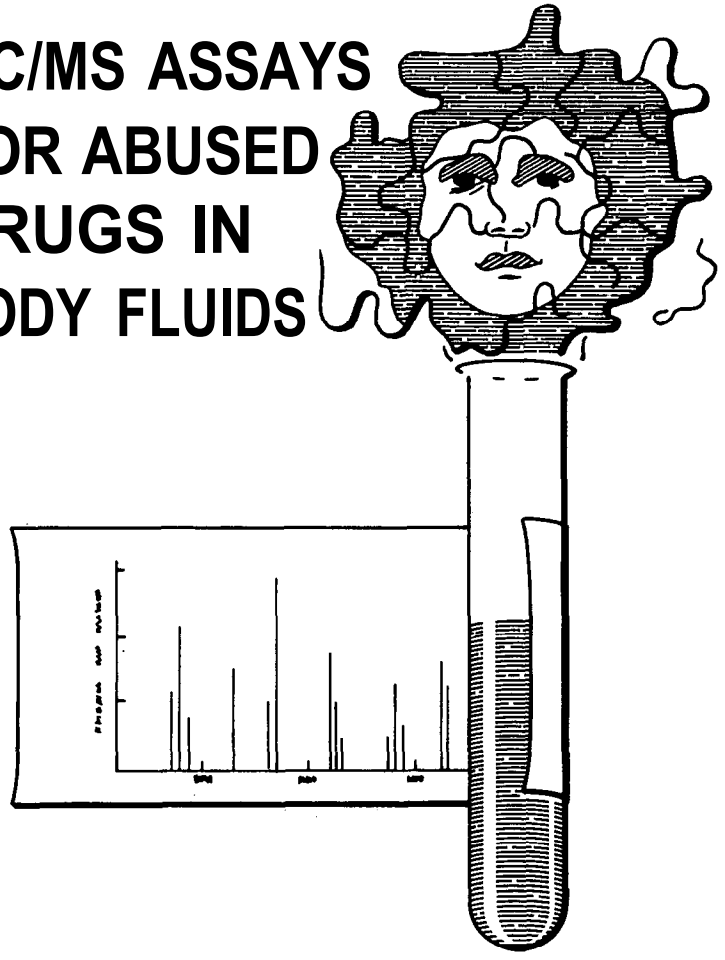
National Institute on Drug Abuse

Research 32

MONOGRAPH SERIES

GC/MS ASSAYS FOR ABUSED DRUGS IN BODY FLUIDS



U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES • Public Health Service • Alchol, Drug Abuse, and Mental Health Administration

GC/MS Assays for Abused Drugs in Body Fluids

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GC/MS Assays for Abused Drugs in Body Fluids

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Foreword

This monograph presents a collection of methods for the quantitative analysis of several important drugs of abuse by the technique of gas chromatography-mass spectrometry (GC/MS). The material is arranged textbook fashion, by chapters on specific drugs, to assist researchers in setting up GC/MS assay procedures in their own laboratories.

These assays are particularly suited to basic studies on the pharmacology and pharmacokinetics of drugs of abuse. Forensic toxicology, which sometimes requires the highly sensitive and specific sample analysis provided by GC/MS, is another important area of application. Even when an alternate method such as immunoassay is available for such analyses, GC/MS is often the only means of providing a confirmation assay. The sensitivity and specificity of GC/MS are especially critical to studies on the impact of drugs on traffic safety. In addition, demand for legislation relating to drugs and highway safety is increasing, and enforcement of such laws will necessitate appropriate sample assays.

The unique characteristics of the GC/MS analytical method make it the only means of determining drug levels under certain circumstances. The inherent complexity of the technique, however, often dissuades investigators from embarking on studies that involve its use. At a time when basic questions underlying drug abuse call for increasingly sophisticated methods of data acquisition, this monograph should provide the information needed to make one of these procedures easier to establish and to encourage new areas of research.

Marvin Snyder, Ph. D.
Director, Division of Research
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Preface

Soon after the establishment of the National Institute on Drug Abuse in 1974, the development of an analytical program emphasizing gas chromatography/mass spectrometry (GC/MS) was initiated. A major part of this program was directed at the development, testing, and eventual publication of GC/MS methods suitable for a number of abused drugs of interest to this Institute. It was felt that by making available carefully described quantitative procedures for these drugs, more researchers would be encouraged to move into several critically important areas of investigation.

A major segment of this development and testing program was carried out by Battelle (Columbus Laboratories and at the Center for Human Toxicology at the University of Utah. These GC/MS methods emphasize chemical ionization techniques which frequently offer significant advantages in sensitivity and specificity over techniques of electron impact ionization.

Each of the techniques described in this monograph has been field tested by one or more independent laboratories in order to optimize both the procedure itself and the clarity of its presentation. This field testing was carried out by Battelle and consisted of sending the participating laboratories a series of known standard samples along with the information brochures included herein as chapters. The participating laboratories then set up the method in their own facilities and analyzed the spiked samples. Problems with either the instructional material or the method allowed Battelle to make many improvements to insure the highest probability of an inexperienced lab's success with one of these assays.

This monograph represents the combined efforts of many individuals and has evolved to its present state over several years. We hope that it will save research investigators valuable time in establishing quantitative essays of these drugs in their own laboratories. The basic principles of GC/MS quantitative analysis as presented here should be useful in setting up procedures for other drugs as well.

Richard L. Hawks , Ph.D.
Division of Research

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Chapter 1

Introduction

The combination of gas chromatography and mass spectrometry (GC/MS) became widely recognized in the 1960's as the most sensitive and versatile tool available for the identification of volatile organic compounds. It was not until the 1970's, however, and the development of selected ion monitoring techniques, that the potential of GC/MS for quantitative analysis was generally appreciated. It now seems certain that the quantitative measurement of specific organic compounds in complex mixtures will become the major application of GC/MS simply because its sensitivity, accuracy, and versatility are unequalled by any other technique, including the popular immunological assays.

The rapid increase in the use of GC/MS has occurred in spite of several severe limitations and deterrents. The major limitation is that for a compound to be analyzed by GC/MS it must have sufficient volatility and thermostability to pass through the gas chromatographic column intact in the vapor state, or be capable of conversion to a derivative which can do so; this requirement prohibits GC/MS analysis for 80 to 90 percent of known organic compounds. Fortunately, this is not a serious problem for the toxicologist, because most drugs and their metabolites are among the structures which can be analyzed by GC/MS. More serious deterrents to wider use of GC/MS in toxicological and clinical laboratories are the expense of GC/MS instrumentation and the relatively high level of training and skill required to make effective use of these instruments. Although the former is unlikely to be reduced in the future, the latter will undoubtedly become less burdensome as the reliability of GC/MS instrumentation is improved and its design simplified.

The purpose of this monograph is to assemble, within a single volume, detailed descriptions of GC/MS procedures for quantitative measurement of some of the drugs and their metabolites which are of particular concern to the National Institute on Drug Abuse (NIDA). The procedures were developed at Battelle Colunbus Laboratories and the Center for Human Toxicology at the University of Utah, and have been extensively evaluated at both of these laboratories. All of the assays make use of deuterium-labeled internal standards and packed column gas chromatography combined with chemical ionization mass spectrometry. This combination was chosen for the following reasons:

- Stable isotope labeled analogs constitute nearly ideal internal standards because their chemical and physical properties are nearly identical to those of the respective unlabeled compounds.

- Most analytical laboratories with GC/MS systems are more familiar with the use of packed columns than with capillary columns.

- Chemical ionization mass spectrometry, using the technique of selected ion monitoring, is the most sensitive method of detection available. With chemical ionization most drugs are efficiently ionized, the sample ion current is concentrated at one or two m/z values, and the likelihood of interference from ions generated by column bleed or components of the biological extract is minimized.

Each drug was originally the subject of a separate analytical manual. A decision was subsequently made, however, to combine the manuals into a single volume, with each chapter devoted to a specific drug and its metabolites. As a result of this evolution of manuscripts, each chapter is relatively self-contained and includes its own list of literature references and its own number sequence for figures, tables, and structural drawings. For this reason, anyone who has the tenacity to read the monograph from cover to cover will find excessive repetition of certain descriptions and explanations. Most readers, however, will not be concerned with all of the drugs included in the monograph, and will choose to read only selected chapters. Nevertheless, in order to keep repetition to a minimum, many of the considerations and operations which are common to all of the assays have been removed from the individual chapters and combined in Chapter 2. We therefore recommend that the reader begin with Chapter 2 and only then proceed to the chapters which deal with the drugs of particular interest.

On the premise that an analyst who must determine the concentration of a drug in biological specimens should know something about the drug, we have included in each chapter background information which we believe to be relevant and useful. This background includes:

- a brief history of the drug and its uses

- a summary of the pharmacology and metabolism of the drug

- a survey of literature procedures for the quantitative analysis of the drug and its metabolites, with particular emphasis on other GC/MS assays for the drug, or assays which may be considered as alternatives to GC/MS analysis

- a compilation of physical, chemical and spectroscopic data on the drug

Although GC/MS instruments are now prominent fixtures in many clinical and toxicological laboratories, too often their use is limited to the identification of unknowns by matching their electron impact

mass spectra to reference spectra. We hope that this monograph will encourage more analysts to use the power of GC/MS as a quantitative technique. It is important, of course, to recognize when to use GC/MS for a particular drug analysis and when to use some other technique. The assays described in this monograph are intended to be used either as reference methods for validation and quality control of other methods, or as the technique of choice when other available methods do not offer adequate sensitivity, specificity or accuracy. The latter situation is often a factor in forensic toxicology, because the outcome of court litigation may depend upon the reliability of a drug assay.

GC/MS can also reduce the cost of implementing a new drug assay. On several occasions we have attempted to use a "less expensive" technique for a particular drug assay, only to find that the assay did not have adequate sensitivity or specificity. Each time we resolved the problem by using GC/MS, but valuable time and money were expended.

Another reason why GC/MS can often be economic relative to other chromatographic techniques is that its specificity frequently permits use of simple and rapid extraction procedures. Not only does a simple extraction procedure lower cost by saving time, but it also reduces the opportunity for human error. The degree to which an extraction procedure can be simplified is dependent, however, upon the level of sensitivity required. For example, tetrahydrocannabinol is psychoactive at blood concentrations of 10 ng/ml, and less. At these low concentrations a multi-step extraction and purification procedure is required in order to eliminate interference by endogenous components in the biological medium. In contrast, pharmacologically significant blood concentrations of a drug such as methaqualone are much higher (> 100 ng/ml); and therefore a simple, direct extraction of bases without further purification or concentration is adequate for GC/MS analysis, provided the chromatographic and mass spectrometric conditions are chosen properly.

The exclusive use of chemical ionization (CI) in each of the drug as says, rather than electron impact (EI) ionization, deserves comment. In qualitative analysis the major advantage of CI is that it normally generates a prominent peak from which the molecular weight of a compound can be calculated. In quantitative analysis of drugs it offers two advantages over EI ionization: 1) the ion current of the analyte is usually concentrated at one or two compound-characteristic masses which are particularly suitable for selected ion monitoring, and 2) most drugs and their metabolites can be efficiently ionized by a selective reagent gas such as ammonia. These features usually permit better effective sensitivity with CI than with EI ionization. Nevertheless, analysts in many laboratories have been reluctant to use chemical ionization. Part of this reluctance undoubtedly reflects a lack of familiarity with CI. However, it is also true that there arc more experimental variables in CI than in EI ionization, and the task of optimizing and controlling these variables may discourage some analysts. For this reason we have included in Chapter 2, and in the discussions of each of the drug assay procedures, suggestions and guidelines for use of CI.

A number of newer chromatographic and mass spectrometric techniques are in various stages of development and evaluation for analysis of drugs. Glass capillary columns are rapidly increasing in popularity and will continue to be used in place of packed columns whenevcr greater separating power and higher sensitivity are needed. The commercial availability of combined liquid chromatograph/mass spectrometer (LC/MS) systems makes it feasible to analyze polar drugs and metabolites directly, without derivatization. The new technique of MS/MS, in which a mass analyzer is used in place of a gas chromatograph to separate the components of a mixture prior to identification or quantitation by mass spectrometry, may ultimately supplant GC/MS where large numbers of samples must be analyzed. Finally, negative ion chemical ionization has been shown to be capable of dramatically higher sensitivity than positive ion CI for the GC/MS analysis of compounds which have high electron affinities. The application of negative ion CI to the analysis of diazepam and N-desmethyldiazepam is discussed in Chapter 9.

None of these newer techniques are employed in the GC/MS drug assays in this monograph, primarily because at the time the assays were developed techniques such as LC/MS, negative ion CI, and MS/MS were in the early stages of development and were available at only a few research laboratories. It is now evident, however, that each of these techniques offers exciting capabilities that will uniquely satisfy specific analytical needs. It is likely that by the time this monograph is distributed, many readers will already have had experience with one or more of the techniques.

The authors gratefully acknowledge the contributions of the many individuals and institutions that have assisted in the development of these assays and the preparation of this monograph. First and foremost, we thank the National Institute on Drug Abuse (NIDA) for financial support. We especially appreciated the guidance, encouragement, and many helpful discussions with the Project Monitors, Drs. Richard L. Hawks and Robert E. Willette. Additionally, we would like to thank the Insurance Institute for Highway Safety for their financial support which enabled us to further evaluate and refine several of these assays. Those who contributed to the laboratory research include Dr. David A. Knowlton, Dr. Pauline A. Clarke, Dr. Denis C.K. Lin, Dr. Edith G. Leighty, Dr. Bruce A. Petersen, Mr. Warren E. Bresler, Mr. Bruce J. Hidy, and Dr. Frank W. Crow at Battelle Columbus Laboratories, and Mr. Dennis M. Chinn and bliss Cheryl L. Jackson at the Center for Human Toxicology. Others who helped evaluate the methods include: Dr. Dan S. Pearce, Laboratory of Criminalistics, Santa Ana, CA; Dr. Jack D. Henion, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY; Dr. James Ferguson, Toxicology Department, The Ohio State University, Columbus Ohio; Dr. Daniel R. DiFeo, Jr., Analytical Chemistry Center, The University of Texas Health Science Center, Houston, Texas; and Miss Pamela Ahearn, State of Rhode Island Department of Health, Providence, Rhode Island.

Finally, special thanks are due to Dr. Bryan S. Finkle at the Center for Human Toxicology for his invaluable assistance in preparing and reviewing the monograph manuscript.

Chapter 2

Experimental Considerations and Operations Common to All of the Assays

Each of the GC/MS assays described in this monograph includes the following steps:

1. Addition of a deuterium labeled internal standard to the biological specimen
2. Solvent extraction of the biological specimen
3. GC/MS analysis of the extract using chemical ioniznt ion and selected ion monitoring
4. Calculation of the drug concentration in the biological sample based on the ratio of ion currents generated by the drug and the internal standard.

This chapter discusses general considerations relevant to each of these operations, and presents our recommendations regarding the preparation of calibration graphs and procedures for monitoring the quality of the analytical data.

Even though all of the assays utilize chemical ionization (CI) , the procedures can be modified for electron impact (EI) ionization. 'Ibis change will usually result in lower sensitivity and specificity because the most abundant ions in the EI mass spectra of these drugs are generally not as satisfactory for selected ion monitoring as the abundant ions in the chemical ionization mass spectra (1). A more rigid requirement of these assays is that the GC/MS system must be capable of continuously monitoring the ion currents at two or more selected ion masses. The usefulness of selected ion monitoring (also called "multiple ion detection" and "mass fragmentography") is now so widely recognized that virtually all GC/MS systems intended for biomedical applications have this capability, either in the form of an interactive data system or a hardwired selected ion monitor.

TYPES AND SOURCES OF SPECIMENS

Because the blood concentration of a drug usually gives the best correlation with the pharmacological effect of the drug, the preferred biological specimens for drug analysis are whole blood, plasma, or serum. All of the GC/MS assays in this monograph are

capable of measuring drug concentrations in blood at least as low as the minimum level at which the drug exerts a detectable physiological effect.

If a choice of specimen is available, plasma is usually preferred. The blood should be collected in evacuated tubes containing sodium fluoride to inhibit bacterial and enzymatic action, and sodium oxalate or heparin to prevent coagulation. The blood should be centrifuged as soon as possible to separate the plasma from the red blood cells, and the plasma transferred into silylated glass tubes equipped with Teflon-lined screw caps. If storage or shipment of the plasma is necessary, it should be frozen and maintained in the frozen state until the extraction can be performed. Several guaranteed 24-hour parcel delivery services are now available to most cities in the United States. These services are well worth the additional cost for shipment of biological specimens.

The GC/MS assays described here are also applicable to other physiological fluids and tissues, including urine, saliva, cerebrospinal fluid, gastric fluid, and homogenates from adipose tissue, liver, kidney, etc. Some specimens, particularly those with a high fat content, may require additional purification in order to remove interfering endogenous compounds. This can be accomplished in the case of basic drugs by a "back extraction" procedure in which the drug is extracted from an organic solvent into aqueous acid. The acid solution is then made basic and the drug reextracted into an organic solvent.

Tetrahydrocannabinol (Chapter 6) is unique among the drugs discussed in this monograph in that it is a highly lipophilic, nonbasic drug, and therefore difficult to separate from the lipids present in physiological specimens. For this reason, the THC assay is a particularly striking example of the power of GC/MS to selectively measure a specific compound within a complex mixture of similar materials.

