction into the chromatograph pumping system.

(d) *Preparation of working standard and sample solutions*—(1) *Working standard solution.* Place approximately 20 milligrams of the working standard, accurately weighed, into a 50-milliliter volumetric flask. Add 25 to 30 milliliters of mobile phase. Shake until dissolved. Dilute to volume with mobile phase.

(2) *Sample solution*—(i) *Micrograms per milligram.* Place approximately 20 milligrams of the sample, accurately weighed, into a 50-milliliter volumetric flask. Add 25 to 30 milliliters of mobile phase. Shake until dissolved. Dilute to volume with mobile phase.

(ii) *Milligrams per vial.* Reconstitute as directed in the labeling. Withdraw the total contents and dilute with mobile phase to a concentration of 0.4 milligram of piperacillin per milliliter.

(e) *Procedure.* Use the equipment, reagents, mobile phase, and working standard and sample solutions described in paragraphs (a), (b), (c), and (d) of this section and proceed as directed in paragraph (e) of this section.

(1) *Systems suitability test.* Chromatograph three replicate samples of ampicillin-piperacillin solution as directed in paragraph (e)(2) of this section. Allow an elution time sufficient to obtain satisfactory separation of expected components after each injection. Record the peak responses and calculate the resolution factor as described for system suitability tests in the United States Pharmacopeia XX General Chapter 621 for gas chromatography. The resolution factor between ampicillin and piperacillin is not

less than 15. If the resolution factor does not meet this limit, adjustments must be made to the system to obtain satisfactory operation before proceeding as described in paragraph (e)(2) of this section.

(2) *Determination of the chromatogram.* Operate the high-pressure liquid chromatograph at ambient temperature at a flow rate of one milliliter per minute. Use a detector sensitivity setting that gives a peak height for the reference standard that is at least 50 percent of scale. Purge the column with mobile phase until a steady baseline is established. Inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(f) *Calculations*—(1) Calculate the piperacillin content in micrograms per milligram as follows:

$$\text{Micrograms of piperacillin per milligram of sample} = \frac{A \times \text{Weight of standard in milligrams} \times \text{Potency of working standard in micrograms per milligram}}{B \times \text{Weight of sample in milligrams}}$$

where:
A=Area of the sample peak (at a retention time equal to that observed for the standard);
B=Area of the standard peak.

(2) Calculate the piperacillin content in grams per vial as follows:

$$\text{Grams of piperacillin per vial} = \frac{A \times \text{Milligrams of standard in milligrams per milliliter} \times \text{Potency of working standard in micrograms per milligram} \times d}{B \times 1{,}000 \times 1{,}000}$$

where:
A=Area of the sample peak (at a retention time equal to that observed for the standard);
B=Area of the standard peak;
d=Dilution factor.

[47 FR 15768, Apr. 13, 1982; 47 FR 33493, Aug. 3, 1982]

§ 436.335 High-pressure liquid chromatographic assay for chloramphenicol palmitate.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 280 nanometers;

(4) A suitable recorder of at least 25.4-centimeter deflection;

(5) A suitable integrator; and

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter, U.S.P. XX.

(b) *Mobile phase.* Mix methanol:water:glacial acetic acid (170:30:1). Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the reference standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the initial baseline.

(d) *Preparation of sample and working standard solutions.* Accurately weigh approximately 65 milligrams of sample

or chloramphenicol palmitate working standard each into a 50-milliliter volumetric flask. Add approximately 35 milliliters of methanol and 1 milliliter of glacial acetic acid. Place in an ultrasonic bath for 10 minutes and dilute to volume with methanol.

(e) *Procedure.* Using the equipment, mobile phase, and operating conditions listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(f) *Calculations.* Calculate the chloramphenicol content as follows:

$$\frac{\text{Micrograms of chloramphenicol}}{\text{per milligram}} = \frac{(A)(W_s)(f)}{(B)(W_u)}$$

where:

A=Area of chloramphenicol palmitate sample peak (at a retention time equal to that observed for the standard);

B=Area of the working standard peak;

W_s=Weight of standard in milligrams;

W_u=Weight of sample in milligrams; and

f=Micrograms of chloramphenicol activity per milligram of chloramphenicol palmitate working standard.

[49 FR 6091, Feb. 17, 1984]

§ 436.336 Thin layer chromatographic identity test for azlocillin.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, equipped with a glass solvent trough in the bottom and a tight-fitting cover for the top.

(2) *Iodine vapor chamber.* A rectangular tank approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, with a suitable cover, containing iodine crystals.

(3) *Plates.* Use 20 x 20 centimeter thin layer chromatography plates coated with Silica Gel G or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Buffer.* Dissolve 9.078 grams of potassium phosphate, monobasic (KH_2PO_4) in sufficient distilled water to make 1,000 milliliters (solution A). Dissolve 17.88 grams of sodium phosphate, dibasic, heptahydrate ($Na_2HPO_4.7H_2O$) in sufficient distilled water to make 1,000 milliliters (solution B). Place 12.1 milliliters of solution B into a 100-milliliter volumetric flask and dilute to volume with solution A.

(2) *Developing solvent.* Place 50 milliliters of *n*-butyl acetate, 9 milliliters of *n*-butanol, 25 milliliters of glacial acetic acid, and 15 milliliters of buffer into a separatory funnel. Shake well and allow the layers to separate. Discard the lower phase and use the upper phase as the developing solvent.

(c) *Preparation of spotting solutions.* Prepare solutions of the sample and working standard, each containing 20 milligrams of azlocillin per milliliter in distilled water.

(d) *Procedure.* Pour developing solvent into the glass trough on the bottom of the chromatography tank to a depth of about 1 centimeter. Use the chamber immediately. Prepare plate as follows: Apply spotting solutions on a line 2.5 centimeters from the base of the silica gel plate and at points 2.0 centimeters apart. Apply approximately 10 microliters of the working standard solution to points 1 and 3. When these spots are dry, apply approximately 10 microliters of sample solution to points 2 and 3. Place spotted plate in a desiccator until solvent has evaporated from spots. Place the plate into the glass trough at the bottom of the chromatography tank. Cover the tank. Allow the solvent to travel about 15 centimeters from the starting line. Remove the plate from the tank and allow to air dry. Warm the iodine vapor chamber to vaporize the iodine crystals and place the dry plate in the iodine vapor chamber until the spots are visible, usually about 10 minutes.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The azlocillin sample and the standard should have spots of corresponding R_f values (approximately 0.4), and standard and sample combined should appear as a single spot for

azlocillin. The penicilloate and penilloate of azlocillin as well as ampicillin appear as additional spots with R_f values of approximately 0.15, 0.3, and 0.25, reectively.

[47 FR 53348, Nov. 26, 1982]

§ 436.337 High-pressure liquid chromatographic assay for cephradine.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 8 millimeters;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder that is compatible with the detector output;

(5) A suitable integrator (optional); and

(6) A 25-centimeter column having an inside diameter of 4.6 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 10 micrometers in diameter, U.S.P. XX.

(b) *Reagents.* (1) 4 percent glacial acetic acid.

(2) 3.86 percent sodium acetate.

(c) *Mobile phase.* 4 percent glacial acetic acid:3.86 percent sodium acetate:methanol:distilled water (3:15:200:782). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase prior to its introduction into the chromatograph pumping system. The distilled water:methanol ratio may be varied to obtain acceptable operation of the system.

(d) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.2 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the cephradine in the cephradine working standard that is about 75 percent of full scale.

(e) *Preparation of working standard and sample solutions*—(1) *Preparation of cephradine working standard solution.* Place an accurately weighed portion of the cephradine working standard into a suitably sized container. Add 5.0 milliliters of distilled water and place in an ultrasonic bath to facilitate dissolution. Dilute with a sufficient amount of mobile phase to obtain a solution containing 0.8 milligram of cephradine activity per milliliter.

(2) *Preparation of cephalexin working standard solution.* Dissolve an accurately weighed portion of the cephalexin working standard with mobile phase to obtain a solution containing 0.02 milligram of cephalexin activity per milliliter. Place in an untrasonic bath to facilitate dissolution.

(3) *Preparation of sample solutions*—(i) *Product not packaged for dispensing (micrograms of cephradine per milligram*). Dissolve an accurately weighed portion of the sample with mobile phase to obtain a solution containing 0.8 milligram per milliliter. Place in an ultrasonic bath to facilitate dissolution. Using this sample solution, proceed as directed in paragraph (f)(1) of this section.

(ii) *Product packaged for dispensing.* Determine both micrograms of cephradine per milligram of the sample and milligrams of cephradine per container. Use separate containers for preparation of each sample solution as described in paragraphs (e)(3)(ii) (*a*) and (*b*) of this section.

(*a*) *Micrograms of cephradine per milligram.* Dissolve an accurately weighed portion of the sample with mobile phase to obtain a solution containing 0.8 milligram per milliliter. Place in an ultrasonic bath to facilitate dissolution. Using this sample solution, proceed as directed in paragraph (f)(1) of this section.

(*b*) *Milligrams of cephradine per container.* Reconstitute the sample as directed in the labeling. Then, using a suitable hypodermic needle and syringe, remove all of the withdrawable contents if it is represented as a single-dose container; or, if the labeling specifies the amount of potency in a given volume of the resultant preparation, remove an accurately measured representative portion from each container. Dilute the solution thus obtained with mobile phase to obtain a solution containing 0.8 milligram per milliliter. Using this sample solution,

proceed as directed in paragraph (f)(1) of this section.

(f) *Procedure*—(1) *Cephradine content.* Using the equipment, reagents, mobile phase, and operating conditions as listed in paragraphs (a), (b), (c), and (d) of this section, inject 10 microliters of the cephradine working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution prepared as described in paragraph (e)(3)(i) of this section into the chromatograph and repeat the procedure described for the working standard solution. The elution order is void volume, cephalexin, and cephradine. If the sample is packaged for dispensing, repeat the procedure for each sample solution prepared as described in paragraphs (e)(3)(ii) (*a*) and (*b*) of this section.

(2) *Cephalexin content.* Proceed as directed in paragraph (f)(1) of this section, except:

(i) Use a detector sensitivity setting that gives a peak height for the cephalexin in the cephalexin working standard that is about 75 percent of full scale; and

(ii) Use the cephalexin working standard in lieu of the cephradine working standard.

(g) *Calculations.* (1) Calculate the micrograms of cephradine per milligram of sample as follows:

$$\text{Micrograms of cefoperazone per milligram of milligram} = \frac{A_u \times P_s \times 100}{A_s \times C_u \times (100 - m)}$$

where:

A_u=Area of the cephradine peak in the chromatogram of the sample (at a retention time equal to that observed for the standard);

A_s=Area of the cephradine peak in the chromatogram of the cephradine working standard;

P_s=Cephradine activity in the cephradine working standard solution in micrograms per milliliter;

C_u=Milligrams of sample per milliliter of sample solution; and

m= Percent moisture content of the sample.

(2) Calculate the cephradine content of the vial as follows:

$$\text{Milligrams of cefoperazone per vial} = \frac{A_u \times P_s \times d}{A_s \times 1{,}000}$$

where:

A_u=Area of the cephradine peak in the chromatogram of the sample (at a retention time equal to that observed for the standard);

A_s=Area of the chephradine peak in the Chromatogram of the cephradine working standard;

P_s=Cephradine activity in the cephradine working standard solution in micrograms per milliliter;

C_s=Milligrams of the standard per milliliter; and

d=Dilution factor of the sample.

(3) Calculate the percent cephalexin content of the sample as follows:

$$\text{Percent cephalexin} = \frac{A_a \times W_b \times P_b \times 10}{A_b \times W_u \times (100 - m)}$$

where:

A_a=Area of the cephalexin peak in the chromatogram of the sample (at a retention time equal to that observed for the standard);

A_b=Area of the cephalexin peak in the chromatogram of the cephalexin working standard;

W_b=Milligrams of cephalexin per milliliter of cephalexin working standard solution;

W_u=Milligrams of cephradine per milliliter of sample solution;

P_b=Micrograms of cephalexin per milligram of cephalexin working standard; and

m=Percent moisture content of the sample.

[49 FR 47483, Dec. 5, 1984]

§ 436.338 High-pressure liquid chromatographic assay for cefoperazone.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A suitable integrator;

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and

packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter, United States Pharmacopeia XX.

(b) *Mobile phase.* Mix 1.2 milliliters 1*M* triethylammonium acetate, 2.8 milliliters 1*M* acetic acid, and 120 milliliters acetonitrile in a one liter flask and dilute to volume with distilled water. Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatographic pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale.

(d) *Preparation of working standard solution*. Dissolve approximately 40 milligrams of working standard, accurately weighed, with mobile phase to obtain a solution containing 0.16 milligram of cefoperazone activity per milliliter.

(e) *Preparation of sample solutions*—(1) *Product not packaged for dispensing (micrograms of cefoperazone per milligram*). Dissolve an accurately weighed portion of the sample with sufficient mobile phase to obtain a solution containing 0.16 milligram of cefoperazone activity per milliliter. Using this sample solution, proceed as directed in paragraph (f) of this section.

(2) *Product packaged for dispensing.* Determine both micrograms of cefoperazone per milligram of the sample and milligrams of cefoperazone per container. Use separate containers for preparation of each sample solution as described in paragraph (e)(2)(i) and (ii) of this section.

(i) *Micrograms of cefoperazone per milligram.* Dissolve and accurately weighed portion of the sample with sufficient mobile phase to obtain a solution containing 0.16 milligram of cefoperazone activity per milliliter. Using this sample solution, proceed as directed in paragraph (f) of this section.

(ii) *Milligrams of cefoperazone per container.* Reconstitute the sample as directed in the labeling. Then using a suitable hypodermic needle and syringe, remove all of the withdrawable contents if it is represented as a single-dose container; or, if the labeling specifies the amount of potency in a given volume of the resultant preparation, remove an accurately measured representative portion from each container. Further dilute and aliquot of this solution with mobile phase to a concentration of 0.16 milligram of cefoperazone activity per milliliter. Using this sample solution, proceed as directed in paragraph (f) of this section.

(f) *Procedure.* Using the equipment, reagents, and operating conditions as listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution prepared as described in paragraph (e)(1) of this section into the chromatograph and repeat the procedure described for the working standard solution. If the sample is packaged for dispensing, repeat the procedure for each sample solution prepared as described in paragraphs (e)(2)(i) and (ii) of this section.

(g) *Calculations*—(1) Calculate the micrograms of cefoperazone per milligram of sample as follows:

$$\text{Micrograms of cefoperazone per milligram} = \frac{A_u \times P_s \times 100}{A_s \times C_u \times (100 - m)}$$

where:

A_u=Area of the cefoperazone sample peak (at a retention time equal to that observed for the standard);

A_s=Area of the cefoperazone working standard peak;

P_s=Cefoperazone activity in the cefoperazone working standard solution in micrograms per milliliter;

C_u=Milligrams of sample per milliliter of sample solution; and

m= Percent moisture content of the sample.

(2) Calculate the cefoperazone content of the vial as follows:

$$\text{Milligrams of cefoperazone per vial} = \frac{A_u \times P_s \times d}{A_s \times 1{,}000}$$

where:

A_u=Area of the cefoperazone sample peak (at a retention time equal to that observed for the standard);

A_s=Area of the cefoperazone working standard peak;

P_s=Cefoperazone activity in the cefoperazone working standard solution in micrograms per milliliter; and

d=Dilution factor of the sample.

[48 FR 789, Jan. 7, 1983; 48 FR 7439, Feb. 22, 1983; 48 FR 28250, June 21, 1983]

§ 436.339 High-pressure liquid chromatographic assay for bleomycin fractions.

(a) *Equipment.* A high-pressure liquid chromatograph equipped with:

(1) Two solvent pumps;

(2) A solvent programmer;

(3) A low dead volume cell 8 to 20 microliters;

(4) A light path length of 1 centimeter;

(5) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(6) A suitable recorder;

(7) A suitable integrator; and

(8) A suitable-sized column approximately 25 centimeters in length having an inside diameter of 4.6 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter, USP XX.

(b) *Reagents*—(1) *0.005M 1-pentanesulfonic acid in 0.5 percent acetic acid adjusted to pH 4.3 with concentrated ammonium hydroxide.* Filter and degas before using.

(2) *Methanol, spectrophotometric grade.* Filter and degas before using.

(3) *Mobile phase.* Adjust the solvent programmer for linear gradient development starting with a mixture of 0.005*M* 1-pentanesulfonic acid:methanol (9:1) and ending with a mixture of 0.005*M* 1-pentanesulfonic acid:methanol (6:4) in 1 hour at a flow rate of 1.2 milliliters per minute. Minor flow rate and gradient changes can be made as necessary depending on column and instrument conditions. Disodium ethylenediaminetetraacetic acid USP at a concentration of 0.005*M* may be added to the mobile phase if necessary for satisfactory performance.

(c) *Preparation of sample solution.* Reconstitute the vial with 6 milliliters of deaerated water.

(d) *Procedure.* Using the equipment and reagents listed in paragraphs (a) and (b) of this section, start pumping the mobile solvent at the initial conditions. Inject 10 microliters of the sample solution into the chromatograph and begin the linear gradient pumping program. After the final mobile phase conditions are reached (1 hour) continue to pump the solvent mixture for an additional 20 minutes or until the demethylbleomycin A_2 is eluted. The elution order is void volume, bleomycinic acid, bleomycin A_2, bleomycin A_5, bleomycin B_2, bleomycin B_4, and demethylbleomycin A_2.

(e) *Calculations.* Calculate the percentage of each bleomycin by comparing its peak area contribution to that of the total response of all the bleomycins.

[48 FR 51912, Nov. 15, 1983]

§ 436.340 High-pressure liquid chromatographic assay for tetracycline hydrochloride content and 4-epitetracycline hydrochloride content.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4-centimeter deflection;

(5) A suitable integrator; and

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles.

(b) *Mobile phase.* Dissolve 0.55 gram of monobasic ammonium phosphate in 900 milliliters of water. Adjust the pH to 1.8 with concentrated phosphoric acid and dilute to 1 liter with water. Mix 800 milliliters of this solution with 200 milliliters of methanol. Filter the mobile

phase through a suitable glass fiber filter that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatography pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.0 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the 4-epitetracycline peak that is at least 50 percent of scale.

(d) *Preparation of working standard and sample solutions*—(1) *Working standard solution.* Accurately weigh approximately 18 milligrams of the tetracycline hydrochloride working standard into a 50-milliliter volumetric flask. Into the same flask, accurately weigh approximately 38 milligrams of the 4-epitetracycline working standard. Dissolve and dilute to volume with a methanol:water mixture (7:18).

(2) *Sample solution.* Reconstitute the sample as directed in the labeling. Transfer 10.0 milliliters of the reconstituted sample into a 50-milliliter volumetric flask and dilute to volume with a methanol:water mixture (7:18).

(e) *Procedure.* Using the equipment, reagents, and operating conditions as listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution. The elution order is 4-epitetracycline followed by tetracycline.

(f) *Calculations.* Calculate the tetracycline hydrochloride and 4-epitetracycline hydrochloride content as follows:

$$\text{Milligrams of tetracycline hydrochloride per milliliter of sample} = \frac{A_1[(W_1 \times B)+(W_e \times C)]}{A_2 \times 1{,}000}$$

$$\text{Milligrams of 4-epitetracycline hydrochloride per milliliter of sample} = \frac{A_3[(W_e \times D)+(W_t \times E)]}{A_4 \times 1{,}000}$$

where:

A_1=Area of the tetracycline sample peak (at a retention time equal to that observed for tetracycline in the tetracycline working standard);

A_2=Area of the tetracycline peak in the tetracycline working standard;

A_3=Area of the 4-epitetracycline sample peak (at a retention time equal to that observed for the 4-epitetracycline peak in the 4-epitetracycline working standard);

A_4=Area of the 4-epitetracycline peak in the 4-epitetracycline working standard;

W_t=Milligrams of the tetracycline working standard;

W_e=Milligrams of the 4-epitetracycline working standard;

B=Percent tetracycline hydrochloride in the tetracycline working standard;

C=Percent tetracycline hydrochloride in the 4-epitetracycline working standard;

D=Percent 4-epitetracycline hydrochloride in the 4-epitetracycline working standard; and

E=Percent 4-epitetracycline hydrochloride in the tetracycline working standard.

[48 FR 51290, Nov. 8, 1983]

§ 436.341 High-pressure liquid chromatographic assay for plicamycin.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 280 nanometers;

(4) A suitable recorder of at least 25.4-centimeter deflection;

(5) A suitable integrator; and

(6) A 25-centimeter column having an inside diameter of 4.6 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 micrometers to 10 micrometers in diameter, U.S.P. XX.

(b) *Reagents*—(1) 0.01*M* phosphoric acid.

(2) *Mobile phase.* Mix acetonitrile (high-pressure liquid chromatography grade):0.01*M* phosphoric acid (350:650). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.0 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale.

(d) *Preparation of working standard and sample solutions*—(1) *Preparation of working standard solution.* Place approximately 5 milligrams of the plicamycin working standard, accurately weighed, into a 50-milliliter, amber volumetric flask and dilute to volume with mobile phase and mix.

(2) *Preparation of sample solution.* Prepare the sample solution as described in the individual monograph for the drug being tested.

(e) *Procedure.* Use the equipment, reagents, operating conditions, and working standard and sample solutions described in paragraphs (a), (b), (c), and (d) of this section, and proceed as directed in paragraph (e)(1) of this section.

(1) *System suitability test.* Equilibrate and condition the column by passage of about 10 to 15 void volumes of mobile phase followed by two or more replicate injections of the working standard solution. Allow an elution time sufficient to obtain satisfactory separation of expected components after each injection. Record the peak responses and calculate the relative standard deviation as described for system suitability tests in the U.S.P. XX General Chapter 621 chromatography. Proceed as directed in paragraph (e)(2) of this section if the minimum performance requirement for the relative standard deviation is not more than 2.0 percent. If the minimum performance requirement is not met, adjustment must be made to the system to obtain satisfactory operation before proceeding as described in paragraph (e)(2) of this section.

(2) *Determination of the chromatogram.* Inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. After separation of the working standard has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(f) *Calculations.* Calculate the plicamycin content as described in the individual monograph for the drug being tested.

[49 FR 24017, June 11, 1984, as amended at 50 FR 5749, Feb. 12, 1985]

§ 436.342 High-pressure liquid chromatographic assay for cefazolin.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection; and

(5) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter, USP XX.