INTERNAL STANDARDS

The sensitivity of a GC/MS system depends on a large number of factors, some of which are difficult to control. Consequently, it is essential to use an internal standard if accurate, quantitative measurements are to be made. An ideal internal standard behaves identically to the analyte throughout the extraction, chromatographic separation, and ionization processes, so that the weight ratio of added internal standard to analyte will remain unaffected by any of these operations. Stable isotope labeled analogs come the closest to meeting the characteristics of ideal internal standards. The isotope label exerts a very slight, but usually insignificant effect on the physical and chemical properties of the compound, yet the higher mass of the isotope labeled ions of the internal standard permits them to be separated from the analyte ions by the mass spectrometer.

The major deterrent to the use of stable isotope labeled drugs as internal standards is their limited availability and the high cost of those that are available. In recognition of this problem, the

National Institute on Drug Abuse has contracted the synthesis of stable isotope labeled analogs of many of the abused drugs and their metabolites. Milligram quantities of these compounds are available without charge to authorized investigators engaged in research requiring the quantitative analysis of these drugs. Enquiries regarding the availability of specific isotope labeled drugs and metabolites should be sent to:

National Institute on Drug Abuse
Division of Research/Research Technology Branch
5600 Fishers Lane
Rockville, MD 20857
301-443-5280

Several commercial firms specialize in the synthesis and sale of stable isotope labeled compounds. These include:

Merck Sharp & Dohme Canada Limited
Isotope Division
P.O. Box 899
Pointe Claire-Dorval, Quebec H9R 4P7
Canada
514-697-2823

KOR Isotopes
56 Rogers Street
Cambridge, MA 02142 USA
517-661-8220

Prochem
19, Ox Bow Lane
Summit, NJ 07901 USA
201-273-0440

Chromacol Ltd.
73 a Friern Barnet Lane
London N20 OXT England
368- 7666

Stohler Isotope Chemicals, Inc.
49 Jones Road
Waltham, MA 02154 USA
617-891-1827

Unfortunately, the problems associated with selling controlled substances have discouraged some of the suppliers from any involvement with this category of drugs. Merck Sharp & Dohme Canada synthesizes and sells stable isotope labeled controlled drugs, but purchasers in the United States must obtain an American Government Permit to Import before Merck can ship the product.

Because the market for stable isotope labeled compounds is still relatively small, their cost is high when purchased from commercial sources. For this reason laboratories with organic synthesis expertise may elect to synthesize their own isotope labeled internal

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standards. Experimental procedures have been published for preparation of many deuterium labeled drugs and metabolites (2,3).

Several factors arc important to keep in mind when selecting an isotope labeled compound for use as an internal standard. Most importantly, the isotope must not undergo exchange under any of the conditions encountered during the extraction, derivatization, or chromatography steps of the assay, nor those within the ion source of the mass spectrometer. The replacement of a deuterium label by hydrogen within the ion source sometimes occurs unexpectedly under chemical ionization conditions. For example, a drug labeled with three dcuterium atoms attached to a tertiary amino methyl group was found to undergo extensive exchange between deuterium and hydrogen atoms when the drug was subjected to methane chemical ionization (3). Fortunately, the exchange did not occur when ammonia was used as the reagent gas.

Additional potential problems associated with the use of deuterium labeled analogs as internal standards are discussed in Millard's book entitled Quantitative Mass Spectrometry (5). In particular, an example is cited in which a prostaglandin was labeled with four deuterium atoms in apparently stable positions in the molecule, but after prolonged storage in methanol considerable exchange of hydrogen for deuterium was found to have occurred. Based upon this experience, Millard recommends that stock solutions of deuterium labeled compounds should be checked at regular intervals for evidence of hydrogen-dcutcrium exchange.

The problem of exchange has prompted some analysts to utilize ^{13}C or ^{15}N labels rather than dcuterium. However, deuterium is usually far easier to incorporate into a molecule than other heavy isotopes and therefore will undoubtedly continue to be the most frequently used label for internal standards. The important point is that the analyst must be aware of the possible problems associated with the use of deuterium labeled internal standards, and to thoroughly evaluate each application of a particular internal standard hefore embarking on an extensive series of assays.

A second important point regarding isotope labeling is that the isotopic variant should have a molecular weight three or more mass units greater than the unlabeled compound. This is because the naturally occurring heavy isotope content (primarily ^{13}C) of organic compounds gives rise to ions of significant intensity at one and two mass units above each carbon containing ion in the compound's mass spectrum. In principle the contributions of these naturally occurring isotope ions can be calculated and subtracted from the ion currents resulting from the internal standard, hut in practice measurement of the intensity of the isotope labeled ion peak is easier and more accurate if there is no contribution from other ions.

Of course, other types of compounds can bc used as internal standards when a suitable isotope labeled analog is not available. For example, homologs and other structurally similar compounds have been used as internal standards and often give satisfactory results (6-8). Nevertheless, when they can be obtained, stable isotope

labeled analogs are the first choice for internal standards of most practitioners of quantitative mass spectrometry (9).

PREPARATION OF CALIBRATION GRAPHS

The procedure we recommend for preparation of calibration graphs ("standard curves") involves addition of a fixed quantity of internal standard to aliquots of an appropriate body fluid containing known quantities of the analyte. The "spiked standards" are then extracted, derivatized, and analyzed by exactly the procedure to be used for unknown samples. The calibration graph is constructed by plotting the measured ratio of ion current peak heights corresponding to the analyte and the internal standard versus the quantity of analyte added to each sample. Any inaccuracies in measurement that occur in the preparation of a calibration graph will affect the accuracy of all subsequent quantitative determinations which utilize that calibration graph. Consequently, preparation of an accurate calibration graph is extremely important. The two requirements that are most critical are: 1) the quantity of analyte in each sample must be accurately known, and 2) exactly the same quantity of internal standard must be added to each sample used to prepare the calibration graph as is added to each of the unknown samples.

As a general rule the sample medium used for preparing a calibration graph should be as similar as possible to the sample medium to be analyzed. If the unknowns consist of plasma samples, the biological standards to be used in establishing the calibration graph can be prepared by adding precise volumes of standard solutions of the analyte to aliquots of a pool of plasma obtained from a local blood bank. It is important, of course, to establish that the plasma pool is completely free of the analyte, or any other compounds which interfere with the assay.

Ideally, the analyte standard solution and the internal standard solution should be added in such a way that the biological medium is not changed and drug compounds are introduced into a molecular environment which exactly mimics that of the biological specimens to be analyzed. For this reason it is generally preferable to use aqueous standard solutions. However, some drugs have very little solubility in water so that it is difficult to prepare aqueous solutions at the concentrations required. Most drugs are soluble in methanol, and addition of small quantities of methanolic solutions to biological media causes relatively minor physical and chemical alteration. For the sake of uniformity the standard solutions for all of the drugs and metabolites in this monograph are prepared in methanol. We have also chosen 100 μl as the volume of the methanolic standard solutions to be added to 1-ml volumes of the biological specimen. This volume of methanolic solution is a compromise in that it is large enough to be conveniently dispensed with good accuracy, but is not so large that it causes extensive protein precipitation.

As previously stated it is important to add precisely the same amount of internal standard to each sample to be analyzed, i.e., to both standards and unknowns. It is not actually necessary to know the weight of internal standard added, as long as it is exactly the same for all samples. Nevertheless, the normal practice is to decide in advance the approximate quantity of internal standard to be added to each sample, since the ratio of analyte to internal standard can be measured most accurately when they are present in similar concentrations. Consequently, the quantity of internal standard added to a specimen should give a concentration intermediate between the lowest and highest anticipated analyte concentrations, or more precisely, the concentration of internal standard should be such that the lowest anticipated weight ratio of analyte to internal standard should approximately equal the inverse of the highest anticipated ratio. Thus, if one desires to measure concentrations of a drug over the range 1 to 1000 ng/ml, the amount of internal standard added should give a concentration of approximately 32 ng/ml (1:32 = 32:1000).

Some analysts may favor a lower concentration of internal standard on the basis that it is more difficult to measure low concentrations of a drug than high concentrations, and therefore the internal standard concentration should be selected so as to facilitate accurate measurement of low levels of the drug. On the other hand, relatively large quantities of internal standard are sometimes used in order for it to act as a "carrier" to minimize losses of analyte due to adsorption at active sites on the surface of extraction vessels and within the GC column (7). However, the "carrier" effect is not a significant factor except at very low analyte concentrations, usually below about 10 ng/ml (8). Based upon these considerations we have standardized our procedure by specifying the addition of approximately 40 ng of internal standard to each ml of body fluid.

A calibration graph should include measurements at a minimum of four different concentrations of analyte plus a blank, i.e., a sample with no added analyte. At least three samples should be prepared and analyzed at each concentration in order to assess the variance in the measurements at each concentration. The analyte concentrations used in preparing the calibration graph must include the extremes of concentrations expected to be found in the unknowns, since linearity must be demonstrated, not assumed. The remaining points on the graph should correspond to intermediate concentrations.

Since it is important to add the same volume of standard solution to each body fluid sample in preparing a calibration graph, it is necessary to prepare a series of standard solutions containing different concentrations of the analyte. A dilution scheme designed to provide seven analyte concentrations covering the range 1 to 1000 ng/ml is shown in Table 1. To use the scheme, prepare standard stock solutions of the analyte and the internal standard having accurately measured concentrations of approximately 0.10 mg/ml in methanol as described in the Experimental Procedure sections within each of the drug chapters. The dilution scheme requires the

use of seven 10-ml volumetric flasks, one 1-ml volumetric pipette, three 2-ml volumetric pipettes, and three 5-ml volumetric pipettes.

TABLE 1. DILUTION SCHEME FOR PREPARING ANALYTE STANDARD SOLUTIONS

Dilute 1 ml of the 0.10-mg/ml analyte stock solution to 10 ml to give a 10.0-µg/ml solution.
Dilute 5 ml of the 10.0-µg/ml solution to 10 ml to give a 5.0-µg/ml solution.
Dilute 2 ml of the 5.0-µg/ml solution to 10 ml to give a 1.0-µg/ml solution.
Dilute 5 ml of the 1.0-µg/ml solution to 10 ml to give a 500-ng/ml solution.
Dilute 2 ml of the 500-ng/ml solution to 10 ml to give a 100-ng/ml solution.
Dilute 5 ml of the 100-ng/ml solution to 10 ml to give a 50.0-ng/ml solution.
Dilute 2 ml of the 50.0-ng/ml solution to 10 ml to give a 10.0-ng/ml solution.

Exactly 100 µl of each of these standard solutions is added to 1 ml of drug-free body fluid along with 100 µl of a 400-ng/ml solution of the internal standard. Prepare the 400-ng/ml standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of internal standard to 250 ml with methanol. Each of the body fluid samples will then contain 40 ng of internal standard and the concentration of analyte shown in the right hand column in Table 2.

Prepare three or more spiked body fluid samples at each of the listed concentrations. Extract and analyze each sample as described in the Experimental Procedure section in the appropriate drug chapter. Plot the ratio of peak heights of the analyte and the internal standard to the quantity of analyte added to the body fluid sample. A calibration graph is then obtained by constructing the straight line which best fits the plotted points. The best fit straight line can be determined by visual estimation, or more accurately, by the method of "least squares" in which that straight line is drawn which minimizes the sum of the squares of the vertical deviations from the line.

Figure 1 is an example of a computer-generated calibration graph. The significant features of the graph are the slope, the y-intercept, the linear range of the assay, and the variance of the measurements at different analyte concentrations. In this example the

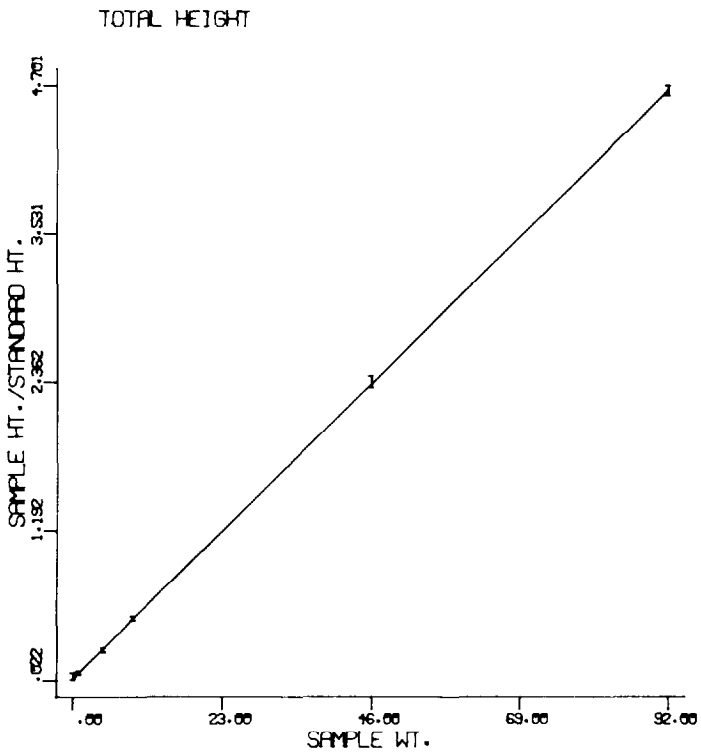


FIGURE 1. A CALIBRATION GRAPH FOR ANALYSIS OF TETRAHYDROCANNABINOL IN PLASMA

TABLE 2. SCHEME FOR ADDITION OF ANALYTE AND INTERNAL STANDARD (IS) TO BODY FLUID SAMPLES FOR PREPARING CALIBRATION GRAPHS

Add to 1 ml of Body Fluid		Resulting Analyte Concentration (ng/ml)
100 µl of the 400-ng/ml IS solution	+ No analyte	0
"	+ 100 µ1 of the 10-ng/ml analyte solution	1
"	+ 100 µ1 of the 50-ng/ml analyte solution	5
"	+ 100 µ1 of the 100-ng/ml analyte solution	10
"	+ 100 µ1 of the 500 ng/ml analyte solution	50
"	+ 100 µl of the 1-µg/ml analyte solution	100
"	+ 100 µ1 of the 5-µg/ml analyte solution	500
"	+ 100 µl of the 10-µg/ml analyte solution	1000

variance is indicated by vertical bars representing the relative standard deviation at each concentration measured. The slope and the y-intercept are the functions used to calculate the quantity of analyte in an unknown based on the ion current response ratio. Assays using stable isotope labeled internal standards usually give calibration lines with positive y-intercepts. The magnitude of the y-intercept reflects the proportion of internal standard molecules containing no heavy isotopes (unlabeled molecules). A large y-intercept can significantly limit the sensitivity of the assay.

An important advantage to the use of stable isotope labeled internal standards is that the slope of the calibration line should remain constant as long as the compositions of the standard solutions remain unchanged and the same volumes of standard solutions are added to the body fluid samples. Because of the nearly identical chemical and physical properties of the analyte and its isotope labeled analog, their weight ratio should not be affected to any

significant extent by variations in the extraction, derivatization, chromatographic and mass spectrometric processes. For this reason the practice in our laboratories is to determine new calibration graphs only when new standard solutions arc prepared, or when the analysis of control specimens containing known concentrations of the drug shows unacceptable variance from the true values.

If the concentration of a drug in an unknown specimen is found to be greater than the highest concentration used in preparing the calibration graph, the specimen should be reanalyzed after dilution with water or saline (typically 1:10) in order to bring its concentration within the extremes of the calibration graph. The alternative practice of adding a larger quantity of internal standard to the specimen is less satisfactory because it requires preparation of a new calibration graph.

SAMPLE EXTRACTION

One of the important criteria that guided our selection and evaluation of sample preparation procedures was that they should be as simple and rapid as possible while still providing adequate recovery and separation of the analyte from compounds that could interfere with the GC/MS quantitation. The very high sensitivity and selectivity of GC/MS often permits use of relatively simple extraction procedures, and we have attempted to fully exploit this feature.

A second major concern was to minimize loss of analyte due to adsorption, evaporation, or decomposition. Even though use of stable isotope labeled internal standards can compensate for loss of a portion of the analyte through these processes, any loss of analyte will reduce the sensitivity of the assay. Loss of analyte due to adsorption on glass surfaces is particularly a problem when the quantity of analyte is in the low nanogram range. Adsorption can be minimized by silylation of all glass surfaces with which solutions of the analyte will come in contact. Commercial formulations are available for liquid-phase silylation of glass surfaces (for example, Glas-Treet, Regis Chemical Co., Morton Grove, IL 60053). However, vapor-phase silylation is more convenient and less expensive if large quantities of glassware must be silylated on a regular basis (10).

All glassware should be thoroughly cleaned before silylation. The easiest and most thorough method of removing organic contaminants from glassware is to bake it off in a muffle furnace. Unfortunately, a muffle furnace of sufficient capacity to handle normal laboratory glassware is expensive to purchase and operate. Chromic acid baths can also be used to remove all traces of organic material from glassware, but they are messy and are a safety hazard. Consequently, in most laboratories glassware is cleaned by washing with a detergent solution followed by thorough rinsing with distilled water. A final rinse with acetone helps to assure removal of any remaining organic residue (11).

It is the practice in our laboratories to use high purity solvents for preparation of standard solutions and all sample extractions, even though interference from solvent impurities is not nearly as much of a problem in GC/MS assays as it is in most other methods for drug analysis. High purity solvents from several commercial sources have proven satisfactory. We have not found it necessary to redistill the solvents.

Some of the drugs discussed in this monograph are quite volatile, so care must be taken to avoid evaporative loss of the drug during concentration of the extract. Steps that have been taken to minimize evaporative loss of analyte include:

1. Eliminating the need for concentration of extracts by using a very small quantity of extraction solvent (see assays for methaqualone, cocaine, and diazepam).

2. Using highly volatile solvents such as methylene chloride, hexane, or 1-chlorobutane, which can be removed by heating at mild temperatures (∿ 40 to 60°C) under a gentle stream of air or nitrogen.

3. Adding a small quantity (10 to 20 µl) of a high boiling liquid to the extract to act as a "keeper solvent" during evaporative concentration.

Procedures for analysis of volatile basic drugs such as amphetamine often specify addition of an acid prior to evaporative concentration in order to convert the drug to a nonvolatile salt. In our experience the use of a keeper solvent such as dimethylformamide (DMF) is a more effective means of reducing loss due to evaporation of such drugs (see assays for phencyclidine, methadone, tetrahydrocannabinol, amphetamine, methamphetamine, mescaline, and DOM). However, it is important to establish that the keeper solvent does not exert adverse effects on the GC/MS assay. For example, we found that DMF could not be used in combination with trifluoroacetic anhydride for derivatization of amphetamine. Apparently the DMF suppressed removal by evaporation of the trifluoroacetic acid formed in the derivatization reaction, and subsequent injections of the highly acidic mixture adversely affected the gas chromatography of the trifluoroacetamide derivative. This problem was avoided by using N-methyl-bis-(trifluoroacetamide) instead of trifluoroacetic anhydride to prepare the amphetamine trifluoroacetamide derivative (see assays for amphetamine, methamphetamine, mescaline and DOM).

CALIBRATION AND PERFORMANCE EVALUATION OF THE GC/MS

In spite of substantial improvements in stability and reliability, gas chromatograph/mass spectrometers remain one of the most attention-requiring of analytical instruments. In addition to having complex electronic circuitry, the mass spectrometer includes key components which can only be operated under high vacuum. If leaks develop in the vacuum system, or if the ionizer, mass analyzer, or detector become contaminated, the performance of the instrument

deteriorates. Yet some contamination is inevitable, since samples are introduced and effectively decomposed within the instrument's vacuum system. Likewise, gas chromatographic columns have a finite lifetime which often is reduced to only a few weeks when large numbers of crude biological extracts are analyzed. For these reasons it is imperative to evaluate the total system performance at regular and frequent intervals.

The specific manner in which a GC/MS system is tuned up and calibrated, and its performance evaluated, will vary with different instruments, with the level of performance required for the particular analyses, and according to the personal preferences of the operator. The procedures described here have proven satisfactory in our laboratories, and are intended as a guide to assist readers in establishing a systematic scheme appropriate for their own daily instrument "check-out."

A thorough discussion of tune-ups and performance evaluation procedures is beyond the scope of this chapter. Instead, the emphasis here is on procedures and suggestions which may not be included in operator and maintenance manuals , but which we have found to be useful in preparing to perform the types of analyses described in this monograph.

Performance Evaluation Procedure

Before turning on the ionizer filament and the electron multiplier voltage to obtain mass spectra it is good practice to check the pressures in the ion source ($< 10^{-3}$ mm) and in the analyzer ($< 10^{-6}$ mm), as well as the GC column head pressure, to assure that no major leaks have occurred. Following this preliminary check, set the GC column at the maximum temperature to be used for the assay, and observe the electron impact mass spectral background with and without the helium carrier gas entering the ion source. Some increase in background will occur when the carrier gas is introduced into the mass spectrometer. However, a very large increase in backrround is cause for concern. If the intensities of ions due to air [m/z 28. (N_2^+), 32 (O_2^+), 40 (Ar^+), and 34 (CO_2^+)] become abnormally large; a leak in the GC system is indicated. The most common locations of air leaks within the GC system are the injector septum and the colunm connector fittings. Under methane or ammonia chemical ionization conditions the air peaks will not be seen, and yet an air leak can significantly affect the instrument sensitivity. Therefore it is important to check for an air leak before switching to chemical ionization. The appearance of abnormally intense ion peaks at higher masses suggests that the GC column should be further conditioned or replaced.

Next, introduce methane into the ion source to serve as the CI reagent gas. This is normally done by changing the GC carrier gas from helium to methane, although some chemical ionization mass spectrometers require use of a separator in the GC/MS transfer line, in which case the methane reagent gas is introduced into the ion source through a separate inlet. Reduce the electron multiplier

voltage (to ∿1.2 KV) and the filament emission current (to ∿ 50 microamps) to prevent possible damage to the electron multiplier, and observe the methane CI mass spectrum. It should be similar to the spectrum shown in Figure 2. The peak at m/z 19 is due to protonated water and is often quite large, particularly just after the instrument has been down for cleaning and maintenance. The size of the water peak can usually be reduced by an overnight system bakeout. However, if ammonia is to be used as the reagent gas, the adsorbed water in the system is unlikely to affect the ionization of sample molecules.

Return the electron multiplier voltage and filament current to their normal operating valves, and bleed a small quantity (about 10^{-5}mm partial pressure) of a suitable reference compound such as perfluorotributylamine (often referred to as PFTBA or FC-43) into the ion source and observe its methane CI mass spectrum. The relative ion intensities in CI mass spectra are often strongly affected by the ion source temperature. At a source temperature of approximately 150°C the methane CI mass spectrum of perfluorotributylamine should look similar to that shown in Figure 3. At higher source temperatures the high-mass ions tend to decrease in abundance relative to the low-mass ions. Either of the very abundant ions at m/z 219 and 414 in the methane CI mass spectrum of perfluorotributylamine can be used to optimize source potentials, depending on which is closest to the analyte and internal standard ions that will be monitored.

In mass spectrometry, resolution and sensitivity are inversely related. Normally the mass spectrometer is adjusted to give unit resolution, i.e. base-line separation between consecutive integral mass peaks. However, in quantitative analysis using selected ion monitoring, a gain in sensitivity can be achieved by decreasing the mass resolution and thereby increasing the ion current passing through the mass analyzer. The degree to which the resolution can be lowered is limited by the specificity required, and since specificity is directly related to resolution, a decrease in resolution will increase the likelihood of interference from other ions. In our laboratories the mass spectrometer is normally adjusted to unit resolution if the ions to be monitored have masses below 300, and the resolution is reduced by about a factor of 2 when ions above mass 300 are to be monitored.

After the mass spectral resolution has been set and the ion source potentials optimized, the system's mass calibration should be checked and recalibrated if necessary.

If the assay to be performed involves the use of ammonia as reagent gas, it is best to introduce the ammonia into the ion source through an inlet line which is adjacent to or concentric with the GC/MS transfer line. We have found that introducing the ammonia gas through an inlet into the ion source at a location opposite to the entering carrier gas gives less satisfactory results.

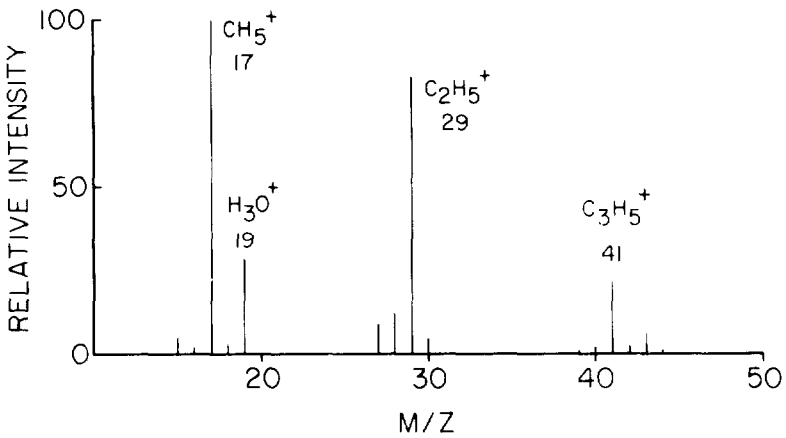


FIGURE 2. METHANE REAGENT GAS IONS (ION SOURCE TEMPERATURE, 150°C; PRESSURE, 1 x 10^{-3} MM)

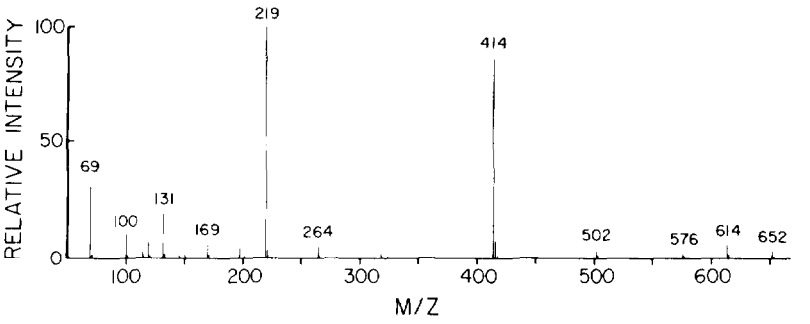


FIGURE 3. METHANE CI MASS SPECTRUM OF PERFLUOROTRIBUTYLAMINE (QUADRUPOLE MASS ANALYZER. ION SOURCE TEMPERATURE, 150°C; METHANE PRESSURE, 1 x 10^{-3} MM; PFTBA PRESSURE, 1 x 10^{-5} MM)

Slowly increase the rate of flow of ammonia into the ion source while observing the ion intensities at m/z 18 and 35. As the ammonia flow rate is increased, the intensity of the m/z 18 peak (NH_4^+) should at first increase and then reach a plateau, at which point a peak at m/z 35 ($NH_3 \cdot NH_4^+$) will appear and begin to increase in intensity. The optimum ammonia flow rate is the point at which the m/z 18 peak reaches a maximum and the m/z 35 peak just begins to increase in height. At this point the methane reagent gas ions will be completely suppressed. The total reagent gas pressure in the ion chamber should be between 0.3 and 1.0 x 10^{-3} mm. If a separator is used, it may be necessary to bleed in methane along with the ammonia to achieve this total pressure. Figure 4 shows an example of the reagent gas ions observed when methane is used as carrier gas and ammonia is introduced into the ion source as described.

Set the gas chromatograph and mass spectrometer ion source operating parameters to those that will be used for the assay to be performed. Inject into the GC/MS system a solution containing a specific quantity of ,analyte which is sufficient to yield a good quality mass spectrum. (Usually 100 to 200 ng is sufficient for this purpose; therefore, it is convenient to inject 1 or 2 µ1 of the 0.1 mg/ml standard stock solution called for in each of the drug assay experimental procedures). Repetitively scan the mass range from 50 daltons to above the protonated molecule ion of the analyte. Compare the size and shape of the analyte peak in the total ion current chromatogram with similar data acquired previously, in order to determine if there has been a significant change in sensitivity or gas chromatographic resolution. Also, compare the acquired CI mass spectrum of the analyte with the corresponding CI mass spectrum in this monograph.

As a final check of the GC/MS performance, set up the system for selectcd ion monitoring and inject a standard solution containing a quantity of analyte close to the lowest level of analyte that the assay must be capable of measuring. The instrument operator's manual should provide guidance as to the selected ion monitoring parameters that will provide the highest signal-to-noise ratio and the best dynamic range. Typically the system will sequentially sample the ion current, at each mass to be monitored, for 100 msec intervals. It is essential, of course, that the ion currents be measured at the precise center of each of the monitored ion masses.

If the analyte peak signal obtained from the selected ion monitoring analysis of the standard solution is sufficiently strong relative to the background noise level to permit reasonably accurate measurement of the peak height (i.e., a signal-to-noise-ratio of at least 3:1), proceed with the analysis of biological extracts.

Since the primary purpose of conducting the foregoing instrument checkout is to assess the current performance of the system, it is extremely useful to maintain a record of the data from these evaluations. The ability to compare current performance with data acquired previously under identical experimental conditions will be a great help in identifying likely causes of any changes that may occur in instrument performance.

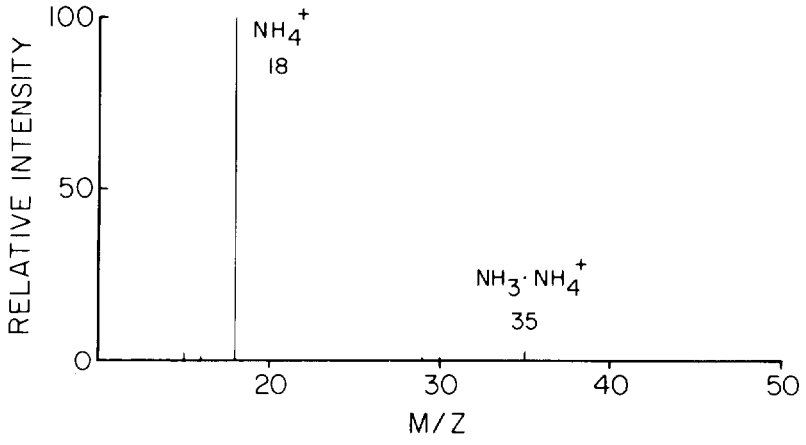


FIGURE 4. METHANE-AMMONIA REAGENT GAS IONS (ION SOURCE TEMPERATURE, 150°C; AMMONIA PRESSURE, 2 x 10^{-4} MM; METHANE PRESSURE, 8 x 10^{-4} MM)

A common cause of poor sensitivity in GC/MS assays is adsorptive loss of the analyte within the GC column. Often the sensitivity improves after 3 number of injections of analyte solution. "Priming" of GC columns in this manner is a common practice in gas chromatography. The effect of priming is most noticeable when a column was previously. used for analysis of chemically different compounds. Ideally a separate column should be reserved for each of the different drugs commonly assayed. If this is not practical, columns used for quantitation of basic compounds should at least not be interchanged with those used for analysis of acidic compounds.

An inherent danger in column priming is that some of the adsorbed analyte may be "washed off" the column during subsequent injections, resulting in an erroneously high value for the concentration of analyte measured. This potential source of error can be easily checked by analyzing a sample blank (i.e. a body fluid extract containing the internal standard but none of the analyte) before beginning analysis of sample unknowns.

QUALITY CONTROL AND SOURCES OF ERROR

In addition to determining that the total GC/MS system performance is satisfactory before beginning the analysis of sample unknowns, it is important intersperse spiked standards among sample unknowns so that intra-day changes in instrument performance will be evident and documented. Control charts are effective means of maintaining a continuous record of the accuracy experienced with each drug assay (12,13).

Probably the largest source of error in GC/MS quantitative drug assays is that involved in preparing the standard solutions used in establishing calibration graphs (5). Once the internal standard is added to the body fluid and becomes equilibrated, there are few things which will change the weight ratio of the drug to its stable isotope labeled analog.

Incomplete equilibration of the internal standard in the body fluid is a potential cause of poor precision. Unfortunately the time and the conditions required for internal standards to achieve complete equilibration in terms of protein binding are difficult to determine and undoubtedly vary for each drug and type of specimen. The assay procedures in this monograph specify equilibration for 15 min before beginning the extraction, but we have no convincing data upon which to base this recommendation. Others routinely allow as long as 12 hr for equilibration (5).

In spite of the high specificity of the selected ion monitoring technique, particularly when ammonia chemical ionization is used, ions from endogenous compounds will occasionally interfere. Usually a visual examination of the ion current profile plots will indicate when an analyte or internal standard peak contains a contribution from another compound; that is, the peak width will be broader than

normal or its retention time will be slightly altered. However, we have seen a few rare cases where an endogenous compound had an identical retention time and yielded ions at the same mass as either the analyte or the internal standard. When this situation occurs it can easily go undetected and result in erroneous data.

The experimental procedures can be altered in several ways if an unusually high degree of specificity is required. For example, the GC/MS analysis can be repeated using a GC column with a different polarity liquid phase, or a high resolution glass capillary column can be used. Also, prominent fragment ions can be monitored in addition to the protonated molecule ions. This may require an alteration of the ionization conditions, such as the substitution of methane for ammonia as the CI reagent gas.

To conclude this chapter, it is appropriate to reemphasize the remarkable versatility of the gas chromatograph/mass spectrometer as an analytical instrument. The experienced analyst will become aware of the full range of capabilities available and can then choose to modify the procedures in order to best satisfy specific analytical needs.

REFERENCES

1. R.L. Foltz, D.A. Knowlton, D.C.K. Lin, and A.F. Fentiman, Jr. Proceedings of the Second International Conference on Stable Isotopes, Oak Brook, IL, October 20-23, 1975.

2. A.F. Fentiman, Jr. and R.L. Foltz. J. Labelled Compounds, 13, 579 (1977).

3. A.F. Fentiman, Jr. and R.L. Foltz. J. Labelled Compounds, 12, 69 (1976).

4. Personal communication from Dr. Fred Falknier, Pfizer, Central Research, Groton, CT.

5. B.J. Millard. Quantitative Mass Spectrometry. Heyden, London (1978).

6. M.G. Lee and B.J. Millard. Biomed. Mass Spectr., 2, 78 (1975).

7. B. Samuelsson, M. Hamberg, and C.C. Sweeley. Anal. Biochem. , 38, 301 (1970).

8. R. Self. Biomed. Mass Spectr., 6, 315 (1979).

9. M. Claeys, S.P. Markey and W. Maenhaut. Biomed. Mass Spectr., 4, 122 (1977).

10. D.C. Fenimore, C.M. Davis, J.H. Whitford, and C.A. Harrington. Anal. Chem., 48, 2289 (1976).

11. R.R. Watts, ed. Analysis of Pesticide Residues in Human and Environmental Samples. U.S. Environmental Protection Agency, Health Effects Research Laboratory, Environmental Toxicology Division, Research Triangle Park, NC, Section 3A, p. 1-6 (1980).