(b) *Reagents*—(1) *Buffer solution, pH 3.6.* Transfer 0.9 gram of sodium phosphate, dibasic USP and 1.298 grams of citric acid USP to a 1-liter volumetric flask. Dissolve and dilute to volume with distilled water and mix.

(2) *Buffer solution, pH 7.0.* Transfer 5.68 grams of sodium phosphate, dibasic

USP and 3.63 grams of potassium phosphate monobasic to a 1-liter volumetric flask. Dissolve and dilute to volume with distilled water and mix.

(3) *Mobile phase.* Mix buffer solution, pH 3.6: acetonitrile (9:1). Filter through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(4) *Internal standard solution.* Transfer 1.2 grams of salicylic acid to a 200-milliliter volumetric flask. Dissolve in 10 milliliters of methyl alcohol, dilute to volume with buffer solution, pH 7.0, and mix.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 2 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the initial baseline.

(d) *Preparation of working standard and sample solutions*—(1) *Working standard solution.* Place approximately 50 milligrams of cefazolin working standard, accurately weighed, into a 50-milliliter volumetric flask. Dissolve and dilute to volume with buffer solution, pH 7.0, and mix. Transfer 4.0 milliliters of this solution to a 200-milliliter volumetric flask, add 5.0 milliliters of internal standard solution, dilute to volume with buffer solution, pH 7.0, and mix.

(2) *Sample solution.* Place approximately 50 milligrams of the sample, accurately weighed, into a 50-milliliter volumetric flask. Dissolve and dilute to volume with buffer solution, pH 7.0, and mix. Transfer 4.0 milliliters of this solution to a 200-milliliter volumetric flask, add 5.0 milliliters of internal standard solution, dilute to volume with buffer solution, pH 7.0, and mix.

(e) *Procedure.* Using the equipment, mobile phase, and operating conditions listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution prepared as directed in paragraph (d)(1) of this section into the chromatograph. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution prepared as described in paragraph (d)(2) of this section into the chromatograph and repeat the procedure described for the working standard solution. Allow an elution time sufficient to obtain satisfactory separation of the expected components. The elution order is void volume, salicylic acid and cefazolin.

(f) *Calculation.* Calculate the micrograms of cefazolin per milligram of sample as follows:

$$\text{Micrograms of cefazolin per milligram} = \frac{R_u \times P_s \times 100}{R_s \times C_u \times (100 - m)}$$

where:

R_u = Area of the cefazolin peak in the chromatogram of the sample (at a retention time equal to that observed for the standard) /Area of internal standard peak;

R_s = Area of the cefazolin peak in the chromatogram of the cefazolin working standard/Area of internal standard peak;

P_s = Cefazolin activity in the cefazolin working standard solution in micrograms per milliliter;

C_u = Milligrams of sample per milliliter of sample solution; and

m = Percent moisture content of the sample.

[48 FR 33478, July 22, 1983; 48 FR 34947, Aug. 2, 1983]

§ 436.343 High-pressure liquid chromatographic assay for cefuroxime.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A suitable integrator; and

(6) A 15-centimeter column having an inside diameter of 4.6 millimeters and packed with hexyl silane chemically bonded to porous silica or ceramic microparticles, 5 micrometers in diameter.

(b) *Reagents*—(1) *Acetate buffer, pH 3.4.* Place 50 milliliters of 0.1*M* sodium acetate into a 1,000-milliliter volumetric flask and dilute to volume with 0.1*M* acetic acid. Mix.

(2) *Mobile phase.* Mix 0.1*M* acetate buffer, pH 3.4:acetonitrile (10:1). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(3) *Internal standard solution.* Prepare a 1.5 milligram per milliliter solution of orcinol monohydrate in water.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale.

(d) *Preparation of working standard and sample solutions*—(1) *Preparation of working standard solution.* Dissolve an accurately weighed portion of the cefuroxime working standard with sufficient distilled water to obtain a stock solution containing 1.0 milligram of cefuroxime per milliliter. Immediately transfer 5.0 milliliters of the stock solution to a 100-milliliter volumetric flask, add 20.0 milliliters of internal standard solution and dilute to 100 milliliters with distilled water and mix. Store the solution in a refrigerator and use within 6 hours.

(2) *Preparation of sample solutions*—(i) *Product not packaged for dispensing (micrograms of cefuroxime per milligram).* Dissolve an accurately weighed portion of the sample with sufficient distilled water to obtain a stock solution containing 1.0 milligram of cefuroxime per milliliter. Immediately transfer 5.0 milliliters of the stock solution to a 100-milliliter volumetric flask, add 20.0 milliliters of internal standard solution and dilute to 100 milliliters with distilled water and mix. Store the solution in a refrigerator and use within 6 hours. Using this sample solution, proceed as directed in paragraph (e) of this section.

(ii) *Product packaged for dispensing.* Determine both micrograms of cefuroxime per milligram of the sample and milligrams of cefuroxime per container. Use separate containers for preparation of each sample solution as described in paragraphs (d)(2)(ii) (*a*) and (*b*) of this section.

(*a*) *Micrograms of cefuroxime per milligram.* Dissolve an accurately weighed portion of the sample with sufficient distilled water to obtain a stock solution containing 1.0 milligram of cefuroxime per milliliter. Immediately transfer 5.0 milliliters of the stock solution to a 100-milliliter volumetric flask, add 20.0 milliliters of internal standard solution and dilute to 100 milliliters with distilled water and mix. Store the solution in a refrigerator and use within 6 hours. Using this sample solution, proceed as directed in paragraph (e) of this section.

(*b*) *Milligrams of cefuroxime per container.* Reconstitute the sample as directed in the labeling. Then using a suitable hypodermic needle and syringe, remove all of the withdrawable contents if it is represented as a single-dose container; or, if the labeling specifies the amount of potency in a given volume of the resultant preparation, remove an accurately measured representative portion from each container. Dilute the solution thus obtained with distilled water to obtain a stock solution of 1.0 milligram per milliliter. Immediately transfer 5.0 milliliters of the stock solution to a 100-milliliter volumetric flask, add 20.0 milliliters of internal standard solution and dilute to 100 milliliters with distilled water and mix. Store the solution in a refrigerator and use within 6 hours. Using this sample solution, proceed as directed in paragraph (e) of this section.

(e) *Procedure.* Using the equipment, reagents, and operating conditions as listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution prepared as described in paragraph (d)(2)(i) of this section into the chromatograph and repeat the procedure described for the working standard solution. If the sample is packaged for dispensing, repeat the procedure for each sample solution prepared as described in paragraphs (d)(2)(ii)(*a*) and (d)(2)(ii)(*b*) of this section.

(f) *Calculations.* (1) Calculate the micrograms of cefuroxime per milligram of sample as follows:

$$\text{Micrograms of cefuroxime per milligram} = \frac{R_u \times P_s \times 100}{R_s \times C_u \times (100 - m)}$$

where:

R_u=Area of the cefuroxime peak in the chromatogram of the sample (at a retention time equal to that observed for the standard)/Area of internal standard peak;

R_s=Area of the cefuroxime peak in the chromatogram of the cefuroxime working standard/Area of internal standard peak;

P_s=Cefuroxime activity in the cefuroxime working standard solution in micrograms per milliliter;

C_u=Milligrams of sample per milliliter of sample solution; and

m=Percent moisture content of the sample.

(2) Calculate the cefuroxime content of the vial as follows:

$$\text{Milligrams of cefuroxime per vial} = \frac{R_u \times P_s \times d}{R_s \times 1{,}000}$$

where:

R_u=Area of the cefuroxime peak in the chromatogram of the sample (at a retention time equal to that observed for the standard)/Area of internal standard peak;

R_s=Area of the cefuroxime peak in the chromatogram of the cefuroxime working standard/Area of internal standard peak;

P_s=Cefuroxime activity in the cefuroxime working standard solution in micrograms per milliliter; and

d= Dilution factor of the sample.

[48 FR 38460, Aug. 24, 1983; 48 FR 40704, Sept. 9, 1983]

§ 436.344 Thin layer chromatographic identity test for cefuroxime.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23×23×9 centimeters, with a glass solvent trough on the bottom and a tight-fitting cover. Line the inside walls of the tank with Whatman #3MM chromatographic paper or equivalent.

(2) *Plates.* Use 20 × 20 centimeter thin layer chromatography plates coated with Silica Gel F or equivalent to a thickness of 250 microns.

(b) *Developing solvent.* Mix chloroform, methanol, and formic acid in volumetric proportions of 90:16:4, respectively.

(c) *Preparation of the spotting solutions.* Dissolve approximately 200 milligrams each of the working standard and sample in 5 milliliters of a 50 percent aqueous acetone solution.

(d) *Procedure.* Pour the developing solvent into the glass trough at the bottom of the chromatography tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Prepare a plate as follows: On a line 2 centimeters from the base of the plate, and at intervals of 2 centimeters, spot 5 microliters each of the sample and working standard solutions. After all spots are thoroughly dry, place the plate directly into the glass trough of the chromatography tank. Cover and seal the tank tightly. Allow the solvent front to travel a minimum of 15 centimeters from the starting line. Remove the plate from the tank and allow it to air dry. Observe under ultraviolet light (254 nanometers).

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values.

[48 FR 38461, Aug. 24, 1983]

§ 436.345 High-pressure liquid chromatographic assay for ceftizoxime.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A suitable integrator; and

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 to 10 micrometers in diameter, USP XX.

(b) *Reagents*—(1) *pH 3.6 buffer solution.* Transfer 2.31 grams of sodium phosphate diabasic dodecahydrate and 1.42 grams of citric acid monohydrate to a

1-liter volumetric flask. Dissolve and dilute to volume with distilled water.

(2) *pH 7.0 buffer solution.* Transfer 14.33 grams of sodium phosphate dibasic dodecahydrate and 3.63 grams of potassium phosphate monobasic to a 1-liter volumetric flask. Dissolve and dilute to volume with distilled water.

(3) *Mobile phase.* Mix pH 3.6 buffer solution:acetonitrile (9:1). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(4) *Internal standard solution.* Place 1.2 grams of salicyclic acid in a 200-milliliter volumetric flask. Dissolve in 10 milliliters of methyl alcohol, dilute to volume with pH 7.0 buffer solution and mix.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale.

(d) *Preparation of working standard solution.* Dissolve an accurately weighed portion of the ceftizoxime working standard with sufficient pH 7.0 buffer solution to obtain a solution containing 1,000 micrograms of ceftizoxime activity per milliliter. Transfer 2.0 milliliters of this solution to a 100-milliliter volumetric flask, add 5.0 milliliters of internal standard solution, dilute to volume with pH 7.0 buffer solution and mix.

(e) *Preparation of sample solutions*—(1) *Product not packaged for dispensing (micrograms of ceftizoxime per milligram).* Dissolve an accurately weighed portion of the sample with sufficient pH 7.0 buffer solution to obtain a concentration of 1.0 milligram per milliliter. Transfer 2.0 milliliters of this solution to a 100-milliliter volumetric flask, add 5.0 milliliters of internal standard solution, dilute to volume with pH 7.0 buffer solution and mix. Using this sample solution, proceed as directed in paragraph (f) of this section.

(2) *Product packaged for dispensing.* Determine both micrograms of ceftizoxime per milligram of the sample and milligrams of ceftizoxime per container. Use separate containers for preparation of each sample solution as described in paragraphs (e)(2) (i) and (ii) of this section.

(i) *Micrograms of ceftizoxime per milligram.* Dissolve an accurately weighed portion of the sample with sufficient pH 7.0 buffer solution to obtain a concentration of 1.0 milligram of ceftizoxime per milliliter. Transfer 2.0 milliliters of this solution to a 100-milliliter volumetric flask, add 5.0 milliliters of internal standard solution, dilute to volume with pH 7.0 buffer solution and mix. Using this sample solution, proceed as directed in paragraph (f) of this section.

(ii) *Milligrams of ceftizoxime per container.* Reconstitute the sample as directed in the labeling. Then using a suitable hypodermic needle and syringe, remove all of the withdrawable contents if it is represented as a single-dose container; or, if the labeling specifies the amount of potency is a given volume of the resultant preparation, remove an accurately measured representative portion from each container. Further dilute an aliquot of the solution thus obtained with sufficient pH 7.0 buffer solution to obtain a concentration of 1.0 milligram per milliliter. Transfer 2.0 milliliters of this solution to a 100-milliliter volumetric flask, add 5.0 milliliters of internal standard solution, dilute to volume with pH 7.0 buffer solution and mix. Using this sample solution, proceed as directed in paragraph (f) of this section.

(f) *Procedure.* Using the equipment, reagents, and operating conditions as listed in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. The elution order is void volume, ceftizoxime, and internal standard. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution prepared as described in paragraph (e)(1) of this section into the chromatograph and repeat the procedure described for the working standard solution. If the sample is packaged for

dispensing, repeat the procedure for each sample solution prepared as described in paragraphs (e)(2) (i) and (ii) of this section.

(g) *Calculations*—(1) Calculate the micrograms of ceftizoxime per milligram of sample as follows:

$$\text{Micrograms of ceftizoxime per milligram} = \frac{R_u \times P_s \times 100}{R_s \times C_u \times (100 - m)}$$

where:

R_u=Area of the ceftizoxime peak in the chromatogram of the sample (at a retention time equal to that observed for the standard)/Area of internal standard peak;

R_s=Area of the ceftizoxime peak in the chromatogram of the ceftizoxime working standard/Area of internal standard peak;

P_s=Ceftizoxime activity in the ceftizoxime working standard solution in micrograms per milliliter;

C_u=Milligrams of sample per milliliter of sample solution; and

m=Percent moisture content of the sample.

$$\text{Milligrams of ceftizoxime per vial} = \frac{R_u \times P_s \times d}{R_s \times 1{,}000}$$

where:

R_u=Area of the ceftizoxime peak in the chromatogram of the sample (at a retention time equal to that observed for the standard)/Area of internal standard peak;

R_s=Area of the ceftizoxime peak in the chromatogram of the ceftizoxime working standard/Area of internal standard peak;

P_s=Ceftizoxime activity in the ceftizoxime working standard solution in micrograms per milliliter; and

d=Dilution factor of the sample.

[48 FR 46270, Oct. 12, 1983; 48 FR 49656, Oct. 27, 1983]

§ 436.346 High-pressure liquid chromatographic assay for cyclosporine.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A suitable pump capable of reproducibly delivering a liquid to a pressure of 4,500 pounds per square inch and a flow rate of at least 5 milliliters per minute;

(2) A suitable ultraviolet detection system operating at a wavelength of 210 nanometers;

(3) A suitable recorder;

(4) A suitable integrator;

(5) An oven or water bath capable of maintaining the column at an operating temperature of 70° C;

(6) A steel capillary tube, 1 meter in length, having an inside diameter of 0.25 millimeter. This tube is inserted between the injection system and the chromatographic column and is equilibrated to 70° C; and

(7) A sample injection valve on which the loop determines the sample size.

(b) *Columns.* The chromatographic column is packed with microparticulate (3 to 10 micrometers in diameter) reversed phase packing materials that exhibit some degree of polarity such as the hydrocarbon bonded silicas with dimethyl, trimethyl, or octyl groups. Connect a saturation column gravity packed with similarly bonded silica particles 40 to 60 microns in diameter to the inlet of the analytical column.

(c) *Mobile phase.* Mix acetonitrile, water, methanol, and *o*-phosphoric acid (900:525:75:0.075 by volume). Degas by passing through a 0.5-micrometer filter with vacuum and ultrasonicate for no less than 2 minutes before use. The mobile phase may be sparged perceptibly with helium through a 2-micrometer metal filter for the duration of the analysis. Adjust the ratio of acetonitrile to aqueous buffer as necessary to obtain satisfactory retention of the peaks.

(d) *Operating conditions.* Perform the assay at a constant operating temperature of 70° C with a typical flow rate of 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with a typical chart speed of 2.5 millimeters per minute. Obtain chromatograms for performance parameters at a chart speed of not less than 25 millimeters per minute to allow a more accurate measurement of peak geometry.

(e) *Preparation of working standard and sample solutions.* Prepare the working standard and sample solutions as directed in the individual monographs for cyclosporine.

(f) *Systems suitability.* Equilibrate and condition the column by passage of about 10 to 15 void volumes of mobile phase followed by about 5 injections of not less than 10 microliters each of working standard solution. Proceed

with the analysis when the following minimum performance requirements have been met or exceeded.

(1) *Capacity ratio factor.* Calculate the capacity ratio (k) of the cyclosporine peak as follows:

$$k = \frac{t - t_m}{t_m}$$

where:

t=Retention time of solute; and

t_m=Retention time of solvent or unretained substance.

The capacity ratio is satisfactory if it is not less than 3 or not more than 10.

(2) *Coefficient of variation.* The coefficient of variation of at least five replicate injections is less than 1 percent.

(3) *Efficiency.* Calculate the efficiency (n) as follows:

$$n = 5.545\left(\frac{t}{W_{0.5}}\right)^2$$

where:

t=Retention time of solute; and

$W_{0.5}$=Peak width at half height. Both t and $W_{0.5}$ must be measured in the same units.

The efficiency is satisfactory if it is greater than 1,500 theoretical plates when assaying cyclosporine and greater than 700 theoretical plates when assaying finished dosage forms.

(4) *Asymmetry factor.* Calculate the asymmetry factor (A_s) as follows:

$$A_s = \frac{W_{0.1}}{2f}$$

where:

$W_{0.1}$=Horizontal distance measured from a point on the cyclosporine peak ascent 10 percent above the baseline to an intercept with the cyclosporine peak descent; and

f=Horizontal distance from point of 10 percent ascent above the baseline of the cyclosporine peak to point of maximum peak height.

The asymmetry factor is satisfactory if it is not more than 1.5.

(5) *Resolution.* Calculate the resolution (R_s) as follows:

$$R_s = \frac{2\ (t_j - t_i)}{(W_i + W_j)}$$

where:

t=Retention time of solute; and the subscripts i and j designate two different peaks and where t_j is larger than t_i; and

W=Width of peak at baseline as determined by extrapolating the relative straight sides to the baseline. Both t and W must be measured in the same units.

Resolution between the cyclosporine peak and any other peak must be at least 1.1.

(g) *Procedure.* Using the equipment, columns, mobile phase, operating conditions and the working standard and sample solutions listed in paragraphs (a), (b), (c), (d), and (e) of this section, inject 20 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of expected components. After separation of the working standard solution has been completed, inject 20 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(h) *Calculations.* Calculate the cyclosporine content of cyclosporine and its dosage forms as directed in the individual monographs.

[49 FR 22631, May 31, 1984; 49 FR 27489, July 5, 1984]

§ 436.347 High-pressure liquid chromatographic assay for cefoxitin.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4 centimeter deflection;

(5) A suitable integrator; and

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 micrometers to 10 micrometers in diameter, U.S.P. XX.

(b) *Reagents*—(1) *One percent potassium phosphate buffer, pH 6.0.* Prepare as described in § 436.101(a)(1).

(2) *Mobile phase.* Mix distilled water:glacial acetic acid:acetonitrile (800:10:190). Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.0 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale. The minimum between peaks must be no more than 2 millimeters above the baseline.

(d) *Preparation of working standard and sample solutions.* Use the working standard and sample solutions prepared as described in the individual monographs for the drug being tested.

(e) *Procedure.* Using the equipment, reagents, and operating conditions as described in paragraphs (a), (b), and (c) of this section, inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(f) *Calculations.* Calculate the cefoxitin content as described in the individual monographs for the drug being tested.

[49 FR 47827, Dec. 7, 1984]

§ 436.348 High-pressure liquid chromatographic assay for ceforanide.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A low dead volume cell 8 to 20 microliters;

(2) A light path length of 1 centimeter;

(3) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(4) A suitable recorder of at least 25.4-centimeter deflection;

(5) A suitable integrator; and

(6) A 30-centimeter column having an inside diameter of 4.0 millimeters and packed wth octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 micrometers to 10 micrometers in diameter, U.S.P. XX. A particular column used for analysis of ceforanide should not be used for the analysis of other drugs.—

(b) *Mobile phase.* Mix 18.0 milliliters of 10 percent aqueous tetrabutylammonium hydroxide and 8.56 milliliters of 11*N* potassium hydroxide. Add the mixture to approximately 700 milliliters of distilled water. Add 200 milliliters of reagent grade methanol. Adjust the pH of the mixture to pH 7.0 with concentrated phosphoric acid and dilute to 1,000 milliliters with distilled water. Prepare fresh daily. Filter the mobile phase through a suitable glass fiber filter or equivalent which is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase just prior to its introduction into the chromatograph pumping system.

(c) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale.

(d) *Preparation of working standard and sample solutions*—(1) *Preparation of working standard solution.* Prepare a solution containing 1,000 micrograms of ceforanide activity per milliliter in mobile phase. Inject working standard solution within 5 minutes after dissolution.

(2) *Preparation of sample solution.* Prepare the sample solution as directed in the individual monograph for the drug being tested. Inject sample solution within 5 minutes after dissolution.

(e) *Procedure.* Use the equipment, mobile phase, operating conditions, and working standard and sample solutions described in paragraphs (a), (b), (c), and (d) of this section, and proceed as directed in paragraph (e)(1) of this section.

(1) *System suitability test.* Equilibrate and condition the column by passage of about 10 to 15 void volumes of mobile

phase followed by three replicate injections of 10 microliters each of the working standard solution. Allow an elution time sufficient to obtain satisfactory separation of expected components after each injection. Record the peak responses and calculate the tailing factor, efficiency of the column, coefficient of variation, and capacity factor as described for system suitability tests in the U.S.P. XX General Chapter 621 chromatography. Proceed as directed in paragraph (e)(2) of this section if the following minimum performance requirements have been met:

(i) *Tailing factor.* The tailing factor is satisfactory if it is not more than 1.2;

(ii) *Efficiency of the column.* The efficiency of the column is satisfactory if it is greater than 1,900 theoretical plates;

(iii) *Coefficient of variation.* The coefficient of variation of at least three replicate injections is satisfactory if it is not more than 1.5 percent; and

(iv) *Capacity factor.* The capacity factor is satisfactory if it is not less than 1.8 and not more than 5.

If the minimum performance requirements are not met, adjustments must be made to the system to obtain satisfactory operation before proceeding as described in paragraph (e)(2) of this section.

(2) *Determination of the chromatogram.* Inject 10 microliters of the working standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the working standard solution has been completed, inject 10 microliters of the sample solution into the chromatograph and repeat the procedure described for the working standard solution.

(f) *Calculations.* Calculate the ceforanide content as directed in the individual monograph for the drug being tested.

[49 FR 25846, June 25, 1984; 49 FR 34347, Aug. 30, 1984]

§ 436.349 High-pressure liquid chromatographic assay for L-lysine in ceforanide for injection.

(a) *Equipment.* A suitable high-pressure liquid chromatograph equipped with:

(1) A suitable pump capable of reproducibly delivering a liquid to a pressure of 5,000 pounds per square inch;

(2) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers;

(3) A suitable recorder;

(4) A suitable integrator; and

(5) A 25-centimeter column having an inside diameter of 4.6 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles, 5 micrometers to 10 micrometers in diameter, U.S.P. XX.

(b) *Reagents*—(1) 2,4-Dinitrofluorobenzene solution. Weigh accurately approximately 760 milligrams of 2,4-dinitrofluorobenzene into a 50-milliliter volumetric flask. Dissolve and dilute to volume with absolute ethyl alcohol.

(2) Tris (hydroxymethyl) aminomethane (THAM) solution. Weigh accurately approximately 1.44 grams of THAM into a 100-milliliter volumetric flask. Dissolve and dilute to volume with distilled water.

(c) *Mobile phase.* Mix methanol and water (62:38), and adjust to pH 3.0 with glacial acetic acid.

(d) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of 1.5 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the standard that is at least 50 percent of scale with a typical chart speed of 0.2 inch per minute.

(e) *Preparation of standard and sample solutions*—(1) *Preparation of standard solution.* Weigh accurately approximately 36 milligrams of *L*-lysine used as the standard into a 100-milliliter volumetric flask. Dissolve and dilute to volume with distilled water. Transfer 2.0 milliliters of the *L*-lysine solution into a 10-milliliter volumetric flask, add 2.0 milliliters of THAM solution and 3.0 milliliters of 2,4-dinitrofluorobenzene solution. Cap tightly and mix well. Place the flask in a 50° C water bath for 30 minutes. Remove from water bath, allow the flask to cool to room temperature, and dilute to volume with methanol. Mix well.

(2) *Preparation of sample solution.* Weigh accurately approximately 150

milligrams of the sample, ceforanide for injection, into a 100-milliliter volumetric flask. Dissolve and dilute to volume with distilled water. Transfer 2.0 milliliters of the sample solution into a 10-milliliter volumetric flask, add 2.0 milliliters of THAM solution and 3.0 milliliters of 2,4-dinitrofluorobenzene solution. Cap tightly and mix well. Place the flask in a 50° C water bath for 30 minutes. Remove from water bath, allow the flask to cool to room temperature, and dilute to volume with methanol. Mix well.

(f) *Procedure.* Use the equipment, reagents, mobile phase, operating conditions, and standard and sample solutions described in paragraphs (a), (b), (c), (d), and (e) of this section, and proceed as directed in paragraph (f)(1) of this section.

(1) *System suitability test.* Equilibrate and condition the column by passage of about 10 to 15 void volumes of mobile phase followed by three replicate injections of 20 microliters each of the standard solution. Allow an elution time sufficient to obtain satisfactory separation of the expected components after each injection. Record the peak responses and calculate the resolution factor, tailing factor, efficiency of the column, coefficient of variation, and capacity factor as described for system suitability tests in the U.S.P. XX General Chapter 621 chromatography. Proceed as directed in paragraph (f)(2) of this section if the following minimum performance requirements have been met:

(i) *Resolution factor.* The resolution factor between the peak for derivatized *L*-lysine and from the peak for the dinitrofluorobenzene derivatizing reagent is satisfactory if it is not less than 4.5;

(ii) *Tailing factor.* The tailing factor is satisfactory if it is not more than 1.3;

(iii) *Efficiency of the column.* The efficiency of the column is satisfactory if it is greater than 1,500 theoretical plates;

(iv) *Coefficient of variation.* The coefficient of variation of at least three replicate injections is satisfactory if it is not more than 1.5 percent; and

(v) *Capacity factor.* The capacity factor is satisfactory if it is not less than 4 and not more than 6.

If the minimum performance requirements are not met, adjustments must be made to the system to obtain satisfactory operation before proceeding as described in paragraph (f)(2) of this section.

(2) *Determination of the chromatogram.* Inject 20 microliters of the standard solution into the chromatograph. Allow an elution time sufficient to obtain satisfactory separation of the expected components. After separation of the standard solution is completed, inject 20 microliters of the sample solution into the chromatograph and repeat the procedure described for the standard solution.

(g) *Calculations.* Calculate the percent of *L*-lysine per milligram of ceforanide for injection as follows:

$$\text{Percent of } L\text{-lysine} = \frac{A_u \times P_s}{A_s \times C_u \times 10}$$

where:

A_u = Area of the *L*-lysine peak in the chromatogram of the sample (at a retention time equal to that observed for the standard);

A_s = Area of the *L*-lysine peak in the chromatogram of the *L*-lysine standard:

P_s = *L*-lysine content in the *L*-lysine standard solution in micrograms per milliliter; and

C_u = Milligrams of sample per milliliter of sample solution.

[49 FR 25846, June 25, 1984; 49 FR 34347, Aug. 30, 1984; 49 FR 40006, Oct. 12, 1984]

§ 436.350 High-performance liquid chromatographic assay for cefonicid.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in

that monograph, the inlet of this column may be connected to a guard column 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested with a flow rate not to exceed 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the apparatus described in paragraph (a) of this section; and the reagents and working standard and sample solutions described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements as follows:

(c) *System suitability test.* Using the apparatus and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$ = Width of peak at 5 percent height; and
f = Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left[\frac{t_R}{W_h}\right]^2$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of solute; and
w_h=Peak width at half-height.

(3) *Resolution factor.* Calculate the resolution factor (R), between desacetyl cefonicid and cefonicid, as follows:

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where:
t_1=Retention time of desacetyl cefonicid;
t_2=Retention time of cefonicid; and
w_1 and w_2=Widths of the bases of the corresponding peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

(4) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:
$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using the sample solution in lieu of the working standard solution.

[49 FR 34347, Aug. 30, 1984, as amended at 49 FR 44460, Nov. 7, 1984; 50 FR 29209, July 18, 1985]

§ 436.351 High-performance liquid chromatographic assay for amoxicillin and clavulanic acid.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 10 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested with a flow rate not to exceed 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the apparatus described in paragraph (a) of this section; and the reagents and working standard and sample solutions described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. The retention times for amoxicillin and clavulanic acid are about 2.1 and 1.0 minutes, respectively, under these prescribed conditions. Record the peak responses and calculate the prescribed system suitability requirements as follows:

(c) *System suitability test.* Using the apparatus and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factors for the amoxicillin and clavulanic acid peaks.* Calculate the tailing factors (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$=Width of peak at 5 percent height; and
f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left[\frac{t_R}{W_h}\right]^2$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of amoxicillin or clavulanic acid peaks; and
w_h=Corresponding peak width at half-height.

(3) *Resolution factor.* Calculate the resolution factor (R) as follows:

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where:
t_1=Retention time of amoxicillin peak;
t_2=Retention time of clavulanic acid peak; and
w_1 and w_2=Widths of the bases of the corresponding peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

(4) *Coefficient of variation (Relative standard deviation).* Calculate the coefficient of variation (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:
$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using the sample solution in lieu of the working standard solution.

[49 FR 39671, Oct. 10, 1984]

§ 436.352 High-performance liquid chromatographic assay for determining clavam-2-carboxylate content in clavulanate potassium.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, approximately 30 centimeters in length, packed with a material as defined in the monograph for the drug being tested.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested with a flow rate not to exceed 0.5 milliliter per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the apparatus described in paragraph (a) of this section; and the mobile phase and working standard and sample solutions described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. The retention times for clavam-2-carboxylic acid and clavulanic acid are about 10 and 14 minutes, respectively, under these prescribed conditions. The sample solution should be injected at least in duplicate and an average should be taken. For each such series of samples injected, two injections of standard should be made, one before and one after the sample series, and an average should be taken. Record the peak responses and calculate the prescribed system suitability requirements as follows:

(c) *System suitability test.* Using the apparatus and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$=Width of peak at 5 percent height; and

f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left[\frac{t_R}{W_h}\right]^2$$

where:

n=Efficiency, as number of theoretical plates for column;

t_R=Retention time of clavam-2-carboxylic acid peak; and

w_h=Corresponding peak width at half-height.

(3) *Resolution factor.* Calculate the resolution factor (R) as follows:

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where:

t_1=Retention time of clavam-2-carboxylic acid peak;

t_2=Retention time of clavulanic acid peak; and

w_1 and w_2=Widths of the bases of the corresponding peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

(4) *Coefficient of variation (Relative standard deviation).* Calculate the coefficient of variation (S_R

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:
$\overline{X}$ is the mean of N individual measurements of X_i

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using the sample solution in lieu of the working standard solution.

[49 FR 39671, Oct. 10, 1984]

§ 436.353 High-performance liquid chromatographic assay for amdinocillin.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested with a flow rate not to exceed 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the apparatus described in paragraph (a) of this section; and the reagents and working standard and sample solutions described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements as described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Using the apparatus and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$=Width of peak at 5 percent height; and
f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column by either of the following formulas:

$$n = 5.545\left[\frac{t_R}{W_h}\right]^2; \text{ or}$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of solute;
w_h=Peak width at half-height; and
W=Width of the base of the peak obtained by extrapolating the relatively straight sides of the peak to the baseline.

(3) *Resolution factor.* Calculate the resolution factor (R) as follows:

$$R = \frac{2(t_{Rj} - t_{Ri})}{w_i + w_j}$$

where:
t_{Rj}=Retention time for a solute eluting after i (t_{Rj} is larger than t_{Ri});
t_{Ri}=Retention time for any solute;
w_i=Width of peak at baseline for any solute; and
w_j=Width of peak at baseline for any solute eluting after i.

(4) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R

$$S_R = \frac{100}{\overline{X}} \left(\frac{\sum_{i=1}^{n} \left(X_i - \overline{X} \right)^2}{N-1} \right)^{1/2}$$

where:
$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using the sample solution in lieu of the working standard solution.

[50 FR 7764, Feb. 26, 1985; 50 FR 10220, Mar. 14, 1985; 50 FR 18243, Apr. 30, 1985]

§ 436.354 High-performance liquid chromatographic assay for ceftriaxone.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested.

(b) *Procedure.* Perform the assay at the temperature specified in the monograph for the drug being tested with a flow rate not to exceed 2.0 milliliters per minute, Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale. Use the apparatus described in paragraph (a) of this section; and also, use the system suitability requirements, reagents, working standard, test and sample solutions, and calculations as directed in the individual monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of 20 microliters each of the test solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements as described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Using the apparatus and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Capacity factor.* Calculate the capacity factor (k) as follows:

$$k = \frac{t_R - t_M}{t_M}$$

where:
t_R=Retention time of solute; and
t_M=Retention time of solvent or unretained substance.

(2) *Resolution.* Calculate the resolution (R) as follows:

$$R = \frac{2(t_{Rj} - t_{Ri})}{w_i + w_j}$$

where:
t_{Rj}=Retention time for a solute eluting after i (t_{Rj} is larger than t_{Ri});
t_{Ri}=Retention time for any solute;
w_i=Width of peak at baseline for any solute; and
w_j=Width of peak at baseline for any solute eluting after i.

(3) *Asymmetry factor.* Calculate the asymmetry factor (A_s

$$A_s = \frac{a+b}{2a}$$

where:
a=Horizontal distance from point of ascent to point of maximum peak height; and

b=Horizontal distance from point of maximum peak height to point of descent.

(4) *Efficiency of the column.* Calculate the efficiency of the column (reduced plate height) (h_r

(i)

$$n = 5.545\left(\frac{t_R}{W_h}\right)^2;$$

(ii)

$$h = \frac{L}{n}; \text{ and}$$

(iii)

$$h_r = \frac{h}{d_p}.$$

where:

n=Efficiency, as number of theoretical plates for column;

t_R=Retention time of solute;

w_h=Peak width at half-height;

h=Efficiency, as height equivalent to one theoretical plate;

L=Length of column; and

d_p=Average diameter of particle in column.

(5) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:

$\overline{X}$ is the mean of N individual measurements of X_i.

The complete operating system is acceptable for assay if it meets the system suitability requirements of the monograph for the drug being tested. If the complete operating system is acceptable, proceed as described in paragraph (b) of this section using the sample solution in lieu of the test solution. Calculate the drug content as described in the individual monograph for the drug being tested.

[50 FR 9999, Mar. 13, 1985]

§ 436.355 High-performance liquid chromatographic assay for ticarcillin-clavulanic acid.

(a) *Equipment.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being testing;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 10 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested with a flow rate not to exceed 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the equipment described in paragraph (a) of this section; and the reagents and working standard and sample solutions described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. The clavulanic acid peak is sharp and the chromatograms of standard and sample solutions show baseline separations between it and any neighboring peaks. The retention times for clavulanic acid and ticarcillin are approximately 3 minutes and 14 minutes, respectively. Record the peak responses and calculate the prescribed system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Using the equipment and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factors for the ticarcillin and clavulanic acid peaks.* Calculate the tailing factors (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$=Width of peak at 5 percent height; and

f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left(\frac{t_R}{W_h}\right)^2$$

where:

n=Efficiency, as number of theoretical plates for column;

t_R=Retention time of ticarcillin or clavulanic acid peaks; and

w_h=Corresponding peak width at half-height.

(3) *Resolution factor.* Calculate the resolution factor (R) as follows

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where:

t_1=Retention time of clavulanic acid peak;

t_2=Retention time of ticarcillin peak; and

w_1 and w_2=Widths of the bases of the corresponding peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

When using the method to assay clavulanic acid alone, the resolution factor is not applicable.

(4) *Coefficient of variation (Relative standard deviation).* Calculate the coefficient of variation (S_R

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:

$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using the sample solution in lieu of the working standard solution.

[50 FR 33517, Aug. 20, 1985; 50 FR 43384, Oct. 25, 1985]

§ 436.356 High-performance liquid chromatographic assay for ceftazidime.

(a) *Equipment.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, flow rate, and calculations specified in the monograph for the drug being tested. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute.

Use the equipment described in paragraph (a) of this section. Use the reagents, working standard solution, and sample solution described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution for the system suitability test. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Select the system suitability requirements specified in the monograph for the drug being tested. Then, using the equipment and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$=Width of peak at 5 percent height; and
f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left(\frac{t_R}{w_h}\right)^2$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of solute; and
w_h=Peak width at half-height.

(3) *Resolution.* Calculate the resolution (R) as follows:

$$R = \frac{2(t_{Rj} - t_{Ri})}{w_i + w_j}$$

where:
t_{Rj}=Retention time of a solute eluting after i (t_{Rj} is larger than t_{Ri});
t_{Ri}=Retention time of any solute;
w_i=Width of peak at baseline measured by extrapolating the relatively straight sides to the baseline of any solute; and
w_j=Width of peak at baseline measured by extrapolating the relatively straight sides to the baseline of any solute eluting after i.

(4) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:
$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, except alternate injections of the working standard solution with injections of the sample solution.

[50 FR 48397, Nov. 25, 1985]

§ 436.357 Atomic absorption test for sodium carbonate content.

(a) *Equipment.* A suitable atomic absorbance spectrophotometer equipped with:

(1) A suitable sodium hollow-cathode discharge lamp;

(2) An oxidizing air-acetylene flame;

(3) A nebulizer-burner system;

(4) An optical dispersing device capable of isolating a resonance line of sodium from other wavelengths produced by the emission source; and

(5) A suitable radiation detector.

(b) *Ionization buffer.* Dissolve and dilute 19.07 grams of potassium chloride in distilled water to 1,000 milliliters.

(c) *Preparation of reference standard and sample solutions*—(1) *Reference*

standard solution. Accurately weigh approximately 140 milligrams of sodium chloride, which has been previously dried for 40 to 50 minutes at a temperature of 500 to 650 ° C. Dissolve and dilute with sufficient distilled water to obtain a stock solution containing 5.5 micrograms of sodium per milliliter. Mix 10 milliliters of the stock solution with 10 milliliters of ionization buffer and dilute the mixture with distilled water to obtain a solution containing 0.55 microgram of sodium per milliliter.

(2) *Sample solution*. Dilute the stock sample solution, prepared as directed in the monograph for the drug being tested, with distilled water to obtain a solution containing 5.5 micrograms of sodium per milliliter (estimated). Mix 10 milliliters of this solution with 10 milliliters of ionization buffer and dilute the mixture with distilled water to obtain a solution containing 0.55 microgram of sodium per milliliter (estimated).

(3) *Procedure*. Determine the atomic absorbance of the reference standard and sample solutions at a wavelength of 589 nanometers, using the atomic absorbance spectrophotometer and a reagent blank prepared by diluting 10 milliliters of ionization buffer to 100 milliliters with distilled water.

(d) *Calculations*. Calculate the percent sodium carbonate (S) as follows:

$$\text{Percent sodium carbonate} = \frac{A_u \times P_s \times 2.304}{A_s \times C_u \times 10}$$

where:

A_u=Absorbance of sodium in the sample solution;

A_s=Absorbance of sodium in the reference standard solution;

P_s=Sodium concentration in the reference standard solution in micrograms per milliliter; and

C_u=Milligrams of sample per milliliter of sample solution.

[50 FR 48398, Nov. 25, 1985, as amended at 54 FR 20785, May 15, 1989]

§ 436.358 High-performance liquid chromatographic assay for pyridine.

(a) *Equipment*. A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 15 to 25 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure*. Perform the assay and calculate the pyridine content using the temperature, instrumental conditions, flow rate, and calculations specified in the monograph for the drug being tested. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 25 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the equipment described in paragraph (a) of this section. Use the reagents, working standard solution, and sample solution described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed for the system suitability test by five replicate injections of the same volume (between 10 and 20 microliters) of the system suitability test solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test*. Select the system suitability requirements specified in the monograph for the drug being tested. Then, using the equipment and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor*. Calculate the tailing factor for the pyridine peak (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$=Width of peak at 5 percent height; and

f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Resolution.* Calculate the resolution (R) as follows:

$$R = \frac{2(t_{Rj} - t_{Ri})}{w_i + w_j}$$

where:

t_{Rj}=Retention time of *t*-butyl ceftazidime;

t_{Ri}=Retention time of pyridine;

w_i=Width of pyridine peak at the baseline measured by extrapolating the relatively straight sides to the baseline; and

w_j=Width of *t*-butyl ceftazidime peak at the baseline measured by extrapolating the relatively straight sides to the baseline.

(3) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation for the pyridine peak (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:

$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, except alternate injections of the working standard solution with injections of the sample solution.

[50 FR 48398, Nov. 25, 1985; 50 FR 53308, Dec. 31, 1985]

§ 436.360 Gel permeation chromatographic assay for high molecular weight polymer.

(a) *Equipment.* A suitable gel permeation chromatograph equipped with.

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 50 centimeters long and 9 millimeters internal diameter, packed with a material as defined in the monograph for the drug being tested.

(b) *Procedure.* Perform the assay and calculate the high molecular weight polymer content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 10 percent of scale with a typical chart speed of not less than 2.5 millimeters per minute. Use the equipment described in paragraph (a) of this section. Use the reagents, working standard solution, and sample solution described in the monograph for the drug being tested. Equilibrate and condition the column by passage of mobile phase for not less than 18 hours, removing any voids that may form at the top of the column, followed by five replicate injections of the same volume (100 microliters) of the blue dextran system suitability test solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Select the system suitability requirements specified in the monograph for the drug being tested. Then, using the equipment and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:

$W_{0.05}$=Width of peak at 5 percent height; and

f=Horizontal distance from point of ascent to

a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left(\frac{t_R}{w_h}\right)^2$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of solute; and
w_h=Peak width at half-height.

(3) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:
$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using two injections of the same volume (100 microliters) of the working standard solution followed by one injection of the same volume (100 microliters) of the sample solution.

[50 FR 48398, Nov. 25, 1985; 51 FR 1367, Jan. 13, 1986]

§ 436.361 High-performance liquid chromatographic assay for aztreonam.

(a) *Equipment.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 40 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, flow rate, and calculations specified in the monograph for the drug being tested. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the equipment described in paragraph (a) of this section. Use the reagents, working standard solution, and sample solution described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by five replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Select the system suitability requirements specified in the monograph for the drug being tested. Then, using the equipment and procedure described in this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$=Width of peak at 5 percent height; and
f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left[\frac{t_R}{W_h}\right]^2$$

where:

n=Efficiency, as number of theoretical plates for column;

t_R=Retention time of solute; and

w_h=Peak width at half-height.

(3) *Resolution.* Calculate the resolution (R) as follows:

$$R = \frac{2(t_{Rj} - t_{Ri})}{w_i + w_j}$$

where:

t_{Rj}=Retention time of a solute eluting after i (t_{Rj} is larger than t_{Ri});

t_{Ri}=Retention time of any solute;

w_i=Width of peak at baseline of any solute; and

w_j=Width of peak at baseline of any solute eluting after i.

(4) *Coefficient of variation (relative standard deviation).* Calculate the coefficient of variation (S_R in percent) as follows:

$$S_R = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{N-1}\right]^{1/2}$$

where:

$\overline{X}$ is the mean of N individual measurements of X_i.

If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, except alternate injections of the working standard solution with injections of the sample solution.

[52 FR 4611, Feb. 13, 1987; 52 FR 8550, Mar. 18, 1987]

§ 436.362 Thin-layer chromatographic test for free erythromycin content in erythromycin estolate bulk.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank approximately 23 centimeters long, 23 centimeters high, and 9 centimeters wide, equipped with a glass solvent trough in the bottom and a tight-fitting cover for the top.

(2) *Plates.* Use a 20- by 20-centimeter precoated silica gel 60 F–254 thin-layer chromatography plate. Before using, place the plate in an unlined developing chamber containing approximately 100 milliliters of anhydrous methanol and allow the solvent front to travel to the top of the plate, marking the direction of travel. Remove the plate and allow to drip dry. Store in a dry place.

(b) *Reagents*—(1) *Developing solvent.* Mix 15 milliliters of chloroform and 85 milliliters of anhydrous methanol. Use fresh developing solvent for each test.

(2) *Spray solution.* Dissolve 150 milligrams of xanthydrol in a mixture of 7.5 milliliters of glacial acetic acid and 92.5 milliliters of 37 percent hydrochloric acid.