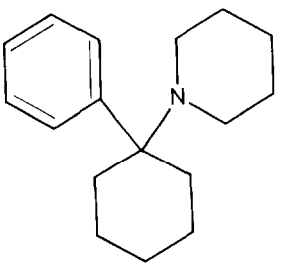
12. D.L. Massart, A. Dijkstra, and L. Kaufman, eds. Evaluation and Optimization of Laboratory Methods and Analytical Procedures. Elsevier, Amsterdam (1978).

13. H.C. Curtius and M. Roth, eds Clinical Biochemistry, Principles and Methods. Walter de Gruyter, New York, NY, 1, p. 594-617 (1974).

Chapter 3

Phencyclidine (PCP)

Phencyclidine (PCP), 1-(1-phenylcyclohexyl)piperidine (I), was originally developed in the 1950's and marketed in 1963 as Sernyl, a surgical anesthetic. However, human clinical use was soon discontinued because of bizarre adverse side effects, notably post-anesthetic delirium. By 1967 the drug was commercially available for veterinary use only (1).



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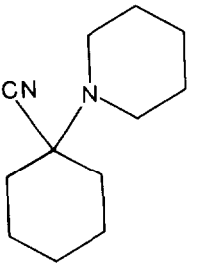


II

PCP appeared as an illicit street drug in 1967, but within a year had fallen out of favor in the drug culture, presumably because of its unpleasant effects. In spite of the early negative response, street use regained momentum and by 1976 PCP was the fourth most common cause of hospitalization for drug abuse in San Francisco (2) and the leading cause of inpatient psychiatric admissions in the Washington, D.C. area (3). Until very recently, PCP sold on the street was rarely represented correctly; either it was disguised by an esoteric name or it was deliberately misrepresented as THC, mescaline, psilocybin, or some other popular hallucinogen. It is often mixed with other drugs such as LSD or amphetamine, and appears in various physical forms including powder, capsules, tablets, or liquid sprayed on plant leaves. Administration among users is by smoking, nasal inhalation, or oral ingestion (3). "Street" PCP is not generally diverted from legitimate veterinary sources, but is synthesized illegally from readily available chemicals (5). An intermediate in the synthesis, 1-piperidinocyclohexane carbonitrile (II), is extremely toxic and is difficult to remove from the final product. Its presence as a contaminant in imperfectly synthesized PCP has been implicated in cases of coma and death following PCP ingestion (6,7).

PHARMACOLOGY

The pharmacology of PCP is dose-dependent and complex. The drug acts primarily on the central nervous system as a depressant, but in a manner distinctly different from that of the sedative-hypnotic class of depressants. Studies in rats have shown it to be a potent competitive inhibitor of catecholamine uptake in both the dopaminergic and noradrenergic regions of the brain. This, combined with its anticholinergic properties, makes PCP an unusual and, therefore, an attractive substance for studies of model psychoses (8). In its original human surgical doses (0.25 mg/kg body weight given intravenously), PCP was found to induce unresponsiveness and complete anesthesia although the patients appeared to he awake. Blood pressure was elevated. With intravenous doses of 0.5-1 mg/kg, agitation and seizure activity appeared. Following even a small oral "street" dose (commonly 1-6 mg), the initial "high," during which the subject is unresponsive but confused and agitated, is followed by depression, irritability, feelings of isolation, and sometimes paranoia. Doses of 10-20 mg, possible even by smoking, are sufficient to induce stupor or coma, and the larger amounts possible with oral ingestion can precipitate fatal epileptic seizures or hypertensive crises. Blood concentrations of PCP as low as 0.10 µg/ml have been associated with behavioral effects leading to injury or death, while concentrations greater than 1.0 µg/ml cause coma in most individuals. Blood concentrations over 2.0-2.5 µg/ml are generally fatal (9). Recovery from sublethal doses usually occurs within a few hours when the drug has been smoked or inhaled, but oral ingestion is characterized by a recovery period of days or weeks (2). It has been suggested that these prolonged effects may be due to the lipophilic nature of PCP; in rats high concentrations of the drug were found in adipose tissue long after its disappearance from plasma and urine (10). Also, the analysis of blood and tissue samples from phencyclidine-related fatalities revealed that in all cases tissue concentrations greatly exceeded those in blood, and blood levels were similar to those found in the plasma from casual users (11). Therefore, since PCP concentrates in tissue, plasma concentrations may not adequately reflect the severity of ingestion.

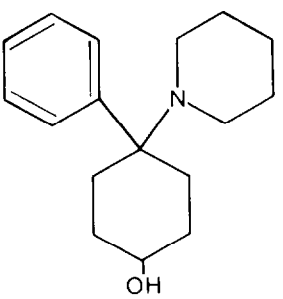
Schizophrenic psychosis, as opposed to short-tern intoxication, can persist for several weeks after the use of PCP, and appears to be related to personality factors and/or individual drug sensitivity. The drug has been found to be teratogenic (12). While deaths resulting from true pharmacologic overdoses of PCP have been reported, in many other cases death has resulted from the behavioral toxicity of the drug; i.e., accidental drowning or violence provoked by aggressive hehavior (13). Clinically significant interactions of PCP with other drugs of abuse have been suggested by animal experiments. For example, in monkeys PCP strongly potentiates the effects of pentobarbital, including respiratory depression. PCP has also been found to reinforce drug self-administration in monkeys, the only hallucinogen which is known to do so, and some human chronic users report psychological dependence and behavioral tolerance. However, no evidence for physical dependence has been reported either in man or animals (14).

The pharmacology and toxicology of PCP are reviewed in papers published in 1974 (1.5) and in 1978 (9). Also, a National Institute on Drug Abuse monograph reviews many aspects of the abuse of PCP, treatment of PCP intoxications, and the pharmacology of the drug (16).

PHARMACOKINETICS AND METABOLISM

Phencyclidine, with a pKa of 8.5, appears to be rapidly metabolized and excreted. About 60 percent of labeled PCP given intravenously to monkeys appeared in the urine within 12 hours and 75 percent within eight days (17). In cases of acute intoxication in humans, urine concentrations have been used to predict the duration of coma (2). A comparison of plasma with urinary PCP concentrations in human "overdose" cases showed that urinary concentrations of PCP were 13-19 times the concurrent concentrations in plasma (18). A plasma elimination half-life of about 11 hours has been reported in a case of human PCP ingestion (19). It has been shown, however, that PCP is excreted much more rapidly in acidic urine, and several severely intoxicated patients have been successfully treated by urine acidification procedures, such as gastric infusion with NH_4Cl (20).

In man, PCP is excreted primarily as monohydroxylated derivatives in the form of conjugates. Two of these metabolites, isolated from human urine after enzymatic hydrolysis, were identified by mass spectrometry as 4-phenyl-4-piperidinocyclohexanol (III) and 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (IV) (21). Both metabolites were found to exhibit weak pharmacological activity in test animals (22)



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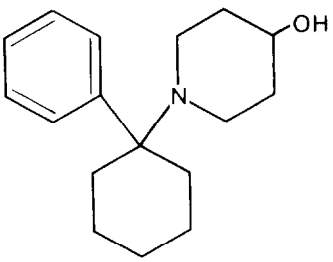

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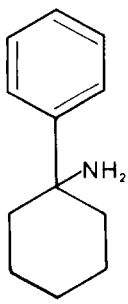

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Traces of a N-dealkylated metabolite, 1-phenylcyclohexylamine (V), have also been detected in the urine of a PCP-intoxicated patient (23). Dihydroxy metabolites are produced in rats, mice, pigeons, sheep, and monkeys, and in these animals PCP tends to be less toxic than in species which have a weaker capacity for hydroxylation (24). In tests on monkeys, no metabolites were detected in central or peripheral tissue, although unchanged PCP was present in sciatic nerve, spinal cord, and brain (17). The detection of PCP metabolites in human blood has not been reported.

ANALYTICAL METHODOLOGY

Generally, the urine of patients seen on an emergency basis contains a sufficiently high concentration of unmetabolized PCP (>0.2 mg/ml) to permit qualitative detection by any of a variety of analytical methods (25). These have included thin-layer chromatography (TLC), with a sensitivity of 0.2 µg/ml (26) and gas chromatography (GC) with a sensitivity of 2.0 µg/ml (27). GC combined with mass spectrometry (28,29) and TLC (30) has been used to identify PCP in confiscated street drug samples. However, both clinical work and pharmacokinetic studies have been hampered by the lack of reliable quantitative methods for analysis of PCP in biological fluids. Hoffmann-La Roche markets a radio-immunoassay (RIA) kit for PCP and Syva is field testing a homogeneous enzyme immunoassay (EMIT) for the drug. To date, however, the sensitivities and specificities of these assays have not been published.

Recently, a method using ultraviolet spectroscopy was reported, which was able to measure PCP concentrations in urine extracts containing as little as 0.01 mg of the drug by comparison with standard absorbance curves. However, when other basic drugs were present in the sample, GC assay with a flame ionization detector (FID) was used. Methadone was chosen as the internal standard. The GC-FID system (Chromosorb W coated with Apiezon L, column temperature 200ºC) detected 0.076 µg/ml of PCP added to urine, and measured a range of 0.1-69.4 µg/ml of the drug in samples from hospital admissions (31).

For rapid analysis of urine for PCP in a clinical setting, temperature-programmed (150- 230°) GC-FID on a column of 5 percent SE-30 plus 1 percent Carbowax 20M on Chromosorb WHP has been suggested. A metabolite, 1- (1-phenylcyclohexyl)-4-hydroxypiperdine (IV) is also observable by this technique, to confirm the presence of PCP even when the drug has been extensively metabolized (32).

A GC-FID method using mepivicaine as the internal standard and an OV-1 or OV-17 column at 180°C, was applied to blood and tissue samples as well as to urine. Blood concentrations in fatal overdose cases ranged from 0.5 to 5.0 µg/ml, and in liver tissue from the same individuals, from 5.0 to 36.0 µg/g (33). Human plasma PCP concentrations in clinical cases, which covered a range of 0.09-0.22 µg/ml, were measured by a similar GC-FID technique (3.8 percent UCW-98 on Gas Chrom Q at 200°C column temperature) using ketamine as the internal standard (19). Improved sensitivity and selectivity were achieved in a GC method by using a nitrogen-phosphorus detector (3 percent OV-17, 165°C isothermal). Meperidine was used as the internal standard because of its chemical similarity to PCP. The method gave a linear calibration curve up to 1 µg/ml and could detect as little as 10 ng/ml of PCP in plasma (11).

The combination of GC and MS with selected ion monitoring and the use of an isotope-labeled variant as the internal standard has been applied to the quantitation of PCP in blood and urine. Since an initial report of the adaptation of GC/MS to the assay of PCP

(21), the technique has been applied to a study of blood concentrations in 26 PCP intoxications where concentrations ranged from 0.007 to 0.250 μg/ml (34), and to studies of serial plasma and urine samples in an effort to correlate PCP concentrations with the duration of psychotropic effects (20). GC/MS analysis of PCP has also been adapted to an automated procedure using probability based matching, in a computerized GC/MS system (Olfax) utilizing a reverse-search algorithm for the measurement of PCP levels in the urine. The method permits analysis of up to eight samples per hr and has a detection limit of approximately 10 ng/ml of PCP (35).

For quantitative studies of PCP, it is important to note that phencyclidine undergoes progressive thermal decomposition to 1-phenylcyclohexene as temperatures rise above 150°C. To minimize pyrolysis during gas chromatograpny, the injection port temperature should be kept below 200°C (21,25,36).

Solvents which have been used for the extraction of PCP from alkaline biological fluids include hexane, with an overall recovery of 70-85; percent (21); cyclohexane, 95 percent (used only for urine) (35); petroleum ether, 76 percent from plasma (19); diethyl ether (34); 1-chlorobutane (33); 95 percent ethanol (for TLC of urine) (30); and chloroform, for urine only, with 83 percent recovery reported (27,31). Increasing the pH of the urine to 9 reportedly allows 94 percent recovery of PCP and quantitative recovery of the nydroxy metabolite with chloroform (32). Extraction from urine by a reverse phase XAD-2 resin procedure, with quantitative recover) of PCP and 89 percent of the metabolite, has also been reported. Back: extraction into aqueous acid is often used in order to separate PCP from neutral and acidic materials, followed by realkalinization and reextraction with an organic solvent.

EXPERIMENTAL PROCEDURE

Because phencyclidine can be psychoactive at low blood concentrations the GC/MS analytical procedure is designed to measure the drug at plasma concentrations as low as 1 ng/ml. The extraction procedure consists of addition of deuterium-labeled phencyclidine and a pH 9.6 buffer to the plasma, followed by extraction with hexane, back extraction into acid, and finally, alkalinization and reextraction with hexane. Chemical ionization with methane and ammonia as reagent gases is used for the GC/MS analysis.

Standards and Reagents

Phencyclidine hydrochloride was purchased from Applied Science Laboratories, State College, PA 1680l. Phencyclidine-$^{2}H_5$ (1-[1-(phenyl-$^{2}H_5$) cyclohexyl]piperidine) was synthesized from bromobenzene-$^{2}H_5$ by a published procedure (38). It was found to be 99 percent pure based on GC/MS analysis. Over 99 percent of the molecules contained five deuterium atoms and less than 0.1 percent of the molecules were undeuterated.

A suitable pH 9.6 buffer can be prepared by dissolving 500 grams of potassium monohydrogen phosphate (K_2HPO_4) in about 500 ml of distilled water. When all of the salt is dissolved, allow the solution to cool to room temperature and add sufficient water to give exactly 1 liter.

Stock solutions of phencyclidine and phencyclidine-$^2H_5$, used for the determination of calibration graphs (Chapter 2) and the preparation of working standards, are prepared as follows. Weigh into a 100-ml volumetric flask 11.5 mg of phencyclidine hydrochloride or 10 mg of the free base. Dissolve the drug in methanol and bring the volume to exactly 100 ml with additional methanol. The resulting stock solution will correspond to a concentration of 0.10 mg/ml of phencyclidine calculated as the free base. A series of working standard solutions can then be prepared by appropriate dilution of the stock solution as described in Chapter 2.

The stock solution of phencyclidine-$^2H_5$ is prepared in the same manner. For the measurement of phencyclidine in body fluids within the concentration range of 1 to 1000 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µ1 of a 400-ng/ml phencyclidine-$^2H_5$ methanolic solution to each ml of sample. Prepare the 400-ng/ml standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of phencyclidine-$^2H_5$ to 250 ml with methanol. Store stock and working standard solutions in well-stoppered or capped glass vessels under refrigeration.

Extraction

Transfer 1 ml of specimen (whole blood, plasma, urine, or cerebrospinal fluid) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately). Add 100 µ1 of the 400-ng/ml phencyclidine-$^2H_5$ internal standard solution to the body fluid and vortex for 10 sec. Allow the sample to equilibrate for about 15 min and then add 1 ml of the pH 9.6 buffer and 5 ml of hexane. Cap the tube and check for a leak around the cap. Gently mix the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min and then transfer the organic layer (top) to a clean culture tube by means of a disposable Pasteur pipette. Add 5 ml of 0.2 N H_2SO_4 and vigorously agitate for about 1 min. Remove and discard the hexane layer (top). Add a sufficient quantity of 1 N NaOH to the aqueous layer to raise the pH to 9.5. Add 5 ml of hexane and again agitate vigorously for about 1 min. Centrifuge and transfer the organic layer (top) to a silylated concentrator at least 5 ml in volume and having a conical or nipple-shaped bottom. Add 20 µ1 of dimethylformamide as a “keeper” solvent to minimize evaporative loss of the phencyclidine. Remove the hexane by evaporation under a gentle stream of nitrogen or filtered air while heating at 40 to 50°C. When the extract volume has decreased to about 20 µ1, stopper the tube or cover the top with Parafilm and store at 0°C until the GC/MS analysis is to be performed. Inmediately prior to the analysis, allow the tube to warm to room temperature.

GC/MS Analysis

The following experimental conditions are satisfactory for GC/MS analysis of phencyclidine:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-1 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Temperatures:	Injector, 200°C Column, 180°C isothermal GC/MS transfer line, 200°C Ion source, 160°C

Under these conditions the phencyclidine and phencyclidine-$^2H_5$ should elute at about 3 min as narrow, symmetrical peaks.

before beginning the GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 244 and 249. They correspond to the protonated molecule ions for phencyclidine and phencyclidine-$^2H_5$, respectively. With the divert valve in the divert position, inject 2 to 6 µl of the hexane extract into the gas chromatograph. After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the phencyclidine peaks have eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of phencyclidine in the plasma sample is determined by measuring the heights (or areas) of the phencyclidine and phencyclidine-$^2H_5$ peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the concentration of phencyclidine in the specimen by dividing the measured quantity of phencyclidine by the exact volume of specimen used in the analysis.

Discussion of the Experimental Procedure

During extraction of phencyclidine from biological fluids, the basic drug can be separated from neutral and acidic materials by a back extraction technique. The biological sample is made alkaline and extracted with an organic solvent; the phencyclidine is then back extracted from the organic solvent with an aqueous acid. However, if chloroform or methylene chloride is used as the organic solvent in these steps and dilute hydrochloric acid is used for the back extraction, most of the phencyclidine will remain in the organic phase, together with the neutral components. The problem is avoided by using hexane as the organic solvent and dilute sulfuric acid for the back extraction. The aqueous acid extract is subsequently made alkaline and reextracted with hexane. With this combination of reagents, the overall recovery of phencyclidine from 1 ml of whole blood containing 5 µg of the drug ranges

between 70 and 85 percent.