(c) *Preparation of spotting solutions*—(1) *Sample solution.* Prepare a solution of the sample in anhydrous methanol to contain 10 milligrams per milliliter.

NOTE: It is advisable to prepare the sample and standard solutions immediately before spotting to minimize the possibility of degradation in solution.)

(2) *Standard solution.* Prepare a solution of erythromycin base reference standard in anhydrous methanol to contain 1 milligram per milliliter. Weigh 99.5, 99.0, and 97.0 milligrams of erythromycin estolate (propionyl erythromycin lauryl sulfate) reference standard and transfer to separate 10-milliliter volumetric flasks. To these flasks add 0.5, 1.0, and 3.0 milliliters, respectively, of the 1-milligram-per-milliliter solution of erythromycin base reference standard and dilute to volume with anhydrous methanol. These solutions contain, respectively, 0.5 percent, 1.0 percent, and 3.0 percent erythromycin base in erythromycin estolate. Prepare a solution of erythromycin estolate reference standard in anhydrous methanol to contain 10 milligrams per milliliter. Prepare a solution of erythromycin base reference standard in anhydrous methanol to contain 0.1 milligram per milliliter.

(d) *Procedure.* Pour 100 milliliters of developing solvent into the glass trough on the bottom of the unlined chromatography tank. Cover and seal the tank. Allow it to equilibrate while the plate is being prepared. Prepare a

plate as follows: On a line 2.0 centimeters from the base of the thin-layer plate, apply 1.0 microliter of each of the following solutions:

(1) 10-milligrams-per-milliliter solution of erythromycin estolate reference standard, equivalent to 10 micrograms of erythromycin estolate;

(2) 0.5 percent base-in-estolate solution, equivalent to 0.05 microgram of base and 9.95 micrograms of estolate;

(3) 1.0 percent base-in-estolate solution, equivalent to 0.10 microgram of base and 9.90 micrograms of estolate;

(4) 3.0 percent base-in-estolate solution, equivalent to 0.30 microgram of base and 9.70 micrograms of estolate;

(5) 0.1-milligram-per-milliliter solution of erythromycin base reference standard, equivalent to 0.1 microgram of erythromycin base; and

(6) Sample solution, equivalent to 10 micrograms of erythromycin estolate. Allow the spots to dry. Place the plate directly in the chromatograph tank. Cover and seal the tank. Allow the solvent front to travel a distance of 7 centimeters (about 27 minutes). Remove the plate from the tank, and allow it to air dry under a hood. With the plate still under the hood, spray uniformly with the spray solution. Heat the sprayed plate in an oven at 100 °C for 5 minutes. (CAUTION: Avoid exposure to the acid fumes while removing the plate from the oven.)

(e) *Evaluation.* Erythromycin base and erythromycin estolate appear as reddish-violet spots on the sprayed and heated plate. Better visualization of the erythromycin base spots may be gained by viewing the plate under long-wavelength (366 nanometers) ultraviolet light, erythromycin base appearing as dark spots on a yellow-green fluorescent background. Erythromycin base has an R_f value of about 0.3. Erythromycin estolate has an R_f value of about 0.7. Compare the size and intensity of any erythromycin base spots in the sample lane with the erythromycin base spots in the erythromycin base reference standard lane and in the 0.5 percent, 1.0 percent, and 3.0 percent base-in-estolate lanes, and report the percentage of erythromycin base (free erythromycin) in the sample. For a more accurate determination of free erythromycin content, it may be necessary to repeat the test using a different set of standards.

[53 FR 1919, Jan. 25, 1988]

§ 436.363 High-performance liquid chromatographic assay for cefmenoxime.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable detection system specified in the monograph for the drug being tested;

(2) A suitable recording device of at least 18-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) An analytical column, 3 to 30 centimeters long, packed with a material as defined in the monograph for the drug being tested; and if specified in that monograph, the inlet of this column may be connected to a guard column, 3 to 5 centimeters in length, packed with the same material of 30 to 60 micrometers particle size.

(b) *Procedure.* Perform the assay and calculate the drug content using the temperature, instrumental conditions, and calculations specified in the monograph for the drug being tested with a flow rate not to exceed 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the working standard that is at least 50 percent of scale with typical chart speed of not less than 2.5 millimeters per minute. Use the apparatus described in paragraph (a) of this section; and the reagents and working standard and sample solutions described in the monograph for the drug being tested. Equilibrate and condition the column by passage of 10 to 15 void volumes of mobile phase followed by 5 replicate injections of the same volume (between 10 and 20 microliters) of the working standard solution. Allow an operating time sufficiently long to obtain satisfactory separation and elution of the expected components after each injection. Record the peak responses and calculate the prescribed system suitability requirements described for the system suitability test in paragraph (c) of this section.

(c) *System suitability test.* Using the apparatus and procedure described in

this section, test the chromatographic system for assay as follows:

(1) *Tailing factor.* Calculate the tailing factor (T), from distances measured along the horizontal line at 5 percent of the peak height above the baseline, as follows:

$$T = \frac{W_{0.05}}{2f}$$

where:
$W_{0.05}$=Width of peak at 5 percent height; and
f=Horizontal distance from point of ascent to a point coincident with maximum peak height.

(2) *Efficiency of the column.* Calculate the number of theoretical plates (n) of the column as follows:

$$n = 5.545\left[\frac{t_R}{W_h}\right]^2$$

where:
n=Efficiency, as number of theoretical plates for column;
t_R=Retention time of solute; and
w_h=Peak width at half-height.

(3) *Resolution.* Calculate the resolution (R) as follows:

$$R = \frac{2\left(t_{RJ} - t_{Ri}\right)}{w_i + w_J}$$

where:
t_{RJ}=Retention time of a solute eluting after i (t_{RJ} is larger than t_{Ri});
t_{Ri}=Retention time of any solute;
w_i=Width of peak at baseline of any solute; and
w_J=Width of peak at baseline of any solute eluting after i.

(4) *Coefficient of variation (Relative standard deviation).*

Calculate the coefficient of variation (S_R) in percent) as follows:

$$\underline{S_R} = \frac{100}{\overline{\underline{X}}}\left[\frac{\sum_{\underline{i}=1}^{\underline{N}}(\underline{X_i} - \overline{\underline{X}})^2}{\underline{N} - 1}\right]^{\frac{1}{2}}$$

where:
X is the mean of N individual measurements of X_i. If the complete operating system meets the system suitability requirements of the monograph for the drug being tested, proceed as described in paragraph (b) of this section, using the sample solution in lieu of the working standard solution.

[53 FR 13401, Apr. 25, 1988; 53 FR 19368, May 27, 1988]

§ 436.364 Atomic absorption test for sodium carbonate content of cefmenoxime hydrochloride for injection.

(a) *Apparatus.* A suitable atomic absorbance spectrophotometer equipped with:

(1) A suitable sodium hollow-cathode discharge lamp;

(2) An oxidizing air-acetylene flame;

(3) A nebulizer-burner system;

(4) An optical dispersing device capable of isolating a resonance line of sodium from other wavelengths produced by the emission source; and

(5) A suitable radiation detector.

(b) *Reagents.* Ionization buffer: Dissolve 19.07 grams of potassium chloride in distilled water and dilute to 1,000 milliliters.

(c) *Preparation of reference standard and sample solutions*—(1) *Reference standard solution.* Accurately weigh approximately 140 milligrams of sodium chloride which has been previously dired for 40 to 50 minutes at a temperature of 500 to 650 ° C. Dissolve and dilute with sufficient distilled water to obtain a stock solution containing 5.5 micrograms of sodium per milliliter. Mix 10 milliliters of the stock solution with 10 milliliters of ionization buffer and dilute the mixture with distilled water to obtain a solution containing 0.55 microgram of sodium per milliliter.

(2) *Sample solution.* Dilute the sample solution used in §442.222(b)(1)(ii)(B)(*1*) of this chapter, with sufficient distilled water to obtain a stock solution containing 5.5 micrograms of sodium per milliliter (estimated). Mix 10 milliliters of the stock solution with 10 milliliters of ionization buffer and dilute the mixture with distilled water to obtain a solution containing 0.55 microgram of sodium per milliliter (estimated).

(3) *Procedure.* Determine the atomic absorbance of the reference standard and sample solutions at a wavelength

of 589 nanometers, using the atomic absorbance spectrophotometer and a reagent blank prepared by diluting 10 milliliters of ionization buffer to 100 milliliters with distilled water.

(d) *Calculations.* Calculate the percent sodium carbonate as follows:

$$\text{Percent sodium carbonate} = \frac{A_u \times P_s \times 100 \times 0.9068 \times d}{A_s \times C_u}$$

where:
A_u=Absorbance of sodium in the sample solution;
A_s=Absorbance of sodium in the reference standard solution;
P_s=Milligrams of sodium chloride per milliliter of the reference standard solution;
C_u=Milligrams of sample per milliliter of sample solution; and
d= Dilution factor of the sample.

[53 FR 13401, Apr. 25, 1988]

§ 436.365 Thin layer chromatographic identity test for rifampin.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23×23×9 centimeters, with a glass solvent trough on the bottom and a tight-fitting cover, lined with Whatman #3MM chromatographic paper or equivalent.

(2) *Plates.* Use 20×20 centimeter thin layer chromatography plates coated with silica gel 60 F–254 or equivalent to a thickness of 250 microns.

(b) *Developing solvent.* Mix chloroform and methanol in volumetric proportions of 90:10, respectively.

(c) *Spotting solutions*—(1) *Preparation of working standard solution.* Dissolve approximately 50 milligrams of rifampin working standard in 5 milliliters of chloroform.

(2) *Preparation of sample solution.* Dissolve the contents of a sample vial in 60 milliliters of chloroform.

(d) *Procedure.* Pour the developing solvent into the glass trough on the bottom of the tank and onto the paper lining the walls of the tank. Cover and seal the tank. Allow it to equilibrate. Prepare a plate as follows: On a line 2.5 centimeters from the base of the thin layer chromatography plate and at intervals of 2.0 centimeters, spot 3 microliters of the working standard solution to points 1 and 3. When these spots are dry, apply 3 microliters of the sample solution to points 2 and 3. After all the spots are thoroughly dry, place the plate into the trough in the bottom of the tank. Cover and tightly seal the tank. Allow the solvent front to travel about 7 centimeters from the starting line. Remove the plate from the tank and air dry.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line, and the distance the red spots are from the starting line. Divide the latter by the former to calculate the R_f value.

[54 FR 38375, Sept. 18, 1989; 54 FR 42886, Oct. 18, 1989]

§ 436.366 High-performance liquid chromatography assay for determining chromatographic purity of vancomycin.

(a) *Apparatus.* A suitable high-performance liquid chromatograph equipped with:

(1) A suitable ultraviolet detection system operating at a wavelength of 254 nanometers or preferably 280 nanometers;

(2) A suitable recording device of at least 25-centimeter deflection;

(3) A suitable chromatographic data managing system; and

(4) A 25-centimeter analytical column having an inside diameter of 4.6 millimeters and packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles; 5 micrometers in diameter.

(b) *Reagents*—(1) *0.2 percent triethylammonium phosphate buffer.* To 2,000 milliliters of distilled water, either add 4 milliliters of triethylamine or 4 grams of triethylammonium chloride. Adjust the pH to 3.2 with phosphoric acid.

(2) *Sample solvents.* (i) Vancomycin hydrochloride: Mobile Phase A.

(ii) Vancomycin base: 5 milliliters Mobile Phase A; add 0.1*N* HCl dropwise with swirling until sample dissolves: dilute to volume with Mobile Phase A.

(c) *Mobile Phases*—(1) *Mobile Phase A.* Add 70 milliliters of acetonitrile and 10 milliliters of tetrahydrofuran to 920 milliliters of 0.2 percent triethylammonium phosphate buffer and mix well. Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase, briefly, just prior to its introduction into the chromatographic pumping system.

(2) *Mobile Phase B.* Add 290 milliliters of acetonitrile and 10 milliliters of tetrahydofuran to 700 milliliters of 0.2 percent triethylammonium phosphate buffer and mix well. Filter the mobile phase through a suitable glass fiber filter or equivalent that is capable of removing particulate contamination to 1 micron in diameter. Degas the mobile phase, briefly, just prior to its introduction into the chromatographic pumping system.

(d) *Operating conditions.* Perform the assay at ambient temperature with a typical flow rate of about 2.0 milliliters per minute. Use a detector sensitivity setting that gives a peak height for the main peak (Vancomycin B) that is at least 50 percent of scale. The run time is 30 minutes per injection and the gradient conditions are as follows: (0, 12, 12.5, 8, 0, 2)

Time (minutes)	Mobile phase A (percent)	Mobile phase B (percent)	Gradient condition
0	100	0	Initial conditions.
12	100	0	Isocratic region.
20	0	100	Linear ramp.
22	0	100	Isocratic region.
23	100	0	Return to initial.
30	100	0	Reequilibration.

(e) *Preparation of resolution and sample solutions*—(1) *Resolution solution.* Prepare a solution of vancomycin hydrochloride reference standard in water containing 0.5 milligram per milliliter. Heat at 65 °C for 24 hours and allow to cool. This procedure generates two desamido-vancomycin isomers. The first desamido isomer elutes during the isocratic period and before the vancomycin B peak; the second desamido isomer elutes during the gradient ramp and is used to demonstrate the effective performance of this stage.

(2) *Sample preparation.* In a volumetric flask either dissolve a representative sample or dilute a representative portion with sample solvent to give a sample preparation containing approximately 10 milligrams per milliliter. Pipet 2 milliliters of this sample solution into a separate 50-milliliter volumetric flask and dilute to volume with sample solvent to give a diluted sample preparation containing approximately 0.4 milligram per milliliter.

(f) *Procedure.* Optimize chromatographic conditions under isocratic conditions by equilibrating the system while pumping 100 percent mobile phase A through the column. Inject 20 microliters of the resolution solution onto the column and record the chromatogram. Adjust the acetonitrile concentration of mobile phase A as needed to provide a retention time for vancomycin B of 7.5 to 10.5 minutes. Use the resolution solution to perform the system suitability tests. The elution order is resolution compound 1, vancomycin B, resolution compound 2. Return the system to the initial gradient operating conditions. Separately inject 20 milliliters of each diluted (0.4 milligram per milliliter) and concentrated (10 milligrams per milliliter) sample solution onto the column and record each chromatogram.

(g) *System suitability test.* Using the resolution solution described in paragraph (e)(1) of this section, test the performance of the chromatographic system as follows:

(1) *Asymmetry factor.* Calculate the asymmetry factor (*A*), measured at a point that is 10 percent of the vancomycin B peak height from the baseline, as follows:

$$A_s = \frac{a+b}{2a}$$

where:

a=Horizontal distance from point of ascent to point of maximum peak height; and

b=Horizontal distance from point of maximum peak height to point of descent.

The asymmetry factor (A_r) is satisfactory if it is not less than 0.8 and not more than 1.8.

(2) *Efficiency of the column.* From the number of theoretical plates (n) calculated as described in § 436.216(c)(2) calculate the reduced plate height (h_r) for the vancomycin B peak as follows:

$$h_r = \frac{(L)(10{,}000)}{(n)(d_p)}$$

where:

L=Length of the column in centimeters;

n=Number of theoretical plates; and

d_p=Average diameter of the particles in the column in micrometers.

The absolute efficiency (h_r) is satisfactory if it is not more than 40 for the vancomycin B peak in the resolution solution.

(3) *Resolution.* The resolution (R) between the vancomycin B peak and the peak for resolution compound 1 is not less than 3.0. Resolution compound 2 is eluted between 3 and 6 minutes after the start of the period when the percentage of mobile phase B is increasing from 0 percent to 100 percent.

(4) *Coefficient of variation (relative standard deviation).* The coefficient of variation (S_R in percent) of five replicate injections of the resolution solution is calculated as described in § 436.216(c)(4) is satisfactory if it is not more than 2.0 percent.

(5) *Capacity factor (k).* Calculate the capacity factor (k) for vancomycin B as follows:

$$k = \frac{t_r - t_m}{t_m}$$

where:

t_r=Retention time of solute; and

t_m=Retention time of solvent or unretained substance, calculated as follows:

$$t_m = \frac{(3.1416)(D^2)(L)(0.75)}{4F}$$

where:

D=Column diameter in centimeters;

L=Column length in centimeters;

0.75=Average total column porosity; and

F=Flow rate in milliliters per minute.

The capacity factor (k) for vancomycin B is satisfactory if it is not less than 2.6 and not more than 3.3.

When the system suitability requirements have been met, then proceed as described in paragraph (f) of this section. Alternate chromatographic conditions are acceptable provided that the system suitability parameters are met. However, the sample preparation described in paragraph (e)(2) of this section should not be changed.

(h) *Calculations.* (1) Calculate the percentage of vancomycin B in the specimen as follows:

$$\text{Percentage of vancomycin B} = \frac{A_B}{A_{Total}} \times 100 \text{ percent}$$

where:

A_B=Area of the vancomycin B peak in the dilute (0.4 milligram per milliliter) sample solution; and

A_{Total}=Area of the vancomycin B peak in the dilute (0.4 milligram per milliliter) solution+[Area of the total related substances peaks (exclude the area of the vancomycin B peak) in the concentrated solution (10 milligrams per milliliter) divided by 25].

(2) Calculate the percentage of each other peak as follows:

$$\text{Percentage of related substance } (i) = \frac{[A_{i/25}]}{A_{Total}} \times 100 \text{ percent}$$

where:

A_i=Area of any given peak, other than the main peak in the concentrated solution (10 milligrams per milliliter); and

A_{Total}=Area of the vancomycin B peak in the dilute (0.4 milligram per milliliter) solution+[Area of the total related substances peaks (exclude the area of the vancomycin B peak) in the concentrated solution (10 milligrams per milliliter) divided by 25].

[54 FR 20383, May 11, 1989; 54 FR 25849, June 20, 1989]

§ 436.367 Thin-layer chromatographic identity test for cephalexin hydrochloride.

(a) *Equipment*—(1) *Chromatography tank.* Use a rectangular tank approximately 23 × 23 × 9 centimeters, with a glass solvent trough in the bottom and a tight-fitting cover. Line the inside walls of the tank with Whatman #3 MM chromatographic paper or equivalent.

(2) *Plates.* Use 20 × 20 centimeter thin layer chromatographic plates coated with silica gel 60F–254 or equivalent to a thickness of 250 microns.

(b) *Developing solvent.* Mix ethylacetate, acetonitrile, water and glacial acetic acid in volumetric proportions of 42:14:18:14, respectively.

(c) *Preparation of the spotting solutions.* Prepare a solution of the sample containing 25 milligrams per milliliter of cephalexin hydrochloride in water. Prepare a solution of cephalexin monohydrate reference material at a concentration of 25 milligrams per milliliter. Add water and 0.1*N* hydrochloric acid in a dropwise mode until the material is completely dissolved.

(d) *Procedure.* Pour the developing solvent into the glass trough at the bottom of the chromatography tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Prepare a plate as follows: On a line 2 centimeters from the base of the plate, and at intervals of 2 centimeters, spot approximately 5 microliters of the standard solution to points 1 and 3 and approximately 5 microliters of the sample solution to point 2. After all spots are thoroughly dry, place the plate directly into the glass trough of the chromatography tank. Cover and seal the tank. Allow the solvent front to travel approximately 15 centimeters from the starting line. Remove the plate from the tank and allow it to air dry.

(e) *Evaluation.* View the dry plate under ultraviolet light (254 nanometers). Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values of approximately 0.35.

[54 FR 48860, Nov. 28, 1989; 54 FR 51816, Dec. 18, 1989]

§ 436.368 Thin layer chromatographic identity test for cefprozil.

(a) *Equipment*—(1) *Chromatography tank.* Use a glass rectangular tank approximately 23 x 23 x 9 centimeters lined with filter paper and equipped with a tight-fitting cover.

(2) *Plates.* Use 20 x 20 centimeter thin layer chromatography plates coated with silica gel GF to a thickness of 250 microns.

(b) *Reagents*—(1) *Diluent.* Mix 0.1N HCl and acetone in volumetric proportions of 1:4.

(2) *Developing solvent.* Mix n-butanol, glacial acetic acid and water in volumetric proportions of 60:20:20.

(3) *Detection reagent.* Iodine vapor.

(c) *Assay solutions*—(1) *Reference standard solution.* Dissolve 50 milligrams of cefprozil (Z) reference standard in 10 milliliters of diluent.

(2) *Sample solution.* Place an amount of sample containing approximately 50 milligrams of cefprozil in a 20-milliliter glass stoppered vial. Add 10 milliliters of diluent. Shake for 5 minutes and allow the solids to settle.

(d) *Procedure.* Pour a suitable quantity of the developing solvent into a glass, chromatographic tank lined with filter paper and allow to equilibrate for 1 hour. On a line 2 centimeters from the bottom edge of the plate, spot 10 microliters each of the reference solution and sample solution. Draw a line indicating the distance to which the developing solvent must travel at a point 12 centimeters from the bottom edge of the plate. Place the plate in the tank and allow the solvent to migrate to the finishing line. Remove the plate and air dry in a fume hood. Place the dried plate in a chamber containing iodine vapors.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line, and the distance the spots are from the starting line. Divide the latter by the former to calculate the R_f value. The identity test is positive if the sample solution produces a yellow spot at the same R_f value and has the same appearance as the spot obtained for the reference solution. The R_f value for cefprozil (Z) is approximately 0.45. Cefprozil (E), has an R_f value of approximately 0.47.Cefprozil (Z) is ''absent'' if the above test is performed and no spots, which correspond to those from the reference solution, are obtained for the sample.

[58 FR 26660, May 4, 1993]

§ 436.369 Thin layer chromatography test for free *N*-isobutylpiperidone content in rifabutin.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 23 X 23 X 9 centimeters, with a glass solvent trough on the bottom and a tight-fitting cover.

(2) *Iodine vapor chamber.* A rectangular tank, approximately 23 X 23 X 9 centimeters, with a suitable cover, containing iodine crystals.

(3) *Plates.* Use 20 X 20 centimeter thin layer chromatography plates coated with silica gel 60F 254 or equivalent to a thickness of 250 microns.

(b) *Reagents*—(1) *Developing solvent.* Mix petroleum ether (b.p. 60 to 80 ° C) and acetone in volumetric proportions of 100:30, respectively.

(2) *Spray solution.* Prepare a 1 percent solution of soluble starch in water (containing 0.01 percent mercuric iodide).

(c) *Preparation of spotting solutions*—(1) *Sample solution.* Prepare a solution of the rifabutin sample in 1:1 chloroform/methanol to contain 10 milligrams per milliliter.

(2) *Standard solution.* Prepare a solution of *N*-isobutylpiperidone standard in 1:1 chloroform/methanol to contain 1 milligram per milliliter. Transfer aliquots of 0.5, 1.0, 2.0, 5.0, and 10.0 milliliters into separate 100-milliliter volumetric flasks and dilute to volume with 1:1 chloroform/methanol. These solutions contain, respectively, the equivalent of 0.05, 0.1, 0.2, 0.5, and 1.0 percent of *N*-isobutylpiperidone.

(d) *Procedure.* Pour 100 milliliters of developing solvent into the glass trough on the bottom of the unlined chromatography tank. Cover and seal the tank. Allow it to equilibrate while the plate is being prepared. Prepare a plate as follows: on a line 2.0 centimeters from the base of the thin layer chromatography plate, and at intervals of 2.0 centimeters, apply 10 microliters of each of the standard solutions and the sample solution prepared as directed above. After the spots are thoroughly dry, place the plate into the trough in the bottom of the tank. Cover and tightly seal the tank, allow the solvent front to travel about 15 centimeters from the starting line and then remove the plate from the tank. Air dry the plate. Warm the iodine vapor chamber to vaporize the iodine crystals and place the dry plate in the iodine vapor chamber until the spots are visible (usually about 5 minutes). Remove the plate from the iodine vapor chamber and spray with 1 percent starch solution.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. *N*-isobutylpiperidone has an R_f value of about 0.3. Rifabutin has an R_f value of about 0.1. Compare the size and intensity of any *N*-isobutylpiperidone spots in the sample lane with the *N*-isobutylpiperidone spots in the standard lanes, and report the percentage of *N*-isobutylpiperidone in the sample.

[59 FR 40806, Aug. 10, 1994]

§ 436.370 Spectrophotometric identity test for rifabutin capsules.

(a) *Equipment.* A suitable spectrophotometer capable of recording the ultraviolet spectrum in the 200 to 400 nanometer range, using suitable quartz cells of 1 centimeter pathlength.

(b) *Preparation of working standard and sample solution*—(1) *Working standard solution.* Suspend approximately 200 milligrams of rifabutin working standard in 20 milliliters of methanol and sonicate for approximately 5 minutes. Filter the resulting solution through a suitable 0.5 micrometer filter. Transfer a 2-milliliter aliquot of the filtered solution to a 100-milliliter volumetric flask and fill to volume with methanol. Further dilute with methanol to obtain a solution containing 20 micrograms of rifabutin activity per milliliter.

(2) *Sample solution.* Empty and combine the contents of five capsules. Suspend a quantity of the capsule contents equivalent to 200 milligrams of rifabutin in 20 milliliters of methanol. Sonicate for about 5 minutes and then filter through an appropriate 0.5 micrometer filter. Transfer a 2-milliliter aliquot to a 100-milliliter volumetric flask and dilute to volume with methanol. Further dilute with methanol to obtain a solution containing 20 micrograms of rifabutin activity per milliliter (estimated).

(c) *Procedure.* Using a suitable spectrophotometer equipped with 1.0 centimeter cells and methanol as the blank, determine the absorbance spectra of the working standard and sample solutions over the ultraviolet range of 250 to 300 nanometers.

(d) *Evaluation.* Compare the spectrum of the sample to that of the working standard. The identity of the rifabutin capsules is confirmed by quantitative comparison of the two spectra with an

absorbance maximum being observed at about 275 nanometers.

[59 FR 40807, Aug. 10, 1994]

Subpart G—Chemical Tests for Nonantibiotic Active Ingredients

§ 436.400 Thin layer chromatographic identity test for iodochlorhydroxyquin.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 9 × 9 × 3.5 inches with a glass solvent trough on the bottom.

(2) *Plates.* Use 20 × 20 centimeter thin layer chromatography plates coated with Silica Gel G or equivalent to a thickness of 250 microns.

(b) *Developing solvent.* Mix benzene and methanol in volumetric proportions of 90:10.

(c) *Preparation of spotting solutions*—(1) *Sample solution.* Use the sample solution prepared as described in the section for the particular product to be tested.

(2) *Reference solution.* Prepare a solution containing 0.5 milligram of iodochlorhydroxyquin U.S.P. reference standard per milliliter in acetone.

(d) *Procedure.* Pour developing solvent into the glass trough on the bottom of the chromatography tank. Cover and seal the tank. Allow it to equilibrate for 1 hour. Spot a plate as follows: Apply approximately 10 microliters each of the sample solution and of the reference solution on a line 2.0 centimeters from the base of the silica gel plate and at intervals of not less than 2.0 centimeters. After all spots are thoroughly dry, place the silica gel plate directly into the glass trough of the chromatography tank. Cover and reseal the tank. Allow the solvent front to travel about 15 centimeters from the starting line, remove the plate from the tank, and allow to air dry. Examine under a strong source of ultraviolet light. The sample and standard are visible as dark spots.

(e) *Evaluation.* Measure the distance the solvent front traveled from the starting line and the distance the spots are from the starting line. Calculate the R_f value by dividing the latter by the former. The sample and standard should have spots of corresponding R_f values (0.55 to 0.60).

Subpart H—Tests for Specific Antibiotic Dosage Forms

§ 436.500 Penicillin in oil and wax.

(a) *Potency.* Proceed as directed in § 440.80a(b)(1) of this chapter except paragraph (b)(1)(ix) thereof and, in lieu of the directions in § 440.80a(b)(1)(iv) of this chapter prepare sample as follows: Liquefy the sample by warming, thoroughly mix, and withdraw 1.0 milliliter using a sterile syringe equipped with an 18-gauge needle. Transfer to a separatory funnel containing approximately 50 milliliters of peroxide-free ether. Shake the separatory funnel vigorously to bring about complete mixing of the material with the ether. Shake with a 25-milliliter portion of 1 percent phosphate buffer at pH 6.0. Remove the buffer layer and repeat the extraction with three 25-milliliter quantites of buffer. Combine the extracts and make the proper estimated dilutions in 1 percent phosphate buffer at pH 6.0. The sample may also be prepared by transferring aseptically 1.0 milliliter of the penicillin in oil and wax to a blending jar containing 100 milliliters of 1 percent phosphate buffer at pH 6.0. Using a high-speed blender, blend this mixture for 1 minute and then make the proper estimated dilutions in 1 percent phosphate buffer at pH 6.0. If the label represents the potency of the penicillin in oil and wax as 200,000 units per milliliter or less, it is satisfactory if it is 85 percent or more of the potency so represented; if represented as more than 200,000 units per milliliter, it is satisfactory if it is 90 percent or more of the potency so represented.

(b) *Sterility.* Proceed as directed in § 436.20, using the method described in paragraph (e)(2) of that section, except using medium B in lieu of medium A.

(c) *Moisture*—(1) *Reagents*—(i) *KarlFischer reagent.* Preserve the reagent in glass-stoppered bottles and use from an all glass automatic burette, protecting the solution from the moisture in the air.

(ii) *Water-methanol solution.* Use methanol containing approximately 1 mg. of water per milliliter. Store the solution in a glass bottle attached to an automatic burette and protect from moisture in the air at all times.

(2) *Standardization of Karl Fischer reagent.* Add a known volume of the Karl Fischer reagent to a suitable titrating vessel which has been previously dried at 105° C. and cooled in a desiccator. Introduce a mechanical stirrer and two platinum electrodes which are connected to a suitable electrometric apparatus for measurement of the endpoint. Start the stirrer and titrate with the water-methanol solution until the endpoint is reached. Calculate the milliliters of Karl Fischer reagent equivalent to each milliliter of water-methanol. Add an accurately weighed quantity of water (approximately 50 milligrams) to a dry titrating vessel, add an excess of the Karl Fischer reagent and back titrate with the water-methanol solution as above. Calculate the milligrams of water equivalent to each milliliter of the Karl Fischer reagent. Standardize the Karl Fischer reagent in this manner daily.

$$e = \frac{W}{V_1 - V_2 f}$$

where:

e=milligrams of water equivalent to 1 ml. Karl Fischer reagent.

w=weight of water in milligrams.

v_1=volume of Karl Fischer reagent used.

v_2=volume of methanol used.

f=volume ratio of Karl Fischer reagent to water-methanol solution.

(3) *Procedure.* Transfer 1.0 milliliter of the penicillin in oil and wax to a dry titrating vessel, add 10 milliliters of dry chloroform and an excess of the Karl Fischer reagent and back titrate with the water-methanol solution until the endpoint is reached. Transfer 10 milliliters of the dry chloroform used to a dry titrating vessel, add an excess of Karl Fischer reagent, and titrate with the water-methanol as above. Calculate the milliliters of Karl Fischer reagent equivalent to 10 milliliters of chloroform.

$$\text{Percent moisture} = \frac{(V_1 - V_2 f - b) \times e \times 100}{s \times 100}$$

where:

b=milliliters Karl Fischer reagent equivalent to 10 ml. of chloroform.

s=volume of the sample in milliliters.

(d) *Measurement of penicillin particle size.* Vigorously shake the container to obtain an even suspension of the penicillin particles and immediately withdraw therefrom approximately 0.5 milliliter of the drug into a clean, dry, tuberculin syringe using a dry 18-gauge needle. Discard approximately the first 5 drops of the mixture extruded from the needle and then extrude approximately 1 minim of the remaining mixture into a test tube containing 3 to 4 milliliters of light mineral oil. Thoroughly mix the contents of the tube and by means of a bacteriological loop (2 millimeters inside diameter, 22 gauge wire), immediately place one loopful of the suspension on each ruled chamber of a bright line hemocytometer. (It is not necessary to use a cover slip.) Confirm by means of the low power objective of the microscope the even distribution of particles over the ruled areas of both chambers and repeat with another loopful of the suspension if even dispersion is not obtained. Use a magnification of 430 or 440 diameters and a calibrated ocular micrometer to measure the penicillin particles. For the purpose of measurement and calculation, the predominant type of crystals observed shall be considered to represent the type of crystals present and the thickness and density of all particles shall be considered constant. Center a large penicillin particle in the microscopic field; measure the particle and all other particles in the field and repeat this operation on other fields until at least 200 particles are measured. Particles of less than 5 microns in length are disregarded. The grouping of the particles by length, the midpoint, the ratio of the midpoints, and the square of the ratio of the midpoints for each group are tabulated below:

Group	Length in microns	Midpoint	Ratio of midpoints	(Ratio)^2
1	5–14	9.5	1.00	1.00
2	15–29	22.0	2.31	5.34
3	30–49	39.5	4.16	17.31
4	50–69	59.5	6.26	39.19
5	70–99	84.5	8.89	79.03
6	100–149	124.5	13.10	171.61
7	150–199	174.5	18.36	337.09
8	200–249	224.5	23.63	558.38
9	250–300	275.0	28.95	838.10

Calculate the percent particles in each group from the total number measured. Determine the percent relative weight for each group as follows:

Plate type particles. Relative weight= (ratio)^2×% of total particles in group.

$$\%\,\text{relative weight} = \frac{\text{Relative weight} \times 100}{\text{Total relative weight}}$$

Rod-shaped particles. In the case of rod-shaped particles measure the width as well as the length.

Relative weight=ratio × average width × % of total particles in group

$$\%\,\text{relative weight} = \frac{\text{Relative weight} \times 100}{\text{Total relative weight}}$$

When examined by the method described in this section not less than 50 percent of the total relative weight of the penicillin in the drug consists of penicillin having a particle size of not less than 50 microns in length.

§ 436.503 Procaine penicillin and buffered crystalline penicillin for aqueous injection.

(a) *Total potency* (*except in single-dose container*), *sterility, moisture, pyrogens, toxicity, pH.* Proceed as directed in § 440.274b(b) of this chapter.

(b) *Buffered crystalline penicillin content*—(1) *Preparation of the solution for assay.* Add the indicated amount of distilled water to the contents of a vial of the sample, and shake well. Withdraw one dose of the suspension with a hypodermic syringe and place in a 10-milliliter volumetric flask. Add 20-percent sodium sulfate solution almost to the mark, centrifuge sufficiently to see the meniscus, make to volume with 20-percent sodium sulfate solution, shake well, and centrifuge to obtain a clear or reasonably clear solution. Dilute a 5.0-milliliter aliquot of this clear solution with 1-percent phosphate buffer, pH 6.0, to give a solution for assay of approximately 2,000 units per milliliter.

(2) *Iodometric assay for total penicillin in the solution for assay.* Determine the quantity of penicillin in the solution for assay by the iodometric assay procedure described in § 440.80a(b)(5)(iv)(*a*) of this chapter.

(3) *Colorimetric determination of procaine penicillin in the solution for assay.* Transfer an aliquot of the solution for assay to a 50-milliliter volumetric flask. Determine the quantity of procaine penicillin in this solution by the following method:

(i) *Reagents*—(*a*) *Sodium nitrite solution.* Dissolve 0.1 gram of sodium nitrite in 100 milliliters of distilled water. Prepare fresh solution every week and store under refrigeration.

(*b*) *Ammonium sulfamate solution.* Dissolve 0.5 gram of ammonium sulfamate in 100 milliliters of distilled water and store under refrigeration.

(*c*) *N-(1-naphthyl)-ethylenediamine solution.* Dissolve 0.1 gram of *N*-(1-naphthyl) ethylenediamine dihydrochloride in 100 milliliters of distilled water. Prepare fresh solutions every week and store under refrigeration.

(*d*) *Standard procaine solution.* Prepare a standard solution containing 27.55 milligrams of procaine hydrochloride U.S.P. in a liter of distilled water (each milliliter of the standard solution is equivalent to 60 units of procaine penicillin).

(ii) *Standards.* Transfer, respectively, 1.0, 2.0, 3.0, 4.0, and 5.0 milliliters of the standard solution and 5.0 milliliters of distilled water to each of six 50-milliliter volumetric flasks. Add 4.0, 3.0, 2.0, and 1.0 milliliters of water to the first four flasks, respectively, to give each a volume to 5.0 milliliters.

(iii) *Procedure.* To each flask for the standards and the solution for assay add 0.5 milliliter of 4 *N* HCl, 1.0 milliliter of the sodium nitrite solution, 1.0 milliliter of the ammonium sulfamate, and 1.0 milliliter of the *N*-(1-naphthyl)-ethylenediamine solution. Mix and wait two minutes after each addition. Make each flask to volume of 50 milliliters with distilled water. Determine the absorbency of the colored solutions at 550 Mμ in a suitable photo electric colorimeter. The instrument is balanced so that the zero concentration reads zero absorbency. Plot the standard curve on coordinate graph paper. Obtain the procaine penicillin content of the solution for assay directly from the point on the standard curve corresponding to its absorbency.

(4) The content of buffered crystalline pencillin in one dose of the product is calculated as follows:

$$A=(B-C)F,$$

where:

A=buffered crystalline penicillin content of the product.
B=total number of units of penicillin per milliliter as determined in paragraph (b)(2) of this section.
C=number of units of procaine penicillin per milliliter as determined in paragaph (b)(3) of this section.
F=appropriate dilution factor depending on the dilution made in the preparation of the solution for assay.

The content of buffered crystalline penicillin in the batch is satisfactory when determined by the method described in this paragraph if it is not less than 85 percent of that which it is represented to contain.

(c) *Procaine penicillin.* The procaine penicillin content of the batch is the difference between the total potency determined by the method described in paragraph (a) or (d) of this section and the content of the buffered crystalline penicillin determined by the method described in paragraph (b) of this section. The procaine penicillin content of the batch is satisfactory when determined by the method described in this paragraph if it is not less than 85 percent of that which it is represented to contain.

(d) *Total potency of a one-dose container.* Wash out the material remaining in the 10-milliliter volumetric flask referred to in paragraph (b)(1) of this section with 1-percent phosphate buffer, pH 6.0. Dilute to give a concentration of approximately 2,000 units per milliliter, and assay by the iodometric method described in § 440.80a (b)(5)(iv)(*a*) of this chapter. Obtain the total potency by adding the number of units found in this solution (units per milliliter × volume) to the number of units found (units per milliliter × volume) in the solution assayed in accordance with paragraph (b)(2) of this section.

§ 436.504 Penicillin-bacitracin ointment.

(a) *Potency*—(1) *Penicillin content.* Proceed as directed in § 540.380a(b)(1) of this chapter, except the last sentence of that paragraph. Its content of penicillin is satisfactory if it contains not less than 85 percent of the number of units it is represented to contain.

(2) *Bacitracin content.* Proceed as directed in § 448.510a(b)(1) of this chapter, except that sufficient penicillinase is added to the sample under test to completely inactivate the penicillin present. Its content of bacitracin is satisfactory if it contains not less than 85 percent of the number of units it is represented to contain.

(b) *Moisture.* Proceed as directed in § 436.201.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§ 436.505 Penicillin-streptomycin-bacitracin ointment; penicillin-dihydrostreptomycin-bacitracin ointment; penicillin-streptomycin-bacitracin methylene disalicylate ointment; penicillin-dihydrostreptomycin-bacitracin methylene disalicylate ointment.

(a) *Potency*—(1) *Content of penicillin, streptomycin, and dihydrostreptomycin.* Proceed as directed in § 536.501(a) of this chapter.

(2) *Bacitracin content.* Proceed as directed in § 448.510a(b)(1) of this chapter, except that:

(i) Sufficient penicillinase is added to the sample under test to completely inactivate the penicillin present.

(ii) Use as the test organism the streptomycin dihydrostreptomycin resistant strain of either Micrococcus flavus (ATCC 10240A)[1] or Sarcina subflava (ATCC 7468/d),[1] grown and maintained in media containing 500 micrograms of streptomycin or dihydrostreptomycin per milliliter of media, or calculate from the quantity of streptomycin or dihydrostreptomycin found, using the method prescribed by paragraph (a)(1) of this section, the quantity that would be present when the sample is diluted to contain one unit of bacitracin (labeled potency) per milliliter. Prepare the bacitracin standard curve by adding the calculated quantity of streptomycin or dihydrostreptomycin to each concentration of bacitracin used for

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

the curve. Use this standard curve to calculate the bacitracin content of the sample.

(3) *Bacitracin methylene disalicylate content.* Proceed as directed in paragraph (a)(2) of this section, except prepare the sample as follows: Place a representative portion of the sample (usually approximately 1 gram, accurately weighed) or the entire contents of a single-dose container in blending jar, add 99 milliliters of a 2.0-percent aqueous solution of sodium bicarbonate and 1 milliliter of a 10-percent aqueous solution of polysorbate 80 and blend for 3 minutes in a high-speed blender. Allow the foam to subside, remove an aliquot of the solution, and dilute to 1 unit per milliliter with 1.0-percent phosphate buffer, pH 6.0.

(b) *Moisture.* Proceed as directed in §436.201.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§436.506 Benzathine penicillin G and buffered crystalline penicillin for aqueous injection.

(a) *Total potency* (*except in single-dose containers*). Proceed as directed in §440.80a(b)(1) of this chapter, except if the bioassay method is used prepare the sample by diluting 1.0 milliliter of the drug suspension with sufficient dimethyl formamide, formamide, or methyl alcohol to dissolve the benzathine penicillin. Make to 100 milliliters with buffer. Shake well and dilute to 1.0 unit per milliliter. If the iodometric method is used, proceed as directed in §440.55a(b) of this chapter, except in preparing the blank solution dilute 1.0 milliliter of the drug suspension to 250 milliliters with 1-percent phosphate buffer at pH 6.0. In preparing the solution for inactivation dissolve 1.0 milliliter of the drug suspension in approximately 20 milliliters of 0.5 *N* NaOH. Allow to stand for 15 minutes. Dilute to 250 milliliters with distilled water. Pipette a 2.0-milliliter aliquot into a 125-milliliter glass-stoppered Erlenmeyer flask and add 2.0 milliliters 1.2 *N* HCl and 10 milliliters 0.01 *N* iodine.

(b) *Buffered crystalline penicillin content.* Place 1.0 milliliter of the drug suspension in a 10-milliliter volumetric flask and add 20 percent sodium sulfate to make 10 milliliters. Shake well and centrifuge to obtain a clear, or reasonably clear, solution. Dilute a 5.0-milliliter aliquot to 50 milliliters with buffer and proceed as directed in §440.80a(b)(1) of this chapter to determine the number of units per milliliter of this solution, and from this value calculate the number of units per milliliter of the drug. The content of buffered crystalline penicillin is satisfactory if it is not less than 85 percent of that which it is represented to contain.

(c) *Benzathine penicillin G content.* The benzathine penicillin G content of the batch is the difference between the total potency as described in paragraph (a) or (d) of this section and the content of buffered crystalline penicillin determined by the method prescribed in paragraph (b) of this section. The content of benzathine penicillin G is satisfactory if it is not less than 85 percent of that which it is represented to contain.

(d) *Total potency of a single-dose container.* Add sufficient distilled water to the material remaining in the 10-milliliter volumetric flask referred to in paragraph (b) of this section to bring the volume back to 10 milliliters and determine the number of units per milliliter of this suspension. If the iodometric method is used, 2.0-milliliter aliquots are placed in 50-milliliter volumetric flasks (one blank and one to be inactivated). Obtain the total potency by adding the number of units found in the 10-milliliter volumetric flask to one-half the content of buffered crystalline penicillin found in paragraph (b) of this section.

(e) *Sterility.* Proceed as directed in §436.20 using the method described in paragraph (e)(2) of that section, except use medium C in lieu of medium A, and medium F in lieu of medium E. During the period of incubation, shake the tubes at least once daily.

(f) *Moisture.* Proceed as directed in §440.74a(b)(5) of this chapter.

(g) *Pyrogens.* Proceed as directed in §436.500.

(h) *Toxicity.* Proceed as directed in §440.55a(b)(3) of this chapter.

(i) *pH.* Proceed as directed in §440.80a(b)(5)(ii) of this chapter, using the suspension resulting when the

product is reconstituted as directed in the labeling.

§ 436.507 Benzathine - procaine - buffered crystalline penicillins for aqueous injection.