During early work with phencyclidine, it was noticed that GC analyses of the drug typically showed a second peak eluting considerably before phencyclidine. The second peak was identified as 1-phenylcyclohexene on the basis of its mass spectrum and comparison with authentic material (Aldrich Chemical Co., Milwaukee, WI). 1-Phenylcyclohexene is a thermal decomposition product of phencyclidine. Consequently, when phencyclidine is gas chromatographed, the size of the 1-phenylcyclohexene peak is dependent on the injection port temperature. Figure 1 shows the flame ionization detector recordings which resulted from gas chromatography of phencyclidine with injection port temperatures of 300, 250, and 200°C. The 1-phenylcyclohexene peak decreased in size as the injection port temperature was lowered. At an injection port temperature of 200°C the 1-phenylcyclohexene peak amounted to only 0.5 percent of the phencyclidine peak height. At an injection port temperature of 150°C the 1-phenylcyclohexene peak was still detectable, but it now represented only 0.1 percent of the phencyclidine peak height. In view of the thermal lability of phencyclidine, it is important to keep all surfaces which may come in contact with the drug at the lowest practical temperature. For example, if the GC/MS separator is heated to 280°C some of the phencyclidine eluting from the gas chromatographic column will be thermally converted to 1-phenylcyclohexene.

The electron impact (EI), methane chemical ionization ($CICH_4$) , and methane-ammonia chemical ionization ($CICH_4$-NH_3) mass spectra of phencyclidine are shown in Figure 2. The EI spectrum of phencyclidine (top) snows an abundant ion at m/z 200 resulting from loss of C_3H_7 from the cyclohexyl ring. Since the deuterium atoms in the internal standard are on the aromatic ring, the corresponding ion in the mass spectrum of the deuterated phencyclidine is shifted to m/z 205; therefore, the ions at m/z 200 and 205 are satisfactory for selected ion monitoring. In the methane CI mass spectrum (middle), the M^+ is more intense than the MH^+, suggesting that charge transfer competes effectively with proton transfer when methane is used as the reagent gas. In the methane-ammonia CI mass spectrum (bottom) the protonated molecule ions give the most intense peak and fragmentation is virtually absent. Relative responses for prominent ions in the various modes of ionization are compared in Table 1. The values in the last column of Table 1 indicate that the best sensitivity is achieved with a mixture of methane and ammonia as the reagent gas. These data were acquired on two separate Finnigan 3200 GC/MS systems, one optimized for EI and the other for CI. If maximum sensitivity is important, each method of ionization should be evaluated on the specific GC/MS system(s) to be used for the analysis. Sensitivities can vary significantly from instrument to instrument.

To evaluate the reproducibility of the method, five independent replicate analyses were performed on 1-ml samples of whole blood containing 100 ng of phencyclidine and 50 ng of the internal standard. Methane was used as the reagent gas and ions were monitored at m/z 159 and 164, and at m/z 243 and 248. The relative standard deviations for the peak height ratios were 2.9 and 2.3, respectively.

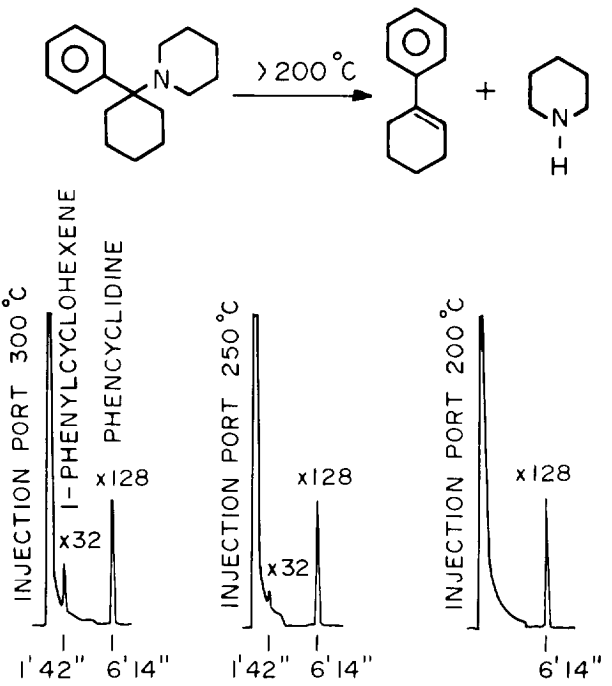


FIGURE 1. GAS CHROMATOGRAMS SHOWING GENERATION OF 1-PHENYLCYCLOHEXENE FROM INJECTION OF PHENCYCLIDINE AT HIGH INJECTION PORT TEMPERATURES

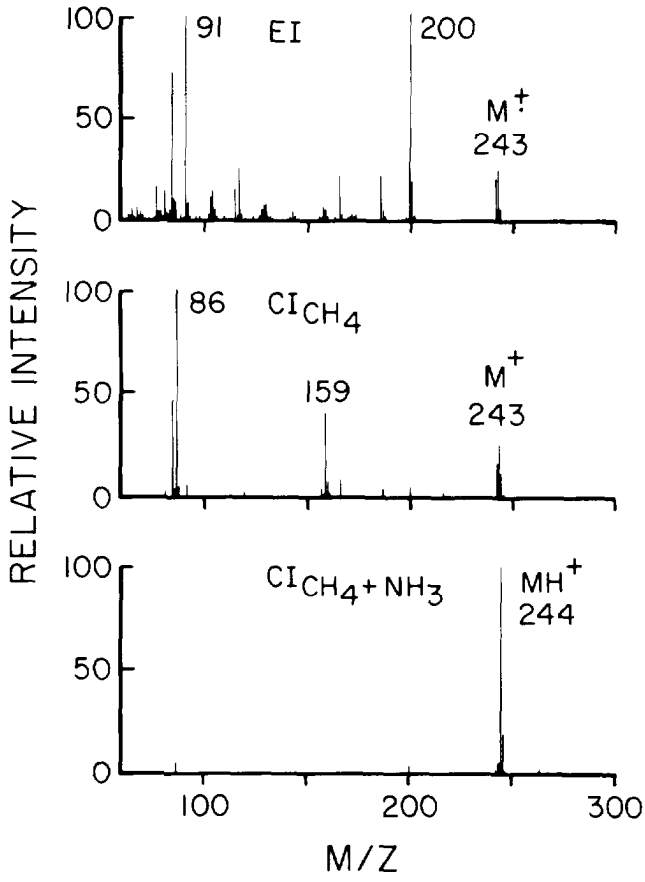


FIGURE 2. MASS SPECTRA OF PHENCYCLIDINE

TABLE 1. RELATIVE RESPONSES FOR PROMINENT IONS IN THE EI AND CI MASS SPECTRA OF PHENCYCLIDINE

Method	m/z Monitored	Percent of Total Ion Current	Relative Response Per Unit Weight of Drug
EI	200 ($M^+ - C_3H_7$)	14	59
CI (CH_4)	243 (M^+)	24	20
CI (CH_4+NH_3)	244 (MH^+)	73	100

Table 2 lists phencyclidine concentrations found in various body fluids from phencyclidine-intoxicated patients. Blood concentrations ranged from 49 ng/ml for a child in a coma, to 2.7 µg/ml found in an adult who died following an overdose.

TABLE 2. CONCENTRATIONS OF PHENCYCLIDINE IN BODY FLUIDS FROM PHENCYCLIDINE INTOXICATED PATENTS

Case Number	Blood	Urine	CSF	Serum
1 (Adult, fatal)	2.7 µg/ml	-	-	-
2 (Child, coma)	49 ng/ml	910 ng/ml	6.5 ng/ml	-
3 (Youth, drowsy)	51 ng/ml	870 ng/ml	-	-
4[a]	-	-	-	171 ng/ml
5[b]	-	-	-	73 ng/ml

[a]The condition of the patient was described as "serious."
[b]The condition of the patient was described as "fairly serious."

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR PHENCYCLIDINE

Nomenclature

Chemical Name: 1-(1-Phencyclohexyl)piperidine

Other Names: Phencyclidine, HOG, PCP, CI-395, Angel dust

Empirical Formula: $C_{17}H_{25}N$

Chemical Abstracts Registry Number: 77-10-1

Trade Names of Hydrochloride: Sernyl, Sernylan

Physical Constants

Appearance: White crystals

Melting Point: 46-46.5°C; hydrochloride, 243-244°C

Boiling Point: 135-137°C (1.0 mm)

Specific Rotation: Optically inactive

Solubility: The hydrochloride is soluble in hater, ethanol and chloroform.

REFERENCES

1. Unsigned. PCP (Phencyclidine): The New Disillusionogen. STASH Press, Madison, WI (1975).
2. Unsigned. Emergency Med. , 8 (3), 265 (1976).
3. P.V. Luisada and B.I. Brown. Clin. Toxicol., 9 (4), 539 (1976).
4. G.D. Lundberg, R.C. Gupta, and S.H. Montgomery. Clin. Toxicol., 9 (4), 503 (1976).
5. A.T. Snulgin and D.E. Mac Lean. Clin. Toxicol., 9 (4), 553 (1976).
6. C. Helisten and A.T. Snulgin. J. Chromatog., 117, 232 (1976).
7. D. Gagne and R.K. Pike. J. Assoc. Off. Anal. Chem., 60, 32 (1977).
8. K.E. Garey and R.G. Heath. Life Sciences., 18, 1105 (1976).
9. R.S. Burns and S.E. Lerner. Clin. Toxicol., 12, 463 (1978).
10. S.H. James and S.H. Schnoll. Clin. Toxicol., 9 (4), 573 (1976).

11. D.N. Bailey, R.F. Shaw, and J.J. Guba. J. Anal. Toxicol., 2, 233 (1978).

12. F. Walker. Science, 184, 189, (1974).

13. R.S. Burns and S.E. Lerner. Clin. Toxicol., 9 (4), 477 (1976).

14. R.L. Balster and L.D. Chait. Clin. Toxicol., 9 (4), 513 (1976).

15. J.C. Munch. Bull. Narcotics, 26 (4), 9 (1374).

16. R.C. Petersen and R.C. Stillman, eds., Phencyclidine (PCP) Abuse: An Appraisal. National Institute on Drug Abuse Research Monograph 21, DHEW Pub. (ADM) 78-728. Supt. of Docs., U.S. Govt. Print. Off Washington, D.C. (August 1978).

17. R.E. Ober, G.W. Gwynn, T. Chang, D.A. McCarthy, and A.J. Glazko. Fed. Proc., 22, pt. 1, 539 (1963).

18. E.F. Domino and A.L. Wilson. Clin. Pharmacol. Therap., 22, 421 (1977).

19. J.A. Marshman, M.P. Ramsay, and E.M. Sellers. Toxicol. Appl. Pharmacol., 35, 129 (1976).

20. A. h. Done, R. Aronow, J.A. Miceli, and D.C.K. Lin, in B.H. Rumack and A.R. Temple, eds., Management of the Poisoned Patient. Science Press, Princeton, NJ, p. 79 (1977).

21. D.C.K. Lin, A.F. Fentiman, Jr., K.L. Foltz, R.D. Forney, Jr., and I. Sunshine. Biomed. Mass Spectr., 2, 206 (1975).

22. E.F. Domino. Int. Rev. Neurobiol., 6, 303 (1964).

23. L.K. Wong and K. Biemann. Biomed. Mass Spectr., 2, 204 (1975).

24. H.B. Hucker. Ann. Rev. Pharmacol., 10, 99 (1970).

25. C.B. Liden, F.H. Lovejoy, Jr., and C.E. Costello. J. Amer. Med. Assoc., 234 (5), 513 (1975).

26. N.C. Jain, R.D. Budd, W.J. Leung, and T.C. Sneath. J. Anal. Toxicol., 1, 77 (1977).

27. B.S. Finkle, E.J. Cherry, and D.M. Taylor. J. Chromatogr. Sci., 9, 393 (1971).

28. J.-E. Lindgren, C.-G. Hammer, R. Hessling, and B. Holmstedt. Am. J. Pharm., 141, 86 (1969).

29. R.C. Shaler, J.M. Parker, and A. Sage. Clin. Toxicol., 7 (3), 307 (1974).

30. J.K. Brown, L. Shapazian, and G.D. Griffin. J. Chromatogr., 64, 129 (1972).

31. R.C. Gupta, I. Lu, G-L. Oei, and G.D. Lundberg. Clin. Toxicol., 8 (b), 611 (1975).

32. P.M. Froehlich and G. Ross. J. Chromatogr., 137, 135 (1977).

33. P.C. Reynolds. Clin. Toxicol., 9 (4), 547 (1976).

34. D.S. Pearce. Clin. Chem., 22, 1623 (1976).

3.5. W.D. MacLeod, Jr., D.E. Green, and E. Seet. Clin. Toxicol., 9 (4), 561 (1976).

36. L.K. Wong and K. Biemann. Clin. Toxicol., 9 (4), 583 (1976).

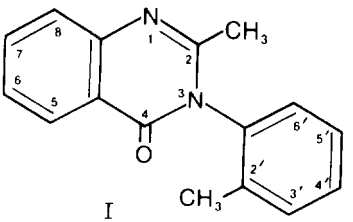
37. A. Kalir, H. Edery, Z. Pelah, D. Balderman, and G. Porath. J. Med. Chem., 12, 473 (1969).

38. A.F. Fentiman, Jr., and R.L. Foltz. J. Labelled Compounds and Radiopharmaceuticals, 12, 69 (1976).

Chapter 4

Methaqualone

Methaqualone (2-methyl-3-o-tolyl-4(3H) -quinazolinone, I) was first synthesized in 1951 after the elucidation of the structure of febrifugine, the active principle of the ancient Chinese antimalarial Ch'ang Shan, stimulated interest in the medicinal chemistry of quinazoline derivatives (1).



I

It was introduced in Europe in 1956 and into the United States in 1965, advertised as a nonbarbiturate, nonaddictive sedative-hypnotic, and marketed under several trade names including Quaalude, Sopor, Optimil, Parest, and Somnafac. Within a few years, it had become the sixth best selling sedative-hypnotic in this country (2). Methaqualone abuse was quick to follow, and by 1972 had reached almost epidemic proportions in the United States, although the first case of fatal poisoning had been reported in Europe at least ten years earlier (3).

PHARMACOLOGY

Even in therapeutic doses (150-500 mg), methaqualone can produce a sensual, euphoric state and a relaxed, intimate mood which explains its popularity and abuse potential. However, the drug may accumulate in the blood following repeated doses and is addictive after prolonged use; abrupt withdrawal can result in death if not properly supervised. Methaqualone is particularly dangerous when consumed with alcohol. Coma and convulsions can result from acute ingestion of 2 grams of methaqualone and death from 8 grams, but in the presence of alcohol these consequences can result from ingestion of considerably smaller quantities of the drug (2). Since 1973, illicit use of methaqualone has dropped dramatically, apparently because of tighter control of its manufacture and distribution.

Methaqualone is a lipophilic, acid-soluble, quinazoline derivative easily prepared from anthranilic acid, o-toludine and an acetyla-

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ting agent. It has a characteristic ultraviolet absorption spectrum and is volatile enough for gas chromatography. Its pKa, calculated from UV spectral data, is 2.5 (4). Although resistant to chemical hydrolysis or oxidation, it is rapidly hydroxylated by hepatic microsomal enzymes to polar metabolites, which are excreted primarily as ß-glucuronic acid conjugates (1). Since metabolites of methaqualone are not present in significant amounts in blood (<0.2 µg/ml) after therapeutic doses, they probably contribute very little to the overall pharmacological response to methaqualone (5) . However, a study of urinary metabolite patterns in six human volunteers given single or repeated therapeutic doses of methaqualone showed a higher urinary level of one metabolite, 2-methyl-3-(2'-hydroxymethylphenyl)-4(3H)-quinazolinone, in individuals who demonstrated the most pronounced physiological response (lower tolerance) to the drug. Since the same metabolite has been found, unconjugated, in the blood of overdose patients, this compound may be pharmacologically active (6).

PHARMACOKINETICS AND METABOLISM

Binding studies by equilibrium dialysis have established that 80 percent of adlministered methaqualone is protein-bound in plasma over the whole therapeutic range of concentration. Based on this observation and on the assumption that only unconjugated methaqualone is pharmacologically active, a comparison of the levels of unbound drug in serum with levels in cerebrospinal fluid (CSF) by gas chromatographic measurement has indicated a passive, uninhibited transfer of drug to the CSF. However, drugs which compete for protein binding sites could theoretically affect the transfer (7). Other workers have also pointed out the susceptibility of the pharmacokinetics of methaqualone to influence by other drugs, especially drugs able to influence hepatic microsomal oxidation. This is not merely of academic interest, since methaqualone is used in many compound formulations for treatment of a wide range of ailments, particularly in Germany (1). In this connection, a study of the kinetics of methaqualone in a formulation containing 250 mg methaqualone, 300 mg carbromal, and 0.33 mg benactyzine, found 6-hr plasma levels of methaqualone to be lower than those usually reported for methaqualone alone. These results may indicate an effect of the other drugs on methaqualone metabolism, lending support to the claim that the pharmaceutical formulation, Staurodorm, alleviates methaqualone “hangover” (8).

The most salient features of the results of pharmacokinetic studies of methaqualone have been the wide variation in the rate of absorption by different subjects and the faster absorption rate for the hydrochloride salt as compared with the free base (1). Plasma levels of methaqualone following a single oral dose of 300 mg generally reach a maximum of 1-8 µg/ml within 2 hr. The average plasma half life has been reported as 2.6 hr for the first, rapid excretory phase (9). A slow excretory phase becomes apparent as much as 100 hr after ingestion, and the half life of this phase is variously reported to be from 19 to 50 hr (10,11).