(a) *Potency*—(1) *Total potency.* Proceed as directed in § 440.80a(b)(1) of this chapter, except if the bioassay method is used prepare the sample by diluting one dose of the drug suspension with sufficient dimethyl formamide or formamide or methyl alcohol to dissolve the benzathine penicillin G. Make to 100 milliliters with 1-percent phosphate buffer, pH 6.0. Shake well, and dilute to 1.0 unit per milliliter with buffer. If the iodometric method of assay is used, add the indicated amount of distilled water to the contents of a vial of the sample, shake well, and proceed as follows (except for single-dose containers):

(i) Using a standardized hypodermic syringe, withdraw one dose and dilute with 1-percent phosphate buffer, pH 6.0, to give a concentration of approximately 2,000 units per milliliter. Use 2.0 milliliters of this suspension as the blank in the iodometric assay procedure described in § 440.80a(b)(5)(iv)(*a*) of this chapter.

(ii) Using a standardized hypodermic syringe, withdraw another dose, place in a flask, and add 20 milliliters of 0.5 *N* NaOH for each 300,000 units of benzathine penicillin, mix well, being sure that all penicillin is in solution, and allow to stand for 15 minutes. Add 1 milliliter of 1.2 *N* HCl for each 2 milliliters of 0.5 *N* NaOH, mix, and dilute with distilled water to the same volume as was used in paragraph (a)(1)(i) of this section. Place 2.0 milliliters in a 125-milliliter glass-stoppered Erlenmeyer flask, add 10 milliliters of 0.01 *N* iodine, allow to stand for 15 minutes, and titrate with 0.01 *N* sodium thiosulfate as directed in the iodometric assay procedure in § 440.80a (b)(5)(iv)(*a*) of this chapter. The total potency of the batch is satisfactory if it contains not less than 85 percent of that which it is represented to contain.

(2) *Procaine penicillin content* (*except for single-dose containers*). Make suitable dilutions of the solution prepared in paragraph (a)(1)(ii) of this section to obtain approximately 60 units of procaine penicillin per milliliter. Determine the procaine penicillin content by the colorimetric procedure described in § 436.503(b)(3). The content of procaine penicillin is satisfactory if it contains not less than 85 percent of the number of units that it is represented to contain.

(3) *Buffered crystalline penicillin content*—(i) *Preparation of the solution for assay.* (*a*) Add the indicated amount of distilled water to the contents of a vial of the sample, and shake well. Withdraw one dose of the suspension with a hypodermic syringe and place in a 10-milliliter volumetric flask. Add 20-percent sodium sulfate solution almost to the mark, centrifuge sufficiently to see the meniscus, make to volume with 20-percent sodium sulfate solution, shake well, and centrifuge to obtain a clear or reasonably clear solution; or

(*b*) If the original product contains more than 600,000 units, place it in a 50-milliliter volumetric flask, add 20-percent sodium sulfate to the mark, shake well, place a 10-milliliter portion in a centrifuge tube, and centrifuge to obtain a reasonably clear solution.

(*c*) Dilute a 5.0-milliliter aliquot of the clear solution obtained in paragraph (a) (3)(i) (*a*) or (*b*) of this section with 1-percent phosphate buffer, pH 6.0, to give a solution for assay of approximately 2,000 units per milliliter.

(ii) *Iodometric assay for total penicillin in the solution for assay.* Determine the total quantity of penicillin in the solution for assay by the iodometric assay procedure described in § 440.80a (b)(5)(iv)(*a*) of this chapter.

(iii) *Colorimetric determination of procaine penicillin in the solution for assay.* Proceed as directed in § 436.503 (b)(3). The content of procaine penicillin in the batch is satisfactory if it is not less than 85 percent of that which it is represented to contain.

(iv) The buffered crystalline penicillin in one dose of the product is calculated as follows:

$$A=(B-C)F,$$

where:

A = the buffered crystalline penicillin content of the product.

B = the number of units of penicillin per milliliter as determined in paragraph (a)(3)(ii) of this section.

C=the number of units of procaine penicillin per milliliter as determined in paragraph (a)(3)(iii) of this section.
F=the appropriate dilution factor depending on the dilutions made in the preparation of the solution for assay.

The content of buffered crystalline penicillin is satisfactory if the batch contains 85 percent of the number of units per milliliter that it is represented to contain.

(4) *Benzathine penicillin content.* The sum of the procaine penicillin content determined under paragraph (a)(2) or (6) of this section and the buffered crystalline penicillin content determined under paragraph (a)(3) of this section, subtracted from the total potency determined in paragraph (a)(1) or (5) of this section, represents the benzathine penicillin G content. The benzathine penicillin G content is satisfactory if it is not less than 85 percent of the number of units that it is represented to contain.

(5) *Total potency of a single-dose container.* Wash out the material remaining in the volumetric flask referred to in paragraph (a)(3)(i)(*a*) of this section, or combine the contents remaining in the 50-milliliter volumetric flask and in the centrifuge tube referred to in paragraph (a)(3)(i)(*b*) of this section. Dissolve the material by adding 10 milliliters of 1 *N* NaOH for each 300,000 units of benzathine penicillin and allow to stand 15 minutes. Add 1 milliliter of 1.2 *N* HCl for each milliliter of 1 *N* NaOH and then dilute with distilled water to give a concentration of approximately 2,000 units per milliliter. Place 2.0 milliliters in a 125-milliliter glass-stoppered Erlenmeyer flask, add 10 milliliters of 0.01 *N* iodine, allow to stand for 15 minutes, and then titrate with 0.01 *N* sodium thiosulfate as directed in §440.80a (b)(5)(iv)(*a*) of this chapter. For the blank determination prepare a separate sample as directed in paragraph (a)(3)(i) (*a*) or (*b*) of this section and in the first sentence of this paragraph (a)(5), then dilute with 1 percent phosphate buffer, pH 6.0, to give a concentration of approximately 2,000 units per milliliter. The total potency of the one-dose container is equal to the sum of the number of units found in this assay (units per milliliter×volume) and the number of units found (units per milliliter ×volume) in the solution for assay in paragraph (a)(3)(ii) of this section.

(6) *Procaine penicillin content of a single-dose container.* Make suitable dilutions of the NaOH-inactivated solution prepared in paragraph (a)(5) of this section to obtain approximately 60 units of procaine penicillin per milliliter. Determine the procaine penicillin content (units per milliliter× volume) of this solution by the colorimetric procedure described under §436.503(b)(3). To this value add per procaine penicillin content (unit per milliliter×volume) of the solution for assay, as found in paragraph (a)(3)(iii) of this section, to obtain the procaine penicillin content of the one-dose container. The content of procaine penicillin in the batch is satisfactory if it is not less than 85 percent of that which it is represented to contain.

(b) *Sterility.* Proceed as directed in §436.20, using the method described in paragraph (e)(2) of that section, except use medium C in lieu of medium A, and medium F in lieu of medium E. During the period of incubation, shake the tubes at least once daily.

(c) *Pyrogens.* Proceed as directed in §440.55a(b)(4) of this chapter.

(d) *Toxicity.* Proceed as directed in §440.55a(b)(3) of this chapter.

(e) *Moisture.* Proceed as directed in §440.74a(b)(5) of this chapter.

(f) *pH.* Proceed as directed in §440.80a(b)(5)(ii) of this chapter, using the suspension resulting when the product is reconstituted as directed in the labeling.

§436.508 Penicillin - bacitracin - neomycin ointment; penicillin-bacitracin-neomycin in oil.

(a) *Potency*—(1) *Penicillin content; bacitracin content.* Proceed as directed in §436.504(a).

(2) *Neomycin content.* Proceed as directed in §448.510d(b)(1)(ii) of this chapter, except that sufficient penicillinase is added to the sample under test to completely inactivate the penicillin present. Its content of neomycin is satisfactory if it contains not less than 85 percent of the number of milligrams per gram that it is represented to contain.

(b) *Moisture.* Proceed as directed in § 436.201.

§ 436.509 Procaine penicillin-streptomycin-polymyxin in oil; procaine penicillin-dihydrostreptomycin-polymyxin in oil; procaine penicillin-streptomycin-polymyxin ointment; procaine penicillin - dihydrostreptomycin - polymyxin ointment.

(a) *Potency*—(1) *Penicillin content.* Proceed as directed in § 540.380a(b)(1) of this chapter. Its content of penicillin is satisfactory if it contains not less than 85 percent of the number of units per milliliter or per gram that it is represented to contain.

(2) *Streptomycin content.* Proceed as directed in § 544.373(b)(1)(i) of this chapter, except inactivate the penicillin in the combined extractives with sufficient penicillinase at 37° C. for 30 minutes. Its content of streptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams per milliliter or per gram that it is represented to contain.

(3) *Dihydrostreptomycin content.* Proceed as directed in paragraph (a)(2) of this section, using the dihydrostreptomycin working standard as a standard of comparison. Its content of dihydrostreptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams per milliliter or per gram that it is represented to contain.

(4) *Polymyxin content.* Proceed as directed in § 444.170a(b)(2)(i) of this chapter, with the following exceptions:

(i) In lieu of the directions for the preparation of the sample described in § 444.170a(b)(2)(i)(*g*) of this chapter, prepare the sample by one of the following techniques:

(*a*) *Extraction.* Place a convenient-sized representative quantity of the sample in a separatory funnel containing approximately 50 milliliters of peroxide-free ether. Shake the sample and ether until homogeneous. Add 25 milliliters of 10-percent potassium phosphate buffer (pH 6.0) and shake. Remove the buffer layer and repeat the extraction with 25-milliliter portions of buffer at least three times and any additional times that may be necessary to insure complete extraction of the antibiotic. Combine the extractives. Inactivate the penicillin with sufficient penicillinase at 37° C. for 30 minutes. Make the proper estimated dilutions in 10-percent potassium phosphate buffer (pH 6.0) to give a concentration of 10 units per milliliter (estimated).

(*b*) *Blending.* Place a convenient-sized representative quantity of the sample in a blending jar containing 1.0 milliliter of polysorbate 80 and sufficient 1-percent phosphate buffer (pH 6.0) to give a final volume of 200 milliliters. If the sample consists of substantially more than 1 gram, use sufficient buffer to give a final volume of 500 milliliters. If the concentration of polymyxin in the blend is less than 200 units per milliliter, 10-percent phosphate buffer (pH 6.0) should be used in lieu of 1-percent phosphate buffer (pH 6.0). Using a high-speed blender, blend the mixture for 2 minutes. Inactivate the penicillin with sufficient penicillinase at 37° C. for 30 minutes and make the proper estimated dilutions in 10-percent phosphate buffer (pH 6.0) to give a concentration of 10 units per milliliter (estimated).

(ii) The standard curve is prepared in the following concentrations: 6.4, 8.0, 10.0, 12.5, and 15.6 units per milliliter in 10-percent potassium phosphate buffer, pH 6.0. The 10 units per milliliter concentration is used as the reference point. Its content of polymyxin is satisfactory if it contains not less than 85 percent of the number of units per milliliter or per gram that it is represented to contain.

(b) *Moisture.* Proceed as directed in § 436.201.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975; 41 FR 10886, Mar. 15, 1976]

§ 436.510 Penicillin-streptomycin-erythromycin ointment; penicillin-dihydro-streptomycin-erythromycin ointment.

(a) *Potency*—(1) *Penicillin content.* Obtain the weight of the content of a syringe by weighing before and after ejecting the content into a beaker. Stir until homogeneous. Remove a representative sample (usually approximately 1.0 gram, accurately weighed) and place in a separatory funnel containing 50 milliliters of peroxide-free

ether. Add 20 milliliters of 0.1 *M* potassium phosphate buffer (pH 8.0) and shake. Remove the buffer layer and repeat the extraction with three additional 20-milliliter portions of the buffer. Place the buffer solution in a second separatory funnel and wash with three 30-milliliter portions of ether. Discard the ether washes. Remove an aliquot of the buffer solution and proceed as directed in §440.80a(b) (1) of this chapter, except §440.80a(b)(1)(iv) and (ix) of this chapter. If the iodometric chemical assay is used, proceed as directed in §440.80a(b)(5)(iv)(*a*) of this chapter, except prepare the sample as directed in §536.501(a)(1) of this chapter. Its content of penicillin is satisfactory if it contains not less than 85 percent of the number of units that it is represented to contain.

(2) *Streptomycin content.* Using an aliquot of the buffer solution prepared as directed in paragraph (a)(1) of this section, proceed as directed in §444.70a (b)(1) through (9) of this chapter, except add sufficient penicillinase to completely inactivate the penicillin present. Its content of streptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(3) *Dihydrostreptomycin content.* Proceed as directed in paragraph (a)(2) of this section, using the dihydrostreptomycin working standard as the standard of comparison. Its content of dihydrostreptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(4) *Erythromycin content.* Proceed as directed in §444.570b(b)(1)(i)(*b*) of this chapter, except prepare the sample as follows: Place a representative sample (usually approximately 1.0 gram, accurately weighed), in a glass blending jar containing 99 milliliters of 0.1 *M* potassium phosphate buffer, pH 8.0, and 1 milliliter of polysorbate 80. Using a high-speed blender, blend for 2 to 3 minutes. Add 100 milliliters of 0.1 *M* potassium phosphate buffer, pH 8.0, and blend for an additional 2 to 3 minutes. Prepare an intermediate dilution by diluting an aliquot of the filtrate with 0.1 *M* potassium phosphate buffer (pH 8.0), and add sufficient penicillinase to inactivate the penicillin. Then further dilute with buffer to give an erythromycin content of 1.0 microgram per milliliter (estimated). Its content of erythromycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is presented to contain.

(b) *Moisture.* Proceed as directed in §436.500(c).

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§ 436.511 Penicillin-streptomycin-bacitracin methylene disalicylate-neomycin ointment; penicillin-dihydrostreptomycin-bacitracin methylene disalicylate-neomycin ointment.

(a) *Potency*—(1) *Penicillin content.* Proceed as directed in §540.380a(b)(1) of this chapter. Its penicillin content is satisfactory if it contains not less than 85 percent of the number of units that it is represented to contain.

(2) *Streptomycin content.* Proceed as directed in §436.105 of this chapter. Its content of streptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(3) *Dihydrostreptomycin content.* Proceed as directed in §436.105 of this chapter. Its content of dihydrostreptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(4) *Bacitracin methylene disalicylate content.* Proceed as directed in §436.505(a)(3). Its potency is satisfactory if it contains not less than 85 percent of the equivalent number of units of bacitracin that it is represented to contain.

(5) *Neomycin content.* Proceed as directed in §436.105 of this chapter. Its content of neomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(b) *Moisture.* Proceed as directed in §436.201.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§ 436.512 Procaine penicillin G-novobiocin-neomycin-dihydrostreptomycin in oil.

(a) *Potency*—(1) *Penicillin G content.* Proceed as directed in §440.180d (b)(1)(i)(*a*) of this chapter, using the

novobiocin-resistant strain of *Staphylococcus aureus* (ATCC 12692),[1] except prepare the sample as follows: Place the equivalent of one dose of sample in a blending jar, add 1.0 milliliter of polysorbate 80 and a quantity of 1 percent potassium phosphate buffer, pH 6.0, sufficient to make a total of 500 milliliters. Blend for 5 minutes with a high-speed blender and make appropriate dilutions, using 1 percent potassium phosphate buffer, pH 6.0. Its content of penicillin G is satisfactory if it contains not less than 85 percent of the number of units that it is represented to contain.

(2) *Novobiocin content.* Proceed as directed in § 440.180d(b)(3)(i), with the following exceptions:

(i) Prepare the sample as follows: Place the equivalent of one dose of sample in a blending jar, add 1.0 milliliter of polysorbate 80 and a quantity of 0.1*M* potassium phosphate buffer, pH 8.0, sufficient to make a total of 500 milliliters. Blend for 5 minutes with a high-speed blender. To an aliquot, add sufficient penicillinase to inactivate the penicillin, further dilute with 10 percent potassium phosphate buffer, pH 6.0 (solution 6) to give a final concentration of 0.5 microgram novobiocin per milliliter (estimated), and allow to stand for ½-hour at 37° C. before filling the plates.

(ii) Aseptically add to the seed agar used for this assay, at the time the bacterial suspension is added, a slurry of Dowex 50 WX–4, Na^+ type 200–400 mesh, sufficient to make a total concentration of 2 percent. Prepare the slurry by adding 50 grams of the resin to 30 milliliters of distilled water and sterilize for 15 minutes at 15 pounds pressure. Mix the slurry thoroughly before adding. Its content of novobiocin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(3) *Neomycin content.* Proceed as directed in § 436.517(b)(1) of this chapter, using the *Staphylococcus epidermidis* (ATCC 12228)[1] procedure, except:

(i) Prepare the sample as follows: Place the equivalent of one dose of sample in a blending jar, add 1.0 milliliter of polysorbate 80 and a quantity of 0.1*M* potassium phosphate buffer, pH 8.0, sufficient to make a total of 500 milliliters. Blend for 5 minutes with a high-speed blender. To an aliquot, add sufficient penicillinase to inactivate the penicillin, further dilute with 0.1*M* potassium phosphate buffer, pH 8.0, to give a final concentration of 1.0 microgram neomycin per milliliter (estimated), and allow to stand for ½-hour at 37° C. before filling the plates.

(ii) Aseptically add to the seed agar used for this assay, at the time the bacterial suspension is added, a slurry of Dowex 1–X8, Cl type 200–400 mesh, to make a total concentration of 1 percent. Prepare the slurry by adding 50 grams of the resin to 30 milliliters of distilled water and sterilize for 15 minutes at 15 pounds pressure. Mix the slurry thoroughly before adding. Its content of neomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(4) *Dihydrostreptomycin content.* Proceed as directed in § 436.105 except prepare the sample by placing the equivalent of one dose in a blender, add 1.0 milliliter of polysorbate 80 and a quantity of 0.1*M* potassium phosphate buffer, pH 8.0, sufficient to make a total of 500 milliliters. Blend for 5 minutes with a high-speed blender. To an aliquot, add sufficient penicillinase to inactivate the penicillin, further dilute with 0.1*M* potassium phosphate buffer, pH 8.0, to give a final concentration of 1.0 microgram dihydrostreptomycin per milliliter (estimated), and allow to stand for ½-hour at 37° C. before filling the plates. Its content of dihydrostreptomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(b) *Moisture.* Proceed as directed in § 436.500(c).

[39 FR 18944, May 30, 1974, as amended at 41 FR 10886, Mar. 15, 1976]

§ 436.513 Chlortetracycline troches; tetracycline hydrochloride troches.

(a) *Potency.* If it is tetracycline hydrochloride proceed as directed in § 446.81a(b)(1) of this chapter and if it is

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

chlortetracycline hydrochloride troches proceed as directed in § 446.10a(b)(1) of this chapter, except § 446.10a(b)(1)(x), and in lieu of the directions in § 446.10a(b)(1)(iv) and (viii)(*c*) of this chapter prepare the sample as follows: Place 12 troches in a glass blending jar containing 500 milliliters of 0.01*N* HCl. Using a high-speed blender, blend for 3 to 5 minutes and then make the proper estimated dilutions in the buffer solution. The average potency of the troches is satisfactory if they contain not less than 85 percent of the number of milligrams they are represented to contain.

(b) *Moisture.* Proceed as directed in § 440.80a(b)(5)(i) of this chapter.

§ 436.514 Chlortetracycline hydrochloride powder topical; tetracycline hydrochloride powder topical.

(a) *Potency*—(1) *Dry powder.* Using a 3.0-gram sample or the entire contents of the immediate container for each determination, prepare the sample as follows: Using a high-speed blender, blend a 3.0-gram sample in a glass blending jar containing 500 milliliters of 0.01 *N* HCl (use 0.1 *N* HCl if it is tetracycline), or reconstitute in the immediate container as directed in the labeling of the drug. Transfer an appropriate aliquot of 1.0 milliliter to 5.0 milliliters to a 100-milliliter volumetric flask and make to mark with 0.01 *N* HCl (use 0.1 *N* HCl if it is tetracycline). Withdraw an aliquot from the volumetric flask, and if it is chlortetracycline hydrochloride dilute to 0.06 µ g. per milliliter, using 0.1 *M* potassium phosphate buffer, pH 4.5, and proceed as directed in § 446.10a(b)(1) of this chapter. If it is tetracycline hydrochloride, dilute to 0.24 µ g. per milliliter, using 0.1 *M* potassium phosphate buffer, pH 4.5, and proceed as directed in § 446.81a(b)(1) of this chapter. The average potency is satisfactory if it contains not less than 85 percent of the number of milligrams of chlortetracycline hydrochloride or tetracycline hydrocloride per gram or per immediate container that it is represented to contain.

(2) *Powder packaged with inert gases.* Spray, as directed in the labeling, the entire contents of each container to be tested into a separate 2-liter Erlenmeyer flask, held in a horizontal position. Add 500 milliliters of 0.1 *N* HCl and shake to dissolve the contents. Immediately remove aliquots of this solution and, using 0.1 *M* potassium phosphate buffer, pH 4.5, for further dilutions, proceed as directed in § 446.10a(b)(1) of this chapter if it is chlortetracycline hydrochloride powder or § 446.81a(b)(1) of this chapter if it is tetracycline hydrochloride powder. Calculate the average total amount of antibiotic expelled from the containers. The total potency is satisfactory if it contains not less than 85 percent of the number of milligrams of chlortetracycline hydrochloride or tetracycline hydrochloride that it is represented to contain.

(b) *Moisture.* Proceed as directed in § 440.80a(b)(5)(i) of this chapter, except if it is packaged with inert gases proceed as directed in § 536.513(c) of this chapter.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§ 436.515 Capsules tetracycline and oleandomycin phosphate; capsules tetracycline and troleandomycin; capsules tetracycline hydrochloride and oleandomycin phosphate; capsules tetracycline hydrochloride and troleandomycin.

(a) *Potency*—(1) *Tetracycline or tetracycline hydrochloride content by turbidimetric assay*—(i) *Test culture and media.* Maintain the test organism *Escherichia coli* (ATCC 10536)[1] on the agar described in § 440.80a(b)(1) (ii)(*a*) of this chapter. For use in the assay, prepare a suspension of the organism every 2 weeks, as follows: Transfer the organism to a fresh agar slant and incubate at 37°C. overnight. Wash the growth from the slant with the aid of 2 milliliters of sterile distilled water and sterile glass beads into a Roux bottle containing 300 milliliters of the maintenance medium. Incubate overnight at 37° C. and then harvest the growth with 50 milliliters of sterile distilled water and sterile glass beads. Standardize this suspension by determining the dilution that will permit 40-percent light

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

transmission in a photoelectric colorimeter using a 650-millimicron filter and an 18-millimeter diameter test tube as an absorption cell. Prepare the daily inoculum by adding 10 milliliters of that dilution to each liter of nutrient broth, prepared as directed in § 440.80a (b)(1)(ii)(*c*) of this chapter, needed for the test.

(ii) *Working standard and solutions.* Dissolve an appropriate amount of the working standard in sufficient 0.1 *N* HCl to give a concentration of 1,000 micrograms per milliliter. This stock solution may be kept in the refrigerator for 1 week. Make daily dilutions of the stock solution with 0.1 *M* potassium phosphate buffer (pH 4.5) to obtain concentrations of 0.146, 0.187, 0.240, 0.308, and 0.395 micrograms per milliliter. Add 1.0 milliliter of each such concentration to each of three 16 millimeters x 125 millimeters test tubes.

(iii) *Preparation of sample.* Dissolve the contents of a representative number of capsules in sufficient 0.1 *N* HCl to give a stock solution of convenient concentration. Further dilute the stock solution with 0.1 *M* potassium phosphate buffer (pH 4.5) to obtain a final concentration of 0.24 microgram per milliliter (estimated). Add 1.0-milliliter of this dilution to each of three 16 millimeters x 125 millimeters test tubes.

(iv) *Procedure.* To each of the 16 millimeters x 125 millimeters test tubes prepared in paragraph (a)(1)(ii) and (iii) of this section, add 9.0 milliliters of the inoculated nutrient broth described in paragraph (a)(1)(i) of this section and place immediately in a 37° C. water bath for 3 to 4 hours. After incubation, add 0.5 milliliter of a 12-percent formaldehyde solution to each tube and read the absorbance values in a suitable photoelectric colorimeter using a wavelength of 530 millimicrons. Set the instrument at zero absorbance with clear uninoculated broth prepared as described in § 440.80a(b)(1)(ii)(*c*) of this chapter.

(v) *Estimation of potency.* Plot the average values for each concentration of the standard on arithmetic graph paper with absorbance values on the ordinate and tetracycline or tetracycline hydrochloride concentrations on the abscissa. Construct the best straightline through the points, either by inspection or by means of the following equations:

$$L=(3a+2b+c-e)/5,$$
$$H=(3e+2d+c-a)/5,$$

where:

L=absorbance value for the lowest concentration of the standard curve.
H=absorbance value for the highest concentration of the standard curve.
a, b, c, d, e=average absorbance values for each concentration of the standard curve.

Plot the values obtained for *L* and *H* and connect the points with a straight line. Average the absorbance values for the sample and read the tetracycline or tetracycline hydrochloride concentration from the standard curve. Multiply the concentration by appropriate dilution factors to obtain the tetracycline or tetracycline hydrochloride content of the sample. Its potency is satisfactory if it contains the equivalent of not less than 85 percent of the number of milligrams of tetracycline hydrochloride that it is represented to contain.

(2) *Oleandomycin content.* (i) If oleandomycin phosphate is used, proceed as directed in paragraph (c)(1) of this section, except prepare the sample as follows: Dissolve the contents of a representative number of capsules in sufficient 0.1 *M* potassium phosphate buffer (pH 8.0) to give a stock solution of convenient concentration. Further dilute with 0.1 *M* potassium phosphate buffer (pH 8.0) to obtain a final concentration of 5.0 µg. of oleandomycin activity per milliliter (estimated).

(ii) If troleandomycin is used, proceed as follows: Dissolve the contents of a representative number of capsules in chloroform to give a stock solution of 1.0 milligram of oleandomycin activity per milliliter. Transfer 30 milliliters of the chloroform solution to a glass-stoppered test tube (200 millimeters x 22 millimeters) and add 20 milliliters of 1 *N* sodium hydroxide. Shake for 1 minute and centrifuge briefly to aid in the separation of the layers. With the aid of a syringe and needle, remove and discard the aqueous layer. Repeat the washing procedure with two more 20-milliter portions of 1 *N* sodium hydroxide solution. Filter the chloroform layer through a pledget of cotton.

Dilute an aliquot of this solution with chloroform to give a solution containing approximately 25 μg. of oleandomycin per milliliter. Transfer a 5.0 milliliter aliquot to a 40 milliliter glass-stoppered centrifuge tube, dilute to 20 milliliters, with chloroform, and determine the oleandomycin content as directed in paragraph (d)(1)(i) of this section.

Its content of oleandomycin is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(b) *Moisture.* Proceed as directed in § 440.80a(b)(5)(1) of this chapter.

(c) *Oleandomycin phosphate used in making the capsules*—(1) *Potency*—(i) *Cylinders* (*cups*). Used cylinders described in § 440.80a(b)(1)(i) of this chapter.

(ii) *Culture media.* (*a*) Use the nutrient agar described in § 440.80a (b)(1)(ii)(*a*) of this chapter for the seed layer and base layer, except that its pH after sterilization is 7.8 to 8.0.

(*b*) Use the nutrient agar described in § 440.80a(b)(1)(ii)(*a*) of this chapter for maintaining the test organism.

(iii) *Working standard.* Dissolve a suitable weighed quantity (usually 25 milligrams or less) of the working standard (obtained from the Food and Drug Administration) in 2 milliliters of ethanol, then add sufficient 0.1 *M* potassium phosphate buffer, pH 8.0, to give a concentration of 1,000 micrograms of oleandomycin base per milliliter. This stock solution may be kept in the refrigerator for 3 days.

(iv) *Preparation of sample.* Dissolve the sample in sufficient 0.1 *M* potassium phosphate buffer (pH 8.0) to give a convenient stock solution. Further dilute in 0.1 *M* potassium phosphate buffer (pH 8.0) to give a final concentration of 5.0 micrograms per milliliter (estimated).

(v) *Preparation of test organism.* The test organism is *Staphylococcus epidermidis* (ATCC 12228)[1] which is maintained on slants or agar described under paragraph (c)(1)(ii)(*a*) of this section. Wash the organism from the agar slant with 3 milliliters of sterile physiological saline solution onto a large agar surface such as that provided by a Roux bottle containing 300 milliliters of the agar described in paragraph (c)(1)(ii)(*a*) of this section. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate for 4 hours at 32° C. and then wash the resulting growth from the agar surface with about 30 milliliters of sterile physiological saline solution. Standardize the suspension by determining the dilution that will give 80-percent light transmission, using a suitable photoelectric colorimeter with a 650-millimicron filter and an 18-millimeter-diameter test tube as an absorption cell. Run test plates to determine the quantity of the diluted suspension (usually 1.5 milliliters) that should be added to each 100 milliliters of agar to give clear, sharp zones of inhibition of appropriate size.

(vi) *Preparation of plates.* Add 21 milliliters of the agar prepared as described in paragraph (c)(1)(ii)(*a*) of this section to each Petri dish (20 millimeters × 100 millimeters). Distribute the agar evenly in the plates and allow it to harden. Use the plates the same day they are prepared. Melt a sufficient amount of the agar described in paragraph (c)(1)(ii)(*a*) of this section, cool to 48° C., add the proper amount of the test organism as described in paragraph (c)(1)(v) of this section and mix thoroughly. Add 4 milliliters of this inoculated agar to each Petri dish. Distribute the agar evenly in the plates, cover with porcelain covers glazed on the outside, and allow to harden. After the agar has hardened, place 6 cylinders on the agar surface so that they are at approximately 60° intervals on a 2.8-centimeter radius.

(vii) *Standard curve.* Prepare the daily standard curve by further diluting the 1,000 micrograms per milliliter stock solution in 0.1 *M* potassium phosphate buffer (pH 8.0) to obtain concentrations of 3.2, 4.0, 5.0, 6.25 and 7.80 micrograms per milliliter. Use 3 plates for the determination of each point on the curve, except the 5.0 micrograms per milliliter concentration, a total of 12 plates. On each of 3 plates fill 3 cylinders with the 5.0 micrograms per milliliter standard, and the other 3 cylinders with the concentration under test. Thus, there

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

will be 36 five-microgram determinations and 9 determinations for each of the other points on the curve. After incubation, read the diameters of the circles of inhibition in the plates. Average the readings of the 5.0 micrograms per milliliter concentration and the readings of the point tested for each set of 3 plates and average also all 36 readings of the 5.0 micrograms per milliliter concentration. The average of the 36 readings of the 5.0 micrograms per milliliter concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 5.0 micrograms per milliliter reading for that set of 3 plates were the same as the correction point. Thus, if in correcting the 4.0-microgram concentration, the average of the 36 readings of the 5.0-microgram concentration were 20.0 millimeters, and the average of the 5.0-microgram concentration of this set of 3 plates were 19.8 millimeters, the correction would be +0.2 millimeter. If the average reading of the 4.0-microgram concentration of these same 3 plates were 19.0 millimeters, the corrected value would be 19.2 millimeters. Plot these corrected values, including the average of the 5.0 micrograms per milliliter concentration, on 2-cycle semilog paper, using the concentration in micrograms per milliliter as the ordinate (the logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points, either by inspection or by means of the following equations:

$$L=(3a+2b+c-e)/5,$$
$$H=(3e+2d+c-a)/5,$$

where:

L=corrected zone diameter for the lowest concentration of the standard curve,

H=corrected zone diameter for the highest concentration of the standard curve,

c=average zone diameter for 36 readings of the 5.0 micrograms per milliliter standard.

a, b, d, e=corrected average values for the 3.2, 4.0, 6.25, and 7.81 micrograms per milliliter standard solutions, respectively.

Plot the values obtained for *L* and *H* and connect with a straight line.

(viii) *Assay.* Use 3 plates for each sample. Fill 3 cylinders on each plate with the standard 5.0 micrograms per milliliter solution and 3 cylinders with the 5.0 micrograms per milliliter (estimated) sample, alternating standard and sample. Incubate all plates, including those containing the standard curve, at 32° C.–35° C. overnight, and measure the diameter of each circle of inhibition. To estimate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on the 3 plates used. If the sample gives a larger zone size than the average of the standard, add the difference between them to the 5.0 micrograms per milliliter zone on the standard curve. If the average sample value is lower than the standard value, subtract the difference between them from the 5.0 micrograms per milliliter value on the curve. From the standard curve, read the potencies corresponding to these corrected values of zone sizes.

(2) *Toxicity.* Proceed as directed in § 440.80a(b)(4) of this chapter, except use physiological salt solution as the diluent, and inject 0.5 milliliter of a solution containing 8 milligrams per milliliter.

(3) *Moisture.* Proceed as directed in § 440.80(b)(5)(i) of this chapter.

(4) *pH.* Proceed as directed in § 440.80a(b)(5)(ii) of this chapter, using a solution containing 100 milligrams per milliliter.

(5) *Crystallinity.* Proceed as directed in § 440.80a(b)(5)(iii) of this chapter.

(d) *Troleandomycin used in making the capsules*—(1) *Potency*—(i) *Chemical method*—(*a*) *Reagents and equipment.* (*1*) Methyl orange reagent: Shake 0.5 *M* boric acid solution for about 12 hours (to insure saturation) with an excess of methyl orange indicators. An alternative method is to heat the mixture to about 50° C. and shake for about an hour. Then allow to cool. Filter the saturated dye solution and wash three times with chloroform. Store the dye solution over chloroform.

(*2*) Acid-alcohol solution: Add 2 milliliters of concentrated sulfuric acid to 98 milliliters of absolute methyl alcohol.

(*3*) Glycerin: Reagent grade.

(*4*) Centrifuge tubes: 40 milliliters, glass-stoppered.

(*b*) *Procedure.* Prepare a chloroform solution containing 50.0 milligrams activity of standard oleandomycin base in 200 milliliters of solution. Transfer 10.0 milliliters of the solution to a 100-milliliter volumetric flask and dilute to volume with chloroform. Transfer 2.0, 4.0, 6.0, and 8.0 milliliters of this solution to glass-stoppered centrifuge tubes (40-milliliter size) and dilute to a total volume of 20.0 milliliters each with chloroform. To the 20.0 milliliters of the solution present in each (40-milliliter size) centrifuge tube add 0.2 milliliter of glacial acetic acid, 0.20 milliliter of glycerin, and 0.40 milliliter of methyl orange reagent. Shake for 5 minutes and centrifuge for 3 minutes. Immediately transfer to another tube a 10.0-milliliter aliquot from the chloroform (lower) layer. Care must be exercised to see that no portion of the dye-glycerin-phase is included with the chloroform aliquot. Add 1.0 milliliter of acid-alcohol solution to this chloroform aliquot, mix well, and read the absorbancy at 535 mμ, using a 1-centimeter cell and a suitable photometer and using chloroform, similarly treated, as a blank. Prepare a standard curve, plotting the absorbance values of the standard solutions against the concentration expressed in micrograms per aliquot. Accurately weigh the sample to be tested to give 50 milligrams (estimated) of oleandomycin activity, dissolve in chloroform, and make to 200 milliliters with chloroform. Transfer 10.0 milliliters to a 100-milliliter volumetric flask and make to volume with chloroform. Transfer 5.0 milliliters to a glass-stoppered centrifuge tube and proceed as above. Determine the potency of the sample from the standard curve.

(ii) *Microbiological assay.* Proceed as directed in paragraph (c)(1) of this section, except:

(*a*) In lieu of the directions in paragraph (c)(1)(ii)(*a*) of this section, use the nutrient agar described in § 440.80a(b)(1)(ii)(*a*) of this chapter for the seed and base layers, except add 2.0 milliliters of polysorbate 80 to each 100 milliliters of agar. Its pH after sterilization is 7.8 to 8.0.

(*b*) In lieu of the directions in paragraph (c)(1)(iii) of this section, dissolve a suitable weighed quantity (usually 25 milligrams or less) of the troleandomycin working standard (obtained from the Food and Drug Administration) in sufficient 80 percent isopropyl alcohol-water solution to give a concentration of 1,000 micrograms per milliliter (estimated). Use the solution the day that it is prepared.

(*c*) In lieu of the directions in paragraph (c)(1)(iv) of this section, dissolve the sample in sufficient 80 percent isopropyl alcohol-water solution to give a convenient stock solution. Further dilute in 0.2 *M* potassium phosphate buffer, pH 10.5 (35 grams of dipotassium phosphate plus 2 milliliters of 10 *N* NaOH, q.s. to 1 liter), to give a final concentration of 15 micrograms per milliliter (estimated).

(*d*) In lieu of the directions in paragraph (c)(1)(vi) of this section, use the agar described in paragraph (d)(1)(ii)(*a*) of this section for both layers. Use the plates as soon after seeding as is practical. If they are not to be used shortly after seeding, then they should be refrigerated until ready for use.

(*e*) In lieu of the directions for preparing the standard curve in paragraph (c)(1)(vii) of this section, prepare the standard curve by diluting the stock solution in 0.2 *M* potassium phosphate buffer, pH 10.5, to give concentrations of 9.6, 12.0, 15.0, 18.8, and 23.4 micrograms per milliliter. The 15.0 micrograms per milliliter is the reference concentration.

(*f*) In lieu of the directions in paragraph (c)(1)(viii) of this section, incubate the plates at 37° C. overnight. The concentration of the sample and standard being tested is 15.0 micrograms per milliliter.

(2) *Toxicity.* Administer orally, by means of a cannula or other suitable device, to each of five mice within the weight range of 18 grams to 25 grams, 0.5 milliliter of a suspension containing 200 milligrams per milliliter in normal saline solution. If no animal dies within 48 hours, the sample is nontoxic. If one or more animals die within 48 hours, repeat the test, using for each test five or more previously unused mice weighing 20 grams (±0.5 gram) each; if the total deaths within 48 hours is no greater than 10 percent of

the total number of animals tested, including the original test, the sample is nontoxic.

(3) *Moisture.* Proceed as directed in § 440.80a(b)(5)(i) of this chapter.

(4) *pH.* Proceed as directed in § 440.80a(b)(5)(ii) of this chapter, using a saturated aqueous-ethanol (1:1) solution prepared by adding 100 milligrams per milliliter.

(5) *Paper chromatograph method*—(i) *Apparatus and reagents*—(*a*) Chromatographic chamber (cylinder glass-stoppered museum jar 11.5 inches × 3.5 inches).

(*b*) Chromatographic paper (8 inches × 8 inches Whatman No. 1).

(*c*) 0.1 *N* hydrochloric acid.

(*d*) Resolving solvent: Butyl acetate, benzene, nitromethane, pyridine (5:5:5:1 by volume).

(*e*) Spray reagent: 15 grams antimony trichloride per 100 milliliters of chloroform.

(ii) *Procedure.* Dissolve the sample in chloroform to give a solution containing 10 milligrams to 20 milligrams per milliliter. Prepare a sheet of chromatographic paper by drawing a line of origin parallel to and 1 inch from the edge of the paper. Wet the paper thoroughly with the 0.1 *N* hydrochloric acid and blot it firmly between sheets of absorbent paper. Starting 2 inches in from the edges and at 1-inch intervals, apply 3 microliters to 5 microliters of the sample solutions to the starting line. Allow a few minutes for the paper to dry partially. While the paper is still damp, form a cylinder by bringing the outer edges together, allowing about 1-inch overlap, and secure with a paper clip. Stand the paper in the chromatographic chamber, which has been filled to a depth of ½-inch with the resolving solvent. After the solvent front rises to a height of 4 inches to 5 inches above the origin, remove the paper from the tank and hang it up to air dry. Spray the dried paper with the antimony trichloride reagent. Hang the paper in a 100° C. oven for 3 minutes. A purple spot becomes visible for trioleandomycin at an R_f value of about 0.85. The approximate R_f values for diacetyloleandomycin, monoacetyloleandomycin, and oleandomycin are, respectively, 0.72, 0.27, and 0.13.

(6) *Acetyl determination*—(i) *Apparatus and reagents.* (*a*) One three-necked Pyrex flask of approximately 45 milliliters capacity, pear-shaped with T-joints, agar inlet tube, glass-stoppered funnel, glass condenser, and bubble counter.

(*b*) 50-milliliter Pyrex Erlenmeyer flask.

(*c*) 10-milliliter burette, calibrated in 0.02 milliliter.

(*d*) Anhydrous methanol, reagent grade.

(*e*) 2 *N* sodium hydroxide solution.

(*f*) Sulfuric acid solution prepared by adding 100 milliliters of concentrated H_2SO_4 to 200 milliliters of water.

(*g*) 1 *N* barium chloride solution.

(*h*) Phenolphthalein solution (1 percent in ethanol).

(*i*) Water-pumped nitrogen.

(*j*) NaOH solution, 0.015 *N*.

(ii) *Procedure.* Weight accurately (to 0.01 milligram) approximately 30 milligrams of the sample into the three-necked acetyl flask. Add 2.0 milliliters of methanol to dissolve the sample, then add slowly with gentle swirling, 1.0 milliliter of NaOH solution. Connect the gas inlet tube with bubble counter attached, and adjust nitrogen flow to about two bubbles a second. Put glass-stoppered funnel in centerneck of acetyl flask and put about 5 milliliters of H_2O in the funnel. Add a boiling chip to the solution and attach condenser in the refluxing position with water cooling. Adjust burner flame under acetyl flask to reflux solution gently. Reflux for 30 minutes. Cool assembly slightly then rinse down condenser (still in reflux position) with a few milliliters of H_2O. Reassemble condenser to the distillation position and add water through the funnel to make a total of approximately 5 milliliters of H_2O added to acetyl flask. Adjust burner flame so that about 5 milliliters of H_2O and methanol is distilled over in approximately 10 minutes. Discard this distillate. Cool acetyl flask slightly. Acidify solution in flask by adding 1 milliliter of the sulfuric acid solution through the funnel. Adjust burner flame and distill over approximately 20

milliliters of distillate into an Erlenmeyer flask in about 20 minutes, adding water through the funnel as necessary. It is important to keep the liquid volume in the acetyl flask around 2 milliliters to 3 milliliters in order to obtain a quantitative recovery of the acetic acid. Collect a second fraction of distillate, about 10 milliliters in volume. As the second fraction is distilling, process the first fraction. Heat the first reaction and boil gently about 20 seconds. Add a few drops of $BaCL_2$ solution to check if any sulfate was distilled over. If the sulfate is present, discard and repeat the whole determination. If the sulfate is absent immediately titrate the solution with the 0.015 *N* NaOH solution to a faint pink endpoint, using one drop of phenolphthalein solution as the indicator. Repeat the above procedure with the second fraction. If the second fraction requires less than 0.10 milliliter of the 0.015 *N* NaOH solution and all the acetic acid has been distilled over, the determination is completed. If greater than this, collect a third fraction of approximately 10 milliliters and titrate this as before. Total volumes of NaOH used and calculate results as follows:

(Milliliters of NaOH × *N* NaOH × 0.043 × 100)/ Weight sample in grams=Percent acetyl.

(7) *Crystallinity.* Proceed as directed in § 440.80a(b)(5)(iii) of this chapter.

§ 436.516 Tetracycline-neomycin complex powder topical; tetracycline hydrochloride-neomycin sulfate powder topical.

(a) *Potency*—(1) *Tetracycline-neomycin complex powder*—(i) *Tetracycline content.* Proceed as directed in § 436.514(a)(2), except use water in lieu of 0.1 *N* HCl for dissolving the sample. Its tetracycline content is satisfactory if it contains not less than 85 percent of the equivalent number of milligrams of tetracycline hydrochloride that it is represented to contain.

(ii) *Neomycin content.* Using 0.1 *M* potassium phosphate buffer, pH 8.0, dilute an appropriate aliquot of the aqueous solution, prepared as directed in paragraph (a)(1) of this section, to a final concentration of 1 μg. per milliliter (estimated), and proceed as directed in § 436.515(c)(1), except that the neomycin standard stock solution described § 436.517(b)(1)(iii) is used to prepare the standard curve, by further diluting with pH 8.0 buffer to final concentrations of 0.64, 0.80, 1.0, 1.25, and 1.56 μg. per milliliter. The 1.0 μg per milliliter solution is the reference concentration. In lieu of the method described in this subparagraph, the neomycin content may also be determined as follows. Using the aqueous solution described, prepare the sample and proceed as directed in § 436.517(b)(1), except use *Staphylococcus aureus* (American Type Culture Collection 12715)[1] as the test organism, which is grown and maintained on agar containing 100 μg. of tetracycline hydrochloride per milliliter of agar. Its neomycin content is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(2) *Tetracycline hydrochloride-neomycin sulfate powder*—(i) *Tetracycline hydrochloride content.* Prepare the sample as directed in § 436.514(a)(2). Use an appropriate aliquot and proceed as directed in § 446.81a(b)(1) of this chapter. Its tetracycline hydrochloride content is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(ii) *Neomycin content.* Use an appropriate aliquot of the solution prepared in paragraph (a)(2)(i) of this section and proceed as directed in paragraph (a)(1)(ii) of this section. Its neomycin content is satisfactory if it contains not less than 85 percent of the number of milligrams that it is represented to contain.