Methaqualone is extensively metabolized in the human body so that very little unchanged drug, usually <5 percent, is excreted in the urine (12). In fact, the validity of even the small amounts reported has been called into question by the recent identification of methaqualone-N-oxide in the urine of human subjects given 250 mg of methaqualone. This metabolite accounted for 5-9 percent of the dose in 24 hr, and since it easily undergoes thermal conversion to methaqualone under gas chromatographic conditions, its presence could result in mistakenly high calculations for free methaqualone (13).

Metabolic pathways involving formation of dihydroxy and hydroxy methoxy derivatives of methaqualone exist, but they are minor in terms of relative concentrations of methaqualone metabolites in urine. The major pathway for methaqualone is monohydroxylation in the liver at any one of ten possible sites. However, hydroxylation is semi-specific in that five of the possible monohydroxy metabolites predominate over the others in human urine; i.e., those with substitutions at the 3', 4', and 6 positions and on the 2 and 2'-methyl groups (14). The 3-hydroxy metabolite has only recently been confirmed in human urine (15) . Stillwell and co-workers identified five dihydrodiol and two hydroxydihydrodiol metabolites of methaqualone in the unhydrolyzed (unconjugated) fraction of human urine and also detected several unidentified triols. They interpreted these finding as evidence for metabolism of methaqualone in man by way of epoxide intermediates. Mass spectra indicated that three of the dihydrodiol and both hydroxydihydrodiols were formed by epoxidation of the tolyl ring, while the mass spectra of the other two suggested epoxidation of the quinazolinone nucleus. The authors suggested that of the many urinary metabolites of methaqualone which have now been reported, including seven dihydrodiols, nine phenols, two methoxyhydroxyphenols, diols, and several triols, all except the 2- and the 2'-hydroxymethyl derivatives would be likely to involve epoxide intermediates. None of these epoxides have yet been isolated, however (16).

ANALYTICAL METHODOLOGY

Because of the widespread use and abuse of methaqualone and because of its interesting metabolism, virtually every currently popular technique has been applied to the analysis of the drug. Although methaqualone's structure lends itself to spectroptotometric assays, in practice such methods are rarely sensitive enough for the determination of therapeutic levels. However, they have been widely applied in toxicological studies involving gross over-dosage. For spectrophotometric assays, hexane extraction, followed by back extraction with acid to recover methaqualone free from phenolic impurities, is to be preferred to extraction with a more polar solvent such as chloroform because the latter yields a mixture of strongly UV-absorbing materials with UV spectra indistinguishable from that of methaqualone (1).

A commercial kit for radioimmunoassay (RIA) has been evaluated for clinical detection of methaqualone in both urine and blood (17,18). While it proved to be a rapid technique which required no cumbersome

sample preparation, it was unable to distinguish between methaqualone and its metabolites, some of which were as reactive as the parent compound and others less so or not at all. Metabolites present were additive in their effect on the concentration reading. Standard RIA curves prepared in serum for methaqualone and five metabolites demonstrated a detection sensitivity of 2 ng/ml, but because of the contribution of metabolites to the measured levels, RIA in the form tested would be useful as a screening procedure only.

For quantitative studies, gas chromatographic (GC) methods have been developed, some reporting detection levels as low as 0.01 μg/ml for methaqualone in plasma. This degree of sensitivity was achieved in a method reported by Berry in 1969, which involved extraction of 5.0 ml plasma, after pH adjustment by addition of 1.0 ml 1N NaOH, with 15 ml of hexane followed by gas chromatography on a column coated with 3 percent cyclohexanedimethyl succinate. Butobarbital was used as an internal standard. An extraction efficiency of 96 percent was reported for this method (19).

An extraction efficiency of 98-100 percent was reported for a technique in which saturated NaH_2PO_3 solution was added to serum in order to precipitate proteins and lipoproteins and free the drug from binding sites before extraction with n-hexane. After centrifugation, the n-hexane phase was evaporated under nitrogen at 57°C and the residue dissolved in acetone containing hendecyl myristate as an internal standard. Gas chromatography on a 3 percent OV-17 column with nitrogen as the carrier gas provided a sensitivity of 1.0 μg/ml methaqualone (20).

Quantitation of methaqualone and its five most abundant hydroxyl metabolites in urine was done by GC in a recent study designed to characterize metabolite patterns as possible indicators of methaqualone ingestion. Methaqualone was extracted with chloroform from acid-hydrolyzed urine and chromatographed on 3 percent OV-1 columns at 21O°C. To achieve complete separation, the metabolites were silylated and chromatographed on high-resolution SE-30 SCOT columns. Concentrations measured ranged from 0.20 to 1.24 μg/ml methaqualone and 0.56 to 31.5 μg/ml among the five principal hydroxy metabolites. Electron impact mass spectra of the methaqualone and the trimethylsilyl ethers of the metabolites, separated by GC on a 3 percent OV-17 column at 225°C, were used to confirm identification of the metabolites (14).

Selected ion monitoring has come into wide use as a highly sensitive and specific method for measurement of methaqualone as well as for identification and quantitation of its metabolites. An early attempt to use methaqualone labeled with deuterium on the 2-methyl substituent as internal standard was abandoned because of poor isotopic purity. However, the 2-ethyl analog of methaqualone added to plasma before extraction with diethyl ether provided an internal standard suitable for GC/MS analysis. The mass spectrometer monitored the ion current at m/z = 250 for methaqualone and at m/z = 264 for the internal standard. The method permitted measurement of plasma concentrations of the drug as low as 5 ng/ml (10).

More recent utilization of GC/MS in methaqualone studies has led to techniques allowing detection of 0.20 ng/ml methaqualone and its 6-hydroxy metabolite in urine. This degree of sensitivity was achieved by rigorous prepurification of the urine extracts by multiple thin-layer chromatography, use of multilabeled internal standards in which the level of unlabeled compounds was < 0.01 percent, and analysis of the isotopic ratios by field ionization mass spectrometry which produces abundant molecular ions with little or no fragmentation. Heptadeuterated methaqualone was prepared from commercial toluene-$^2H_8$ which was converted to o-toluidine-$^2H_7$ and condensed with N-acetyl-anthranilic acid. Heptadeuterated 6-hydroxy-methaqualone was similarly prepared by condensation of the labeled o-toluidine with 5-methoxy-anthranilic acid followed by demethylation. The internal standards were added to enzyme-hydrolyzed urine samples before adsorption on an Amberlite XAD-2 column and elution with ethyl acetate: chloroform (3: 2, v/v). From the concentrated eluates, methaqualone and the 6-hydroxy metabolite were isolated by chromatography four times on thin-layer plates with, respectively, benzene: n-butanol:methanol (85:10:5); ethyl acetate:methanol:NH_4OH (85:10:1); benzene:acetic acid (9:l); and ether. Other hydroxy metabolites present were apparently eliminated by this procedure. The technique was applied to a study of elimination kinetics of methaqualone over an 11-day period following ingestion of one 250-mg tablet, demonstrating its value in long term investigations when the methaqualone concentration is likely to fall to levels undetectable by conventional methods (10).

EXPERIMENTAL PROCEDURE

Therapeutic doses of methaqualone are relatively high (150-500 mg). Consequently, a simple, direct extraction of 0.1 ml or more of biological fluid is usually sufficient to provide enough of the drug to be measured by GC/MS. Two extraction procedures are described here. Both procedures are relatively simple and rapid. The first procedure should give slightly better recovery and provide a cleaner extract, while the second method is even simpler and completely avoids evaporation of the solvent extract and therefore the possibility of losing small quantities of the drug through evaporation. In our hands, precision and accuracy were comparable with both methods.

Standards and Reagents

The methaqualone hydrochloride used in the development of this method was purchased from Applied Science Laboratories, State College, PA 16801. No impurities were detected by either gas chromatography or mass spectrometry. Methaqualone- $^2H_4$ [2-methyl- 3-o-to1yl -4 (3H)quinazolinone-5,6,7,8-$^2H_4$] was prepared from phthalimide-$^2H_4$ (21). On the basis of GC/MS analysis this material showed a chemical purity of greater than 99 percent, and had the following isotopic composition: $^2H_4$, 97.8 percent; $^2H_3$, 2.1 percent; $^2H_1$ and $^2H_0$ <0.1 percent.

Prepare a pH 9.6 buffer by dissolving 500 grams of Potassium monohydrogen phosphate (K_2HP0_4) in about 500 ml of distilled water. Allow the solution to cool to room temperature and then add sufficient distilled water to make exactly 1 liter.

Stock solutions of methaqualone and methaqualone-$^2H_4$ are prepared as follows for the purpose of determining standard curves and preparing working standards. Weigh into a 100-ml volumetric flask about 10 mg of methaqualone, or about 11.4 mg of methaqualone hydrochloride, and record its weight to the nearest 0.1 mg. If the hydrochloride is used, the equivalent weight of free base is calculated by multiplying the weight of the hydrochloride by 0.874. Dissolve the measured methaqualone in about 75 ml of methanol and then add additional methanol to give exactly 100 ml of solution. 'This solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled with its actual concentration based on the exact measured weight of methaqualone. A series of working standard solutions can then be prepared by appropriate dilution of the stock solution as described in Chapter 2.

The stock solution of methaqualone-$^2H_4$ is prepared in the same manner. For the measurement of methaqualone in body fluids within the concentration range 1 to 1000 ng/ml, addition of approximately 40 ng of the internal standard to each ml of body fluid has proven satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml methaqualone-$^2H_4$ methanolic solution to each ml of body fluid. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of methaqualone- $^2H_4$ to 250 ml with methanol.

Store stock and working standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction—Method 1

Transfer 1 ml of body fluid (whole blood, plasma or urine) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately). Add 100 µl of the 400-ng/ml methaqualone-$^2H_4$ solution and vortex for 10 sec. Allow the sample to equilibrate for about 15 min and then add 1 ml of K_2HPO_4 buffer (pH 9.6) and 5 ml of methylene chloride. Cap the tube and check for a leak around the cap. Gently mix the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min, remove the upper aqueous layer by aspiration, and then transfer by means of a disposable Pasteur pipette the methylene chloride extract (bottom layer) to a silylated concentrator tube (>5 ml volume) having a conical or nipple shaped bottom. Add 20 µl of dimethylformamide to act as a "keeper solvent" to minimize evaporative loss of the methaqualone. Remove the methylene chloride by evaporation under a gentle stream of nitrogen or filtered air while heating at 50 to 60°C. When the volume of extract decreases to about 20 µl, stopper the tube or cover it with Parafilm and store at <0°C until the GC/MS analysis is ready to be performed. Allow the tube to warm to room temperature immediately prior to the analysis.

Extraction—Method 2

Transfer 1 ml of body fluid (whole blood, plasma, or urine) to a 5-ml glass stoppered, conical centrifuge tube. Add 100 µl of the 400-ng/ml methaqualone-$^2H_4$ internal standard solution and vortex for 10 sec. Allow the sample to equilibrate for 15 min and then add 1 ml of K_2HPO_4 buffer (pH 9.6), followed by 100 µl of a solvent consisting of toluene:heptane:isoamyl alcohol in a volume ratio of 70:20:10. Stopper the tube and vortex for at least 30 sec. Separate the phases by centrifugation at 2000 x G for 15 min. The organic solvent should form a narrow upper layer from which an aliquot can be removed with a syringe for injection into the GC/MS.

GC/MS Analysis

The experimental conditions for the GC/MS analysis of methaqualone are as follows:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-1 or OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Reagent gas:	Ammonia, introduced into the ion chamber as described in Chapter 2
Temperatures:	Injector, 270°C Column, 210°C isothermal GC/MS transfer line, 260°C Ion source, 160°C

Under these conditions the methaqualone and methaqualone-$^2H_4$ should elute at about 4 min as narrow, symmetrical peaks.

Prior to beginning GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 251 and 255. They correspond to the protonated molecule ions for methaqualone and methaqualone-$^2H_4$, respectively. With the divert valve in the divert position, inject the organic extract into the gas chromatograph (2 to 4 µl of the extract from Method 1 or 5 to 10 µl of the extract from Method 2). After approximately 1 min switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer and begin data acquisition. When the methaqualone peaks nave eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of methaqualone in the specimen is determined by measuring the heights (or areas) of the methaqualone and methaqualone-$^2H_4$ peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the concentration of methaqualone by dividing the measured quantity of methaqualone by the exact volume of speciman extracted.

Discussion of the Experimental Procedure

The distribution ratios of methaqualone between aqueous phases over a range of pH values and various organic solvents were measured in order to determine the optimum conditions for extraction of the drug from biological fluids. For this purpose a stock solution of methaqualone (100 μg/ml) in 0.1N HCl was prepared. A 5-ml aliquot of the stock solution was adjusted to a specific pH and transferred to a 25-ml screw-cap tube along with 5 ml of the organic solvent. The tube was rocked gently for 30 min and then centrifuged. The optical densities of both the aqueous and organic layers were measured from 350 to 250 nanometers using a Cary 14 recording spectrophotometer. Water or the appropriate solvent was placed in the reference cell.

The results indicated that methaqualone is extracted by chloroform with nearly equal efficiency at all pH values between 2 and 12. At pH 10 chloroform and methylene chloride are almost equally efficient extraction solvents, while hexane and cyclohexane are less effective. At pH 2 all of the solvents investigated extract a significant amount of methaqualone. Consequently, a back extraction into acid, which is often used in drug extractions for removal of non-basic components, would result in loss of an appreciable proportion of the methaqualone. Based on these data, we recommend that the pH of the biological fluid be adjusted to 9-10 before extraction with methylene chloride,

Since methaqualone contains no NH or OH groups, derivatization before gas chromatography is not necessary. The free drug gas chromatographs well on a variety of GC column packings. both 3 percent OV-1 and 3 percent OV-17 on 100/120 mesh Gas Chrom Q have been used successfully, but there is a slight preference for the former because a significantly lower column temperature can be employed.

The electron impact (EI) and chemical ionization (CI) mass spectra of methaqualone are shown in Figure 1. These spectra were obtained on quadrupole mass spectrometers (22). The relative sensitivities achieved by monitoring the ion current at the m/z value corresponding to the most abundant ion generated by each of the ionization processes are shown in Table 1. If maximum sensitivity is desired, CI with methane and ammonia as reagent gases should be used. This combination gives slightly better sensitivity than that provided by methane alone as reagent gas, and is also a more selective mode of ionization. However, since plasma levels following therapeutic doses of methaqualone are moderately high (~5 μg/ml), sensitivity is usually not a problem. At higher ion source temperatures (>160°C) under methane CI conditions fragment ions at m/z 118 become more abundant. Since the corresponding fragment ion in the methane CI mass spectrum of the methaqualone-$^2H_4$ retains the deuterium labels, it appears at m/z 122. Consequently, the ions at m/z 118 and 122 can be monitored in addition to the protonated molecule ions at m/z 251 and 255 to provide confirmational data.

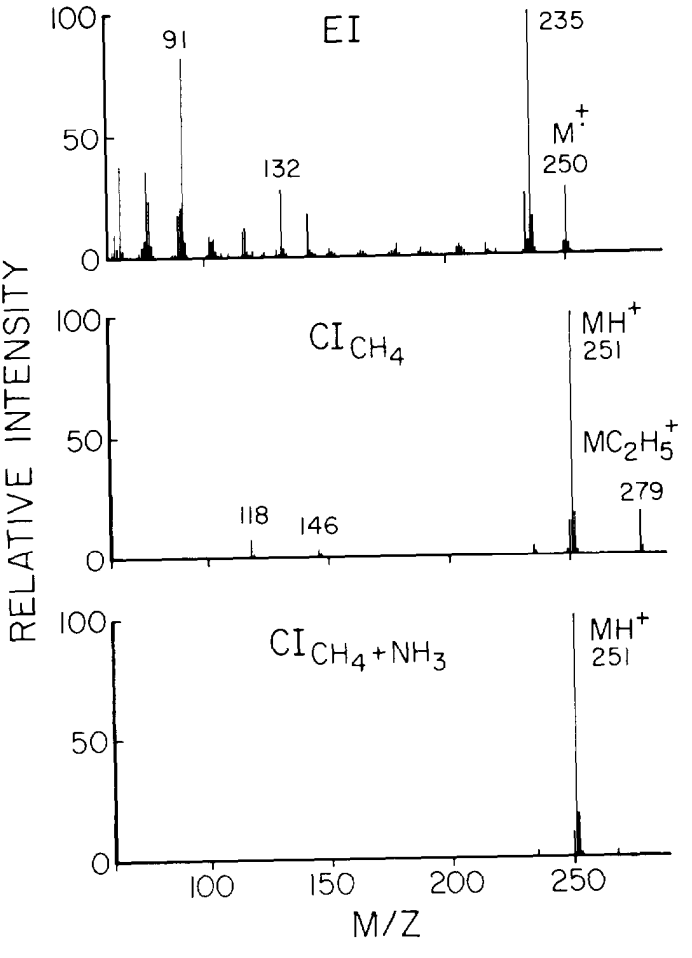


FIGURE 1. MASS SPECTRA OF METHAQUALONE

TABLE 1. RELATIVE RESPONSES FOR PROMINENT IONS IN THE EI AND CI MASS SPECTRA OF METHAQUALONE (22)

Ionization Method	m/z Monitored	Percent of Sample Ion Current	Relative Response Per Unit Weight of Drug
EI	250 (M^+)	4	6
	235 ($M^+ - CH_3$)	1.5	20
CI (CH_4)	251 (MH^+)	57	84
Cl ($CH_4 + NH_3$)	251 (MH^+)	74	100

When solutions of methaqualone and cicosane (internal standard) in methylene chloride were prepared and stored in capped vials at room temperature, 2°C, and -10°C, gas chromatographic analyses of the solutions over a period of six weeks showed no significant changes in the ratio of eicosane and methaqualone peak heights, indicating that methaqualone is stable under these conditions.

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR METHAQUALONE

Nomenclature

Chemical Name: 2-Methyl-3-o-tolyl-4(3H)-quinazolinone

Empirical Formula: $C_{16}H_{14}N_2O$

Chemical Abstracts Registry Number: 72-44-6

Trade Names: Many, including MAOA, MTZ, TR 495, Catendyl, Dormogen, Fadormir, Hyprol, Melsomin, Mebedorm, Normi-Nox, Omnyl, Parminol, Parest, (Quaalude, Roulone, Somnafac, Sonal, Somberol, Soxor, Forinol, Tuazolone

Physical Constants

Appearance: White crystalline powder

Melting Points: Free base: 114-116°C
Hydrochloride: 255-265°C

Specific Rotation: Optically inactive

Solubility: The free base is practically insoluble in water; soluble in ethanol, ether, and chloroform

pKa: 2.54 (4)

REFERENCES

1. S.S. Brown. In L, Reid, ed., Methodological Developments in Biochemistry, Vol. 5, p. 179. North-Holland, Amsterdam (1976).

2. J.A. Ostrenga. Clin. Toxicol., 4, 607 (1973).

3. S.S. Brown and S. Goenechea. Clin. Pharmacol. Ther., 14, 314 (1973).

4. J.J. Zalipsky, D.M. Patel, R.J. Darnowski, and N.H. Reavey-Cantwell. J. Pharm. Sci., 65 (3), 460 (1976).

5. A.F. Delong, R.D. Smyth, A. Polk, R.K. Nayak, and N.H. Reavey-Cantwell. Arch. Int. Pharmacodyn., 222 (2), 322 (1976).

6. L. Kazyak, J.A. Kelley, J.A. Cella, R.E. Droege, L.R. Hilpert, and R.C. Permisohn. Clin. Chem., 3, 2001 (1977).

7. J.M. Christensen and S. Holfort. J. Pharm. Pharmacol., 2, 538 (1975).

8. C. White, E. Doyle, L.F. Chasseaud and T. Taylor. Europ. J. Clin. Pharmacol., 10, 343 (1976).

9. R.N. Morris, G.A. Gunderson, S.W. Babcock, and J.F. Zaroslinski. Clin. Pharmacol. Ther., 13, 719 (1972).

10. G. Alvan, J.E. Lindgren, C. Bogentoft, and O. Ericsson. Europ. J. Clin. Pharmacol., 6, 187 (1973).

11. J.M. McReynolds, H. d'A. Heck, and M. Anbar. Biomed. Mass Spectr., 2, 299 (1975).

12. R. Bonnichsen, K. Dimberg, Y. Marde, and K. Ryhage. Clin. Chim. Acta, 60, 67 (1975)

13. C.N. Reynolds and K. Wilson. Xenobiotica, 6 (2), 113 (1976).

14. R.C. Permisohn, L.K. Hilpert, and L. Kazyak. J. Forensic Sci., 21 (1), 98 (1976).

15. D. Burnett, C.N. Reynolds, K. Wilson, and J.K. Francis. Xenobiotica, 6 (2), 125 (1976).

16. W.G. Stillwell, P.A. Gregory, and M.G. Horning. Drug Metabolism and Disposition, 3 (4), 287 (1975).

17. A.K. Berman, J.P. McGrath, K.C. Permisohn, and J.A. Cella, Clin. Chem., 21, 1878 (1975).

18. R.O. Bost, C.A. Sutheimer, and I. Sunshine. Clin. Chem., 22, 689 (1976).

19. D.J. Berry. J. Chromatogr., 42, 39 (1969).

20. D. Chin and E. Fastlich. Clin. Chem., 20, 1382 (1974).

21. A.F. Fentiman, Jr. and R.L. Foltz. J. Labelled Compounds and Radiopharmaceuticals, 12, 69 (1976).

22. K.L. Foltz, D.A. Knowlton, D.C.K. Lin, and A.F. Fentiman, Jr. Proceedings of the Second International Conference on Stable Isotopes, Oak Brook, IL, October 20-23, 1975.

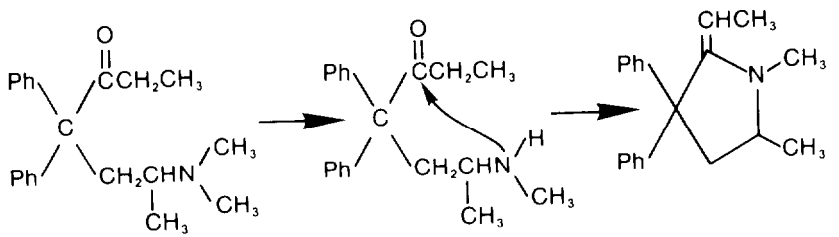
Chapter 5

Methadone

Studies on the pharmacology of methadone (I) began in 1946 at which time the compound was found to be a potent narcotic analgesic (1). Further investigations of the analgesic effects of methadone in man (2) led to its subsequent introduction for the control of severe pain. In contrast to morphine, methadone is effective when taken orally. In 1963 methadone was introduced as an experimental drug for the treatment of heroin dependence (3). Methadone dosage can be maintained or gradually decreased in order to overcome physical dependence. This "detoxification" process is generally conducted over a prolonged period during which counseling and other therapy are also employed. Unfortunately, methadone has considerable abuse potential so that distribution of the drug must be carefully controlled.

METABBOLISM AND PHARMACOKINETICS

The primary metabolic pathway for methadone in man is N-demethylation followed by cyclization to give a pyrrolidine metabolite (II) (4):



I II

Additional metabolites, resulting from further N-demethylation, aromatic ring hydroxylation, and degradation of both side chains, have been identified.

The pharmacokinetics of methadone in man has been extensively studied (5,6,7). One study (5) compared acute and chronic administration of methadone to postaddict volunteers. Following a single 15-mg oral dose, the methadone plasma concentration showed a biexponential decay; the first phase corresponded to a plasma elimination half-life of approximately 14 hr, and the second phase a half-life of approximately 55 hr. The methadone plasma concen-

tration measured at 2 hr after administration was 85 ± 10 ng/ml. During chronic oral administration of the drug, the plasma concentration underwent a single exponential decay with an average half-life of 22 hr. Patients maintained on daily 40-mg doses showed average methadone plasma concentrations of 182 ± 35 ng/ml, while those receiving 80-mg doses showed an average daily methadone concentration of 420 ± 97 ng/ml of plasma. Another study (8) found a plasma elimination half-life of 7.3 hours following intramuscular administration of 10 mg of methadone. More recently, marked variations in the pharmacokinetics of the drug between individual human subjects nave been documented among 12 in-hospital methadone maintenance patients, among whom dose to plasma concentration ratios varied widely from one subject to another, as did the plasma half-life of methadone (7 to 21 hr). Recoveries of the drug from urine ranged from 23 percent to 52 percent of total intake in 24-hour samples (9).

Methadone is eliminated from the body primarily as the parent drug (I) and the pyrrolidine metabolite (II). Following a single dose, urine was found to contain about half as much of the metabolite as of the parent drug, expressed as percentage of daily dose in 24-hour collections, while chronic administration of the drug increased the percentage of the metabolite to twice that of the drug. The metabolite was always the predominant form in feces (5). The increased rate of metabolism and excretion during chronic administration suggests that induction of hepatic microsomal enzymes responsible for drug metabolism may play a significant role in development of tolerance to the drug. However, it has been shown in the rat that methadone does not enhance microsomal drug metabolizing enzyme systems (10). Postmortem examination of tissues from methadone users demonstrated the presence of both the drug and the metabolite in liver, kidney, spleen, and brain; and especially high concentrations in the lung (11).

ANALYTICAL METHODOLOGY

Sensitive and specific methods for analysis of methadone in biological fluids are necessary in order to assess more accurately the pharmacokinetics of the drug, and also to contribute analytical information for correlation with the clinical status of patients who ingest the drug in both maintenance and overdose situations. Thin-layer chromatography (TLC) (12), UV spectrophotometry (13), fluorimetry (14), biological assay (15), radioisotopes (16), gas chromatography (GC) (17-19), and gas chromatography/mass spectrometry (20,21) have been used for detection and quantitation of methadone.

Immunoassay techniques, which are well suited for the analysis of large batches of samples, are available for detection of methadone in urine, and are highly sensitive. Radioimmunoassay (RIA) can detect methadone in urine following doses of as little as 2.5 mg/day, and shows promise as a screening technique because propoxyphene and its metabolites do not interfere as they often do in TLC and GC procedures (22). However, the antibodies used for RIA,

hemagglutination inhibition, or the enzyme multiplied immunoassay (EMIT) do not react with the primary metabolite II at concentrations of 1000 μg/ml or less. Because the concentration of II is often equal to or greater than the concentration of methadone in urine, its detection is important at low drug concentrations. Furthermore, none of the immunological methods are quantitative over the normal range of methadone concentrations found in urine (23).

The most satisfactory methods for quantitation of methadone in biological fluids have been found to involve gas chromatography. The combination of GC with electron impact mass spectrometry (GC/EI-MS) has been used for quantitation of methadone in human plasma (20). A homolog of methadone was used as the internal standard in order that both unlabeled and deuterium-labeled methadone could be administered and simultaneously measured in plasma. The method involved equilibration of 4 ml of plasma with the internal standard, adjustment of the pH to 9.5, and extraction with 1-chlorobutane. The GC column was packed with 1 percent UCW-98 and operated at 190°C. Ions monitored corresponded to $M^+ - CH_3$ for the methadone (m/z 294), methadone-$^2H_3$ (m/z 297), and the internal standard (m/z 308). The sensitivity of the method was limited by the relatively low abundance of the ions monitored. Under electron impact ionization most of the ion current for methadone is carried by low mass fragment ions which are unsuitable for selected ion monitoring. Nevertheless, the method is reported to be capable of measuring methadone concentrations as low as 5 ng/ml with a relative standard deviation of less than 4 percent.

A GC/MS procedure employing chemical ionization has been reported to be about five times more sensitive than the electron impact ionization method because of greater relative abundance of ions in the region of the molecular ion in the CI mass spectrum of methadone (21). Methadone containing five deuterium atoms attached to one aromatic ring was synthesized for use as the internal standard in this assay. Extraction from plasma or urine, after addition of the deuterated standard and adjustment to pH 9, was with ethyl acetate, followed by back extraction into 0.05 N HCl. Gas chromatography was done on a column of 10 percent W-98 on Gas Chrom Q at 180°C. Ion abundance ratios were measured using isobutane chemical ionization in which the protonated molecule ions at m/z 310 for methadone and 315 for $^2H_5$-methadone were monitored.

Methadone-$^2H_{10}$ as well as the corresponding decadeuterated methadone metabolites have been synthesized for application to metabolic studies and as internal standards for GC/MS analysis (24). The labeled compounds have also proven useful in elucidating the electron impact fragmentation processes for methadone and its metabolites.

A GC method reported for quantitation of methadone using an electron capture detector (ECD) involved extraction of methadone from plasma with n-heptane followed by back extraction into an acidic aqueous phase. The aqueous extract was then oxidized with barium oxide to convert the methadone to benzophenone in order to obtain a stronger ECD response. The method was reported to have sensitivity comparable to the GC/EI-MS technique (17).

Several methadone assays using GC with conventional flame-ionization detectors (FID) have been published. In one of them, designed for determination of methadone in the urine of addicts in a methadone maintenance program, 10 ml of urine was adjusted to pH 9.5 and extracted with chloroform- isopropanol (3:1). The GC column was packed with 3 percent OV-17 and operated at 240°C. 4-Benzylbiphenyl was used as the internal standard. The method can quantitate methadone in urine within the concentration range 3 to 30 µg/ml with a relative standard deviation of 7 percent (18). Another GC method measured methadone concentrations in blood, plasma, or urine by a procedure consisting of the addition of n-docosane as the internal standard, extraction of the body fluid (adjusted to pH 9.5) with 1-chlorobutane, and gas chromatography on a 4- ft 1 percent W-98 column maintained at 165°C. The minimum concentration of methadone that could be measured by this procedure was 15 ng/ml using 3 ml of blood or plasma (19). A similar initial extraction procedure (1-chlorobutane) was used in another GC assay, but further purification of the extracted methadone was achieved by back extraction into 0.2 N HCl. The aqueous acid extract was then made alkaline and extracted with chloroform. A drug (SKF 525-A) with a structure similar to methadone was used as the internal standard. The gas chromatographic analysis employed a 3 percent SE-30 column operated at 200°C. This method can detect as little as 15 ng/ml of methadone in 4 ml of plasma (8).

The GC-FID technique using SKF 525-A as an internal standard has been adapted to the simultaneous determination of methadone, the pyrrolidine metabolite, cocaine, methaqualone, phencyclidine, and propoxyphene in small (3 ml) volumes of urine, with detection levels as low as 0.5 µg/ml. The drugs are extracted together at basic pH by toluene: isoamyl alcohol:heptane (76:4:20) and converted to their less volatile hydrochloride salts by addition of 1 percent HCl in methanol; the reported extraction efficiency for methadone and its metabolite is 100 percent. The residue after evaporation is reconstituted in methanol and subjected to GC on a 2 percent W-98/1 percent OV-17 mixed bed column. For optimal accuracy in this procedure, silylated glassware and a fresh buffer solution for the internal standard are required (25). A recently reported improvement in the extraction procedure for methadone increased the sensitivity of a GC-FID assay to 5 ng methadone per ml in a 15-ml sample of whole blood. The method involved extraction of methadone and internal standard (2-dimethylamino-4,4-diphenyl-5-nonanone, specially synthesized for the analysis) with 1-chlorobutane at pH 9.8, back extraction into 0.5M H_2SO_4, alkalinization, and reextraction into chloroform. Sulfuric acid was used instead of HCl because the sulfate salt of methadone has a better partition coefficient (1-chlorobutane:water) than the hydrochloride salt at pH 0 to 2. In some samples, the aqueous phase had to be equilibrated with 2,2,4-trimethylpentane to break the emulsion formed after equilibration of the H_2SO_4 and 1-chlorobutane layers. No evaporation step was required. Recovery of ^{14}C-labeled methadone from whole blood by this procedure was ~93 percent. Chromatography was done on 1.5 percent OV-101 on Gas Chrom Q with temperature programmed from 170- 250°C (26) .

EXPERIMENTAL PROCEDURE

A relatively simple extraction procedure is used for removing methadone from biological fluids. The procedure consists of addition of deuterium-labeled methadone to the specimen together with a pH 9.5 buffer, followed by extraction with 1-chlorobutane. Chemical ionization with methane and ammonia as reagent gases is used for the GC/MS analysis.

Standards and Reagents

d,1-Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) was purchased as the hydrochloride salt from Regis Chemical Company, Morton Grove, IL 60053. Analysis by gas chromatography and mass spectrometry indicated the material was better than 99 percent pure. The methadone-1,1,1-$^2H_3$ hydrochloride was obtained from the National Institute on Drug Abuse. The deuterated methadone was chromatographically pure (> 99 percent) and, based upon its ammonia CI mass spectrum, contained an isotopic composition of $^2H_3$, 96.9 percent; $^2H_2$, 1.9 percent; $^2H_1$, 0 percent; and $^2H_0$, 1.2 percent.

A suitable pH 9.6 buffer can be prepared by dissolving 500 grams of potassium monohydrogen phosphate (K_2HPO_4) in about 500 ml of distilled water. When all of the salt is dissolved, allow the solution to cool to room temperature and add sufficient water to give exactly 1 liter.

Stock solutions of methadone and methadone-$^2H_3$ used for the determination of calibration graphs (Chapter 2) and as reference standards, are prepared as follows. Weigh into a 100-ml volumetric flask 11.2 mg of methadone hydrochloride. Dissolve the methadone hydrochloride in methanol and bring to exactly 100 ml with additional methanol. The resulting stock solution corresponds to a concentration of 0.10 mg/ml of methadone calculated as the free base. A series of standard solutions can then be prepared by appropriate dilution of the stock solution as described in Chapter 2.

The stock solution of methadone-$^2H_3$ is prepared in the same manner. For the measurement of methadone in blood or urine within the concentration range 1 to 1000 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µ1 of a 400-ng/ml methadone-$^2H_3$ methanolic solution to each ml of specimen. Prepare the 400-ng/ml standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of methadone$^2H_3$ to 250 ml with methanol. Store stock and standard solutions in the dark in well-stoppered or capped glass vessels at 0°C or lower. At room temperature, acidic solutions of methadone are unstable; they undergo approximately 40 percent decomposition within two weeks.

Extraction

Transfer 1 ml of specimen (whole blood, plasma, or urine) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately). Add 100 µ1 of the 400-ng/ml methadone-$^2H_3$ internal standard solution to the

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specimen and vortex for 10 sec. Allow the sample to equilibrate for about 15 min and then add 1 ml of pH 9.5 buffer and 5 ml of 1-chlorobutane. Cap the tube and check for a leak around the cap. Gently mix the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min and then transfer the 1-chlorobutane layer (top) to a silylated concentrator tube at least 5 ml in volume and having a conical or nipple-shaped bottom. Add 20 µl of dimethylformamide as a "keeper" solvent to minimize evaporative loss of the methadone. Remove the 1-chlorobutane by evaporation with a gentle stream of nitrogen or filtered air while heating at 40-50°C. When the extract volume has decreased to about 20 µl, stopper the tube or cover the top with Parafilm and store at less than 0°C until the GC/MS analysis is to be performed. Immediately prior to the analysis, allow the tube to warm to room temperature.