(b) *Sterility.* Thoroughly cleanse with a suitable disinfectant the value (do not flame) of each container to be tested. Into each of two empty, sterile Erlenmeyer flasks stoppered with a cotton plug, spray quantitites sufficient to yield a residue of approximately the equivalent of 50 milligrams from 10 separate cans by removing the plug temporarily and using aseptic technique while spraying; allow propellant to evaporate, add 250 milliliters to 500 milliliters of diluting fluid B in lieu of diluting fluid A, and swirl the flasks to dissolve the contents. Then proceed as

[1] Available from: American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

directed in § 436.20 of this chapter using the method described in paragraph (e)(1) of that section.

(c) *Moisture.* Proceed as directed in § 536.513(c) of this chapter.

(d) *Tetracycline-neomycin complex used in making the drug*—(1) *Potency*—(i) *Tetracycline content.* Dissolve the sample to be tested in sufficient water to give a convenient stock solution. Using an appropriate aliquot, proceed as directed in § 446.81a(b)(1).

(ii) *Neomycin content.* Using an aliquot of the stock solution prepared as directed in paragraph (d)(1)(i) of this paragraph, proceed as directed in paragraph (a)(2) of this section, except the last sentence of that subparagraph.

(2) *Toxicity.* Proceed as directed in § 440.80a(b)(4) of this chapter using 0.5 milliliter of a solution prepared by diluting the sample with physiological sodium chloride solution to contain 200 μg. of neomycin per milliliter (estimated).

(3) *pH.* Using a 1-percent aqueous solution, proceed as directed in § 440.80a(b)(5)(ii) of this chapter.

[39 FR 18944, May 30, 1974, as amended at 40 FR 13497, Mar. 27, 1975]

§ 436.517 Bacitracin-neomycin tablets; zinc bacitracin-neomycin tablets; bacitracin methylene disalicylate-neomycin tablets.

(a) *Tablets*—(1) *Potency*—(i) *Bacitracin, zinc bacitracin, or bacitracin methylene disalicylate content.* Proceed as directed in § 448.110a(b)(1). Its content of bacitracin, zinc bacitracin, or bacitracin methylene disalicylate is satisfactory if it contains not less than 85 percent of the number of units per tablet that it is represented to contain.

(ii) *Neomycin content.* Place 5 tablets in a blending jar and add thereto 200 milliliters of a 500-milliliter quantity of 0.10-percent phosphate buffer pH 8.0. After blending for 1 minute with a high-speed blender, add the remainder of the buffer. Blend again for 1 minute and make the proper estimated dilutions in the buffer and proceed as directed in paragraph (b)(1) of this section. Its content of neomycin is satisfactory if it contains not less than 85 percent of the number of milligrams of activity that it is represented to contain.

(2) *Moisture.* Proceed as directed in § 440.80a(b)(5)(i) of this chapter.

(3) *Disintegration time.* Proceed as directed in § 440.180a(b)(3).

(b) *Neomycin used in making the tablets*—(1) *Potency*—(i) *Cylinders* (*cups*). Use cylinders described under § 440.80a(b)(1)(i) of this chapter.

(ii) *Culture medium.* Use the medium described in § 440.80a(b)(1) (ii)(*a*) of this chapter for both the base and seed layers, except its pH after sterilization is 7.8 to 8.0.

(iii) *Working standard.* Dry the working standard (obtained from the U.S.P. Reference Standards Committee, 46 Park Avenue, New York 16, N.Y.) for 3 hours at 60° C. and a pressure of 5 millimeters or less and weigh out a sufficient quantity to make a convenient stock solution by diluting with a 0.1 *M* potassium phosphate buffer, pH 7.8 to 8.0. The stock solution, when stored at a temperature of approximately 15° C., or less, may be used for a period not exceeding 1 month.

(iv) *Standard curve.* Using the stock solution, prepare a daily standard curve as directed in § 444.70a(b)(1)(iv) of this chapter, using solutions of the neomycin working standard in 0.1*M* potassium phosphate buffer, pH 8.0, in concentrations of 6.4, 8.0, 10.0, 12.5, and 15.6 micrograms per milliliter if the test organism *Staphylococcus aureus* (ATCC 6538P),[1] or in concentrations of 0.64, 0.80, 1.0, 1.25, and 1.56 micrograms per milliliter if the test organism is *Staphylococcus epidermidis* (ATCC 12228).[1] The 10.0 micrograms per milliliter and the 1.0 microgram per milliliter concentrations are used as the reference points.

(v) *Preparation of test organism.* The test organism is *Staphylococcus aureus* (ATCC 6538P),[1] which is maintained on agar described in § 440.80a(b)(1)(ii)(*a*) of this chapter. From a stock slant inoculate a Roux bottle containing this same agar and incubate for 24 hours at 32° C.–35° C. Wash the resulting growth from the agar surface with about 50 milliliters of sterile sodium chloride solution. Standardize this suspension by determining the dilution that will permit 80 percent light transmission through a filter at 6500 Angstrom units

[1] See footnote 1 to § 436.516.

in a photoelectric colorimeter. The suspension may be used for 2 weeks if it is stored under refrigeration.*Staphylococcus epidermidis* (ATCC 12228),[1] which is maintained on agar as described in § 440.80a(b)(1)(ii)(*a*) of this chapter, may also be used as the test organism. From a stock slant, inoculate a Roux bottle containing this medium and incubate for 24 hours at 32° C.–35° C. Wash the resulting growth from the agar surface, using approximately 30 milliliters of sterile sodium chloride solution. Standardize the suspension by determining the dilution that will permit 80 percent light transmission through a filter of 6500 Angstrom units in a photoelectric colorimeter. The suspension may be stored for 2 weeks under refrigeration.

(vi) *Preparation of plates.* Using the agar described in subdivision (ii) of this subparagraph and approximately a 0.5 percent inoculum of the suspension described in paragraph (b)(1)(v) of this section, prepare the plates as directed in § 440.80a(b)(1)(v) of this chapter.

(vii) *Assay.* Dissolve volumetrically in 0.1 *M* potassium phosphate buffer, pH 7.8 to 8.0, the sample to be tested to make a convenient stock solution. Further dilute volumetrically this solution with 0.1 *M* potassium phosphate buffer, pH 7.8 to 8.0, to a final concentration of 10.0 micrograms (estimated) per milliliter, if the test organism is *Staphylococcusaureus* or 1.0 microgram per milliliter (estimated) if the test organism is *Staphylococcus epidermidis.*

(2) *Toxicity.* Proceed as directed in § 440.80a(b)(4) of this chapter, using 0.5 milliliter of a solution prepared by diluting the sample to approximately 200 micrograms per milliliter with physiological salt solution.

(3) *Moisture.* In an atmosphere of about 10 percent relative humidity, transfer about 100 milligrams of the finely powdered sample to a tared weighing bottle equipped with ground-glass top and stopper. Weigh the bottle and place it in a vacuum oven, tilting the stopper on its side so that there is no closure during the drying period. Dry at a temperature of 60° C. and a pressure of 5 millimeters of mercury or less for 3 hours. At the end of the drying period fill the vacuum oven with air dried by passing it through a drying agent such as sulfuric acid or silica gel. Replace the stopper and place the weighing bottle in a desiccator over a desiccating agent such as phosphorous pentoxide or silica gel, allow to cool to room temperature, and reweigh. Calculate the percent loss.

(4) *pH.* Proceed as directed in § 440.80a(b)(5)(ii) of this chapter, using a solution containing 33 milligrams per milliliter.

§ 436.542 Acid resistance/dissolution test for enteric-coated erythromycin pellets.

(a) *Equipment.* Use Apparatus 1 as described in the United States Pharmacopeia XX dissolution test.

(b) *Immersion fluids.* All immersion fluids may be degassed by heating immediately prior to use.

(1) *Acid resistance medium.* Use 0.06*N* hydrochloric acid, pH 1.2.

(2) *Dissolution medium.* Dissolve 6.8 grams of monobasic potassium phosphate in 250 milliliters of water. Add 109 milliliters of 0.2*N* sodium hydroxide and 400 milliliters of water and adjust the resulting solution with 0.2*N* sodium hydroxide to a pH of 6.8±0.1. Dilute to 1 liter.

(c) *Procedure.* Warm the immersion fluids to a temperature of 37° ±5.0° C. Place the contents of one capsule into the basket. Lower the basket into 900 milliliters of acid resistance medium contained in the beaker. Ensure that all air is displaced from the immersed basket and that the pellets remain in the basket. Rotate the basket at the speed of 50 revolutions per minute for an accurately timed period of 1 hour. Remove the basket from the fluid and immediately lower the basket into 900 milliliters of dissolution medium contained in the beaker. Again ensure that all air is displaced from the immersed basket and that the pellets remain in the basket. Rotate the basket at 50 revolutions per minute for an accurately timed dissolution period of 45 minutes. Withdraw a 25-milliliter sample of the dissolution medium from a point midway between the stirring shaft and the wall of the vessel and approximately midway in depth. Filter the sample through a Whatman 541 filter paper or equivalent, discarding the first 2 milliliters. Assay for erythromycin using

the filtrate as the test solution as directed in § 436.105. Repeat the test on five additional capsules.

(d) *Evaluation.* Use the interpretation described in the United States Pharmacopeia XX dissolution test.

[46 FR 16678, Mar. 13, 1981, as amended at 50 FR 47213, Nov. 15, 1985; 52 FR 35912, Sept. 24, 1987; 54 FR 41824, Oct. 12, 1989]

§ 436.543 Acid resistance test for pellet-filled doxycycline hyclate capsules.

(a) *Equipment.* Use Apparatus 1 as described in the United States Pharmacopeia XXI dissolution test.

(b) *Acid resistance medium.* Use 0.06*N* hydrochloric acid, pH 1.2. May be degassed by heating immediately prior to use.

(c) *Procedure.* Warm the acid resistance medium to a temperature of 37±2.0 ° C. Place the contents of one pellet-filled capsule into the basket. Lower the basket into a beaker containing 900 milliliters of acid resistance medium. Ensure that all air is displaced from the immersed basket and that the contents of the pellet-filled capsule remain in the basket.

Rotate the basket at the speed of 50 revolutions per minute for an accurately timed period of 20 minutes. Withdraw a 5-milliliter sample of the acid resistance medium from a point midway between the stirring shaft and the wall of the vessel and approximately midway in depth (this is the sample solution). Assay the sample solution for doxycycline as described in paragraph (d) of this section. Repeat the test on five additional pellet-filled capsules.

(d) *Doxycycline content*—(1) *Preparation of working standard solution.* Dissolve an accurately weighed portion of doxycycline hyclate working standard with 0.1*M* hydrochloric acid to obtain a concentration of 0.01 milligram per milliliter.

(2) *Preparation of sample solution.* Dilute the 5-milliliter sample portion to 25 milliliters with 0.1*M* hydrochloric acid.

(3) *Procedure.* Using a suitable spectrophotometer and 0.1*M* hydrochloric acid as the blank, determine the absorbance of each standard and sample solution at the absorbance peak at approximately 345 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(4) *Calculations.* Determine the total amount of doxycycline dissolved as follows:

$$\text{Milligrams of doxycycline dissolved} = \frac{A_u \times c \times d \times 900^*}{A_s}$$

where:

A_u=Absorbance of sample;

A_s=Absorbance of standard;

c=Concentration of working standard in milligrams; and

d=Dilution factor of sample withdrawn from beaker.

*If more than 15 milliliters of dissolution medium is removed, correct for the volume removed.

(e) *Evaluation.* The pellet-filled capsule passes the test if no more than 50 percent of the drug is dissolved at 20 minutes. If one pellet-filled capsule fails to meet this requirement, repeat the test on six additional pellet-filled capsules. No more than 2 pellet-filled capsules in 12 may exceed 50 percent of the drug dissolved at 20 minutes.

[50 FR 41679, Oct. 15, 1985; 50 FR 45603, Nov. 1, 1985]

§ 436.544 Dissolution test for pellet-filled doxycycline hyclate capsules.

(a) *Equipment.* Use Apparatus 1 as described in the United States Pharmacopeia XXI dissolution test.

(b) *Dissolution medium.* Prepare the dissolution medium as follows: Dissolve 10.21 grams of potassium biphthalate and 1.4 grams of sodium hydroxide in approximately 950 milliliters of distilled water and adjust the pH to 5.5 using 1*M* sodium hydroxide solution. Dilute with distilled water to 1,000 milliliters.

(c) *Procedure.* Proceed as directed in the United States Pharmacopeia XXI dissolution test. Ensure that all air is displaced from the immersed basket and that the contents of the pellet-filled capsule remain in the basket. Rotate the basket at the speed of 50 revolutions per minute for an accurately timed period of 30 minutes. Withdraw a 5-milliliter sample of the dissolution medium from a point midway between the stirring shaft and the wall of the

vessel and approximately midway in depth (this is the sample solution). Assay the sample solution for doxycycline as described in paragraph (d) of this section. Repeat the test on five additional pellet-filled capsules.

(d) *Doxycycline content*—(1) *Preparation of working standard solution.* Dissolve an accurately weighed portion of doxycycline hyclate working standard with 0.1*M* hydrochloric acid to obtain a concentration of 0.01 milligram per milliliter.

(2) *Preparation of sample solution.* Dilute the 5-milliliter sample portion to 25 milliliters with 0.1*M* hydrochloric acid.

(3) *Procedure.* Using a suitable spectrophotometer and 0.1*M* hydrochloric acid as the blank, determine the absorbance of each standard and sample solution at the absorbance peak at approximately 345 nanometers. Determine the exact position of the absorption peak for the particular instrument used.

(4) *Calculations.* Determine the total amount of doxycycline dissolved as follows:

$$\text{Milligrams of doxycycline dissolved} = \frac{A_u \times c \times d \times 900^*}{A_s}$$

where:

A_u=Absorbance of sample;
A_s=Absorbance of standard;
c=Concentration of working standard in milligrams; and
d=Dilution factor of sample withdrawn from beaker.

*If more than 15 milliliters of dissolution medium is removed, correct for the volume removed.

(e) *Evaluation.* Use the dissolution acceptance table and interpretation in the United States Pharmacopeia XXI.

[50 FR 41679, Oct. 15, 1985]

§ 436.545 Acid resistance test for erythromycin particles in tablets.

(a) *Equipment.* Use Apparatus 2 as described in the United States Pharmacopeia XXI dissolution test.

(b) *Acid resistance medium.* Use 0.1*N* hydrochloric acid, 500 milliliters.

(c) *Procedure.* Warm the immersion fluid to a temperature of 37±0.5 °C. Place one tablet into a vessel containing 500 milliliters of acid resistance medium. Rotate the paddle at the speed of 50 revolutions per minute for an accurately timed period of 1 hour. Withdraw a 50-milliliter sample of the dissolution medium from a point midway between the stirring shaft and the wall of the vessel and approximately midway in depth. Filter the sample through a Whatman No. 1 filter paper or equivalent, discarding the first 5.0 milliliters. Assay for dissolved erythromycin as directed in paragraph (d) of this section using the filtrate as the sample solution. Repeat the test on five additional tablets.

(d) *Arsenomolybdate colorimetric assay for dissolved erythromycin*—(1) *Apparatus.* Automatic analyzer consisting of (i) a liquid sampler, (ii) a proportioning pump, (iii) suitable spectrophotometers equipped with matched flow cells and analysis capability at 660 nanometers, (iv) a means of recording spectrophotometric readings, and (v) a manifold consisting of the components illustrated in the diagram in paragraph (d)(4) of this section.

(2) *Reagents*—(i) *Arsenomolybdate solutions*—(*a*) *Stock solution.* Dissolve 100 grams of ammonium molybdate in approximately 1,700 milliliters of water contained in a 2-liter volumetric flask. Insert an inert plastic coated stirring bar into the flask, and begin mixing. While mixing, slowly add 84 milliliters of sulfuric acid (temperature of solution should not exceed 50 °C). Dissolve 12 grams of sodium arsenate in 100 milliliters of water, and add to the solution in the flask. Remove the stirring bar, dilute with water to volume, and mix. Store in an amber bottle for 24 hours before using. (This solution should not be allowed to come into contact with rubber.)

(*b*) *Working solution.* Dilute 1 part of stock solution with 2 parts of water, and mix. This solution is freshly prepared on the day of use.

(ii) *Acetate buffer, pH 4.8.* Dissolve 133 grams of ACS grade sodium acetate crystals in about 3.5 liters of water. Adjust the pH to 4.8±0.1 with glacial acetic acid. Dilute with water to 4,000 milliliters, and mix.

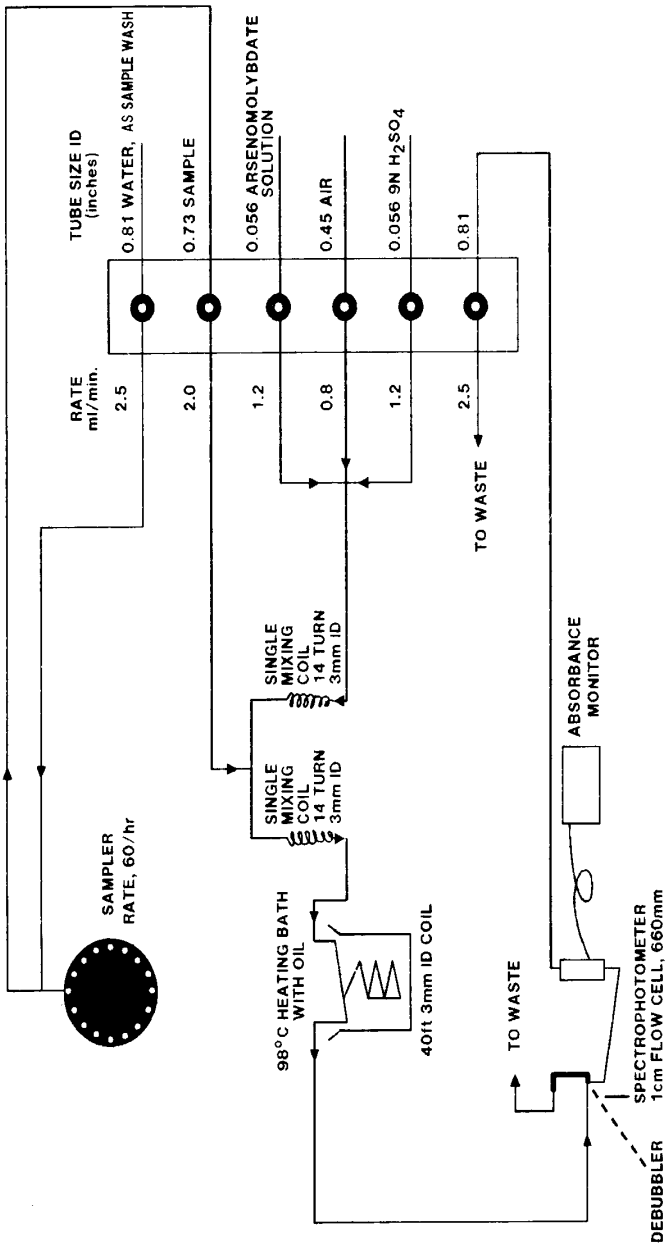
(iii) *9N Sulfuric acid.* Place a 2-liter volumetric flask containing an inert plastic coated magnetic stirring bar and about 1,500 milliliters of water in

an ice bath, and begin mixing. While mixing, cautiously add 300 milliliters of sulfuric acid. Allow the solution to cool. Remove the stirring bar, dilute with water to volume, and mix.

(3) *Preparation of working standard solutions*—(i) *Working standard stock solution.* Accurately weigh approximately 400 milligrams of USP Erythromycin Reference Standard, previously dried at 60 °C for 3 hours under vacuum (pressure of 5 millimeters of mercury or less), and transfer to a 100-milliliter volumetric flask. Dissolve and dilute with acetate buffer, pH 4.8 to volume, and mix.

(ii) *Working standard solutions.* Pipet 5, 10, 15, and 20 milliliters of the standard stock solution into separate 500-milliliter volumetric flasks, add acetate buffer, pH 4.8 to volume, and mix. The approximate concentrations of these solutions (before adjusting for the standard potency) are 40, 80, 120, and 160 micrograms of erythromycin per milliliter, respectively.

(4) *Procedure.* Use the working standard solutions prepared as described in paragraph (d)(3) of this section. The arrangement of the apparatus and flow of the samples and reagents are shown in the manifold diagram set forth following this paragraph. The sampler rate is usually 60 per hour, but may be varied. Establish a steady state by pumping reagents until the record trace becomes constant. Place cups containing the four concentrations of working standard solutions in the sampler followed by no more than 12 cups of sample solutions. Then place four more cups containing the four concentrations of working standard solutions in the sampler. Repeat the sequence above for additional samples by bracketing standards around no more than 12 sample solutions at a time.



(5) *System suitability test.* Perform a linear regression analysis of absorbance versus concentration in micrograms per milliliter of the standards. The system is suitable for calculation if the beginning baseline and the ending baseline after assaying a series of standard and sample solutions

does not vary by more than 2 percent transmittance, and the correlation coefficient for each standard curve is greater than 0.995.

(6) *Calculations.* (i) Calculate the concentration of each standard curve solution in micrograms of erythromycin per milliliter as follows:

$$\text{Concentration of each standard curve solution (micrograms of erythromycin per milliliter)} = \left(\frac{\text{Milligrams of working standard} \times \text{Potency of working standard (micrograms per milligram)}}{100}\right) \times \left(\frac{\text{Milliliters of standard stock solution}}{500}\right)$$

(ii) Calculate the percent of labeled amount of erythromycin released in 60 minutes as follows:

$$\text{Percent of labeled amount of erythromycin released in 60 minutes} = \left(\frac{500}{1{,}000}\right) \times \left(\frac{100}{\text{erythromycin content of tablet}}\right) \times \text{Micrograms of erythromycin per milliliter}$$

[51 FR 37721, Oct. 24, 1986]

PART 440—PENICILLIN ANTIBIOTIC DRUGS

Subpart A—Bulk Drugs

Subpart B—Oral Dosage Forms