GC/MS Analysis

The following experimental conditions are satisfactory for GC/MS analysis of methadone:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Temperatures:	Injector, 260°C Column, 210°C GC/MS transfer line, 210°C Ion source, 160°C

Under these conditions methadone and methadone-$^2H_3$ should elute at between 3 and 5 min as narrow, symmetrical peaks.

Before beginning the GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 310 and 313. They correspond to the protonated molecule ions for methadone and methadone $^2H_3$, respectively. With the divert valve in the divert position, inject 2 to 6 µl of the extract into the gas chromatograph. After approximately 1 min switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the methadone peaks have eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of methadone in the specimen is determined by measuring the heights (or areas) of the methadone and the methadone-$^2H_3$ peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a previously established calibration graph. Calculate the concentration of methadone in the biological sample by dividing the measured quantity of methadone by the exact volume of specimen used in the analysis.

Discussion of the Experimental Procedure

The choice of 1-chlorobutane as the extraction solvent was based primarily on the work of Sullivan and Blake (19) who showed it to be one of the most efficient solvents for removal of methadone from biological fluids. Furthermore, it extracts minimal amounts of endogenous materials. The extraction efficiency is pH dependent, with a maximum recovery of 85 percent when extractions are made at pH 9.5 to 10.0.

Of the GC column packings evaluated, 3 percent OV-17 on 100/120 mesh Gas Chrom Q gave the best combination of good resolution and minimum column bleed, as well as a suitable methadone retention time.

The electron impact (EI), methane chemical ionization (CI_{CH4}), and methane-ammonia chemical ionization ($CI_{CH\text{-}NH3}$) mass spectra of methadone are shown in Figure 1. The EI mass spectrum is dominated by an intense peak at m/z 72 corresponding to the fragment ion $CH_3CH{=}N\ (CH_3)\ 2^+$. This ion is unsuitable for selected ion monitoring because it occurs at a m/z value where interfering ions are likely, and even more important, the corresponding fragment ion from the deuterated methadone does not retain the deuterium label. None of the higher mass ions in the EI mass spectrum are sufficiently abundant to be satisfactory for selected ion monitoring. With methane alone as the reagent gas, the protonated-molecule ion at m/z 310 and the fragment ion at m/z 265 are abundant and are satisfactory for selected ion monitoring. Monitoring an abundant fragment ion in addition to the protonated molecule ion provides additional confidence in the reliability of the quantitative measurements (see Chapter 2). However, addition of ammonia to the methane reagent gas results in approximately a fivefold increase in the ion current generated at m/z 310 by a given amount of methadone (Table 1). Also, the contribution of the methadone-$^2H_3$ internal standard to the m/z 310 ion current is slightly less (1.0 percent vs. 1.9 percent) under ammonia CI than under methane CI. Apparently, in the latter case, some deuteride abstraction occurs. Consequently, small amounts of methadone can be measured more accurately in the presence of large amounts of internal standard when ammonia CI is used. Finally, addition of ammonia to the reagent gas results in a more selective ionization since nonbasic compounds are not ionized. In our experience, when methane alone was used, interfering ions prevented reliable quantitation of low concentrations of methadone (< 10 ng/ml) in Plasma. The interferences were not a problem when a methane-ammonia mixture was used as the reagent gas.

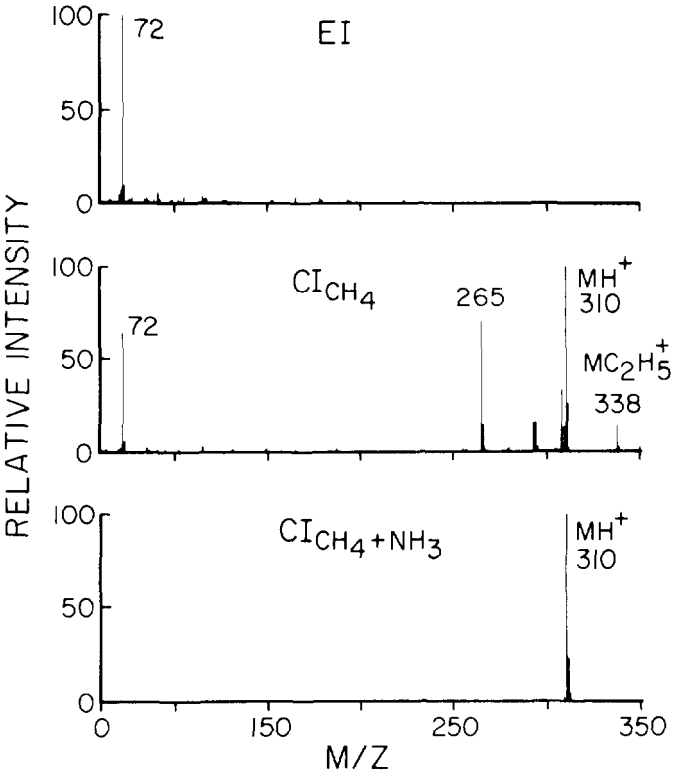


FIGURE 1. MASS SPECTRA OF METHADONE

TABLE 1. RELATIVE RESPONSES FOR PROMINENT IONS IN THE EI AND CI MASS SPECTRA OF METHADONE

Ionization Method	m/z Monitored	Percent of Sample Ion Current	Relative Response Per Unit Weight of Drug
EI	309 (M^+)	< 0.1	< 0.1
	72 (CH=$\overset{+}{N}$(CH3)CH3)	54	750
CI (CH_4)	310 (MH^+)	24	30
CI (CH_4 + NH_3)	310 (MH^+)	76	100

PHYSICAL, CHEMICAL AND SPECTROMEIRIC DATA FOR METHADONE

Nomenclature

Chemical Name: d,1-6-Dimethylamino-4,4-diphenyl-3-heptanone

Empirical Formula: $C_{21}H_{27}NO$

Chemical Abstracts Registry Numbers: d,1-Methadone, 297881
d,1-Methadone hydrochloride, 125564

Trade Names: Amidone hydrochloride, Adanon hydrochloride, Dolophine hydrochloride, Physeptone, Heptadon hydrochloride

Physical Constants

Melting Points: 1-Methadone: 99-100°C
1-Methadone hydrochloride: 241°C
d,1-Methadone: 78°C
d,1-Methadone hydrochloride: 233-236°C

Refractive Index: d,1-Methadone hydrochloride (n^{25}_D) = 1.5299

Specific Rotation: 1-Methadone $[\alpha]_D^{20} = -32^o$ (in ethanol)
1-Methadone HCl $[\alpha]_D^{20} = -169^o$ (C=2.1

pKa: 8.25

Solubility of d,l-Methadone HCl: Water 12 g/100 ml
Ethanol 9 g/100 ml
Isopropanol 2.4 g/100 ml

REFERENCES

1. C.C. Scott and K.K. Chen. J. Pharmacol. Exp. Ther., 87, 63 (1946).

2. H. Isbell, A.J. Eisenman, A. Wikler, and K. Frank. J. Pharmacol. Exp. Ther., 92, 83 (1948).

3. V.P. Dole and M. Nyswander. J. Amer. Med. Assoc., 193, 646 (1965).

4. A.H. Beckett, J.F. Taylor, A.F. Casy, and M.M.A. Hassan. J. Pharm. Pharmacol., 20, 754 (1968).

5. K. Verebely, J. Volavka, S. Mulé, and R. Resnick. Clin. Pharmacol. Ther., 18, 180 (1976).

6. E. Anggård, L.-M. Gunne, J. Holmstrand, R.E. McMahon, C.-G. Sandberg, and H.R. Sullivan. Clin. Pharmacol. Ther., 17, 258 (1975).

7. M.J. Kreek. New York State J. Med., 73, 2773 (1973).

8. C.E. Inturrisi and K. Verebey. J. Chromatogr., 65, 361 (1972).

9. A.K. Dhar and H. Kutt, Fed. Proc., 35, 565 (1976).

10. A.P. Alvares and A. Kappas. J. Lab. & Clin. Med., 79, 439 (1972).

11. A.E. Robinson and F.M. Williams. J. Pharm. Pharmacol., 23, 353 (1971).

12. C.M. Davis and D.C. Fenimore. J. Chromatogr., 104, 193 (1975).

13. H.E. Hamilton, J.E. Wallace, and K. Blum. J. Pharm. Sci., 63, 741 (1974).

14. E.J. McGonigle. Anal. Chem., 43, 966 (1971).

15. C.T. Liu and F.L. Adler. J. Immunol., 111, 472 (1973).

16. A.L. Misra, R. Bloch, N.L. Vadlamani, and S.J. Mulé. J. Pharmacol. Exp. Ther., 188, 34 (1974).

17. P. Hartvig and B. Näslund. J. Chromatogr., 111, 347 (1975).

18. J.L. Valentine, P.E. Wiegert, and K.L. Charles. J. Pharm. Sci., 61, 796 (1972).

19. H.R. Sullivan and D.A. Blake. Res. Commun. Chem. Pathol. Pharmacol., 3, 467 (1972).

20. H.R. Sullivan, F.J. Marshall, R.E. McMahon, E. Anggard, L.M. Gunne, and J.H. Holmstrand. Biomed. Mass Spectr., 2, 197 (1975).

21. D.L. Hachey, M.J. Kreek, and D.H. Mattson. J. Pharm. Sci., 66, 1579 (1977).

22. D.L. Roerig, R.I.H. Wang, M.M. Mueller, D.L. Lewand, and S.M. Adams. Clin. Chem., 22, 1915 (1976).

23. N.C. Jain, W.J. Leung, T.C. Sneath, R.D. Budd, and D. Chinn. J. Anal. Toxicol., 1, 6 (1977).

24. G.I. Kang, F.S. Abbott, and R. Burton. Biomed. Mass Spectr., 6, 179 (1979).

25. N.C. Jain, D.M. Chinn, T.C. Sneath, and R.D. Budd. J. Anal. Toxicol., 1, 192 (1977).

26. R.K. Lynn, R.M. Leger, W.P. Gordon, G.D. Olsen, and N. Gerber. J. Chromatogr., 131, 329 (1977).

Chapter 6

Δ^9-Tetrahydrocannabinol (THC) and Two of its Metabolites, 11-Hydroxy-Δ^9-THC and 11-nor-9-Carboxy-Δ^9-THC

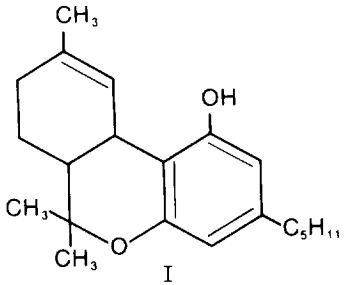
Use of the resin of the hemp plant, Cannabis sativa, for healing and euphoriant purposes is as old as recorded history. From written references to its use as a surgical anesthetic in the Chinese pharmacopeia of the 15th century B.C. until modern times, the history and lore of cannabis have covered a wide range of medical applications, some without value, as in leprosy, and others apparently justified (1). Its use as a hallucinogen and euphoriant has had a colorful history as well; the term "assassin" was brought to Europe by returning crusaders in reference to an 11th century Arabic religious sect whose devotees (hashishin) allegedly used the drug to induce ecstatic visions of paradise before setting out on potentially suicidal missions of political murder. Known in various parts of the world as "ganja," "bhang," "kif," "hashish," or "marijuana," cannabis in its crude form is eaten or smoked. The potency of the preparations varies widely according to botanical strain and the parts of the plant used (2). Cannabis retains its biological activity for long periods when stored in ethanol or in sesame oil, but the activity is reduced by exposure to light and oxygen, which helps account for variations in results of early studies concerning its pharmacology and psychotomimetic effects (3,4).

PHARMACOLOGY

The systematic study of the pharmacology of cannabis has evolved only in the past 12 years, following the total synthesis of $\Delta^{1,9}$-tetrahydrocannabinol (THC*, I) the principal active component of the plant material (5). Recent pharmacological research has shown the drug to have effects that are potentially therapeutic, such as reduction of intraocular pressure, bronchodilation, anticonvulsant action, and retardation of tumor growth (6,7,8). However, the psychotomimetic effects of cannabis, while having some potential for therapeutic use in themselves (analgesia, antidepressant action, etc.), have led to widespread abuse with societal and public health implications. For example, the contribution of cannabis intoxication to traffic accidents is the subject of intensive study (9), because

*In this chapter the term "cannabis" will be used to refer to plant preparations containing a mixture of cannabinoids, while "THC" will represent isolated or synthetic Δ^9-tetrahydrocannabinol, even though at least one other active form, Δ^8-THC, is present in small amounts in some extracts.

the principal subjective effects of THC are distortions of perception and time, and impairment of short term memory. THC acts on the septal area of the limbic system of the brain, where structures controlling emotional behavior are located (10).



I

The interaction of THC with other drugs is also of practical interest. THC attenuates the morphine-abstinence syndrome precipitated by naloxone, but exacerbates alcohol withdrawal symptoms, and it prolongs ether anesthesia. Various cannabis components are known to inhibit liver enzymes, which prevents detoxification of other drugs (3).

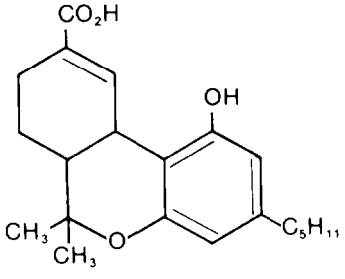
Toxicological studies have indicated carcinogenic potential in smoked cannabis; smoke condensates contain considerably higher amounts of polynuclear hydrocarbons than tobacco smoke (11). Cardiovascular effects of THC include hypotension and tachycardia, and reduction of blood flow to certain areas of the brain has been shown in test animals. Tolerance to the cardiac and blood pressure effects as well as to the psychotropic effects can develop (12). No irreversible pathological changes have been shown to result from THC administration; however, there are reports which suggest possible effects of THC on immune responses, endocrinological function, and cell metabolism. These may have important clinical implications, especially in view of the reported persistence of THC in the body in the form of lipid conjugates (13). Because of its high lipid solubility, THC is slowly eliminated from the body, and it accumulates in tissues. It has been reported that even minute amounts of THC disrupt cell metabolism, preventing formation of DNA, RNA, and protein and retarding cell division. There also appears to be an increased incidence of abnormal forms of spennatoza and a reduced sperm count in male cannabis users (10). The chemistry and pharmacology of THC have been the subjects of extensive recent reviews (14,15,16).

PHARMACOKINETICS AND METABOLISM

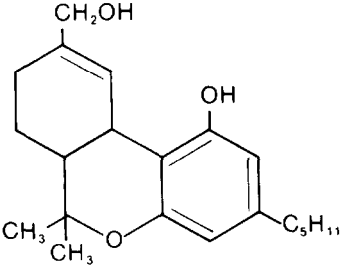
THC has low solubility in water and is poorly absorbed through the intestinal tract. Inhalation or intravenous administration, however, causes a rapid rise in plasma THC concentrations followed by a gradual decline according to a biphasic elimination pattern (17). The physiological "high" corresponds to the time of maximum THC concentration in the blood.

Cannabinoids are readily metabolized in mammals; over 50 cannabinoid metabolites have been identified, with variations among species,

There is some evidence for differential metabolism between lung and liver within the same species (3). Numerous biotransformation pathways for THC are known, including allylic and aliphatic hydroxylations; oxidations to acids, aldehydes, and ketones; conjugations with glucuronic acid or fatty acids; epoxidation of the double bond; and reduction of the terpene double bond. These biotransformations can occur singly to produce monosubstituted metabolites, or in combination to form diols, triols, hydroxy acids, hydroxy ketones, or substituted hexahydrocannabinols (18). In man, the conversion of THC to biologically inactive 11-nor-9-carboxy-Δ^9-THC (II) is a major metabolic pathway. The main initial biotransformation product of THC is its 11-hydroxy derivative (III), which is equally and perhaps more active than THC, but like its precursor it is short-lived in the body and rarely appears in urine. Recent experiments with radiolabeled THC in rats have demonstrated that III penetrates the blood-brain barrier more readily than does THC, possibly because of its high affinity for plasma albumin rather than the lipoprotein to which THC is bound (19).



II



III

Very little unchanged THC is found in urine. One study has reported that 0.005 percent of the administered drug was detectable in the urine of four human subjects up to 6 hours after a 30-mg dose. Excretion of metabolites extended over a period of several days (20) Although the metabolic breakdown of THC is very rapid, the drug can be detected in plasma for at least 72 hr after a single intravenous dose, possibly the consequence of a slowly releasing deep compartment. THC is highly bound to lipoprotein, 97 percent by one method of estimation, and has a reported pka' of 10.6 (21). An effective oral dose of THC in humans is about 50-200 μg/kg body weight, and the effective dose when smoked is 25-50 μg/kg (22).

ANALYTICAL METHODOLOGY

Because there is great interest in the metabolism and pharmacology of THC, and because of the forensic implications of its illicit use, numerous methods have been developed for its detection and quantitation; however, its low concentration in biological fluids

and the potential interference from endogenous lipids have created difficult analytical problems. Fluorometric techniques, based on gallium chelate formation or on cannabinoid dansylation, while only qualitative, have been suitable for some applications such as detection of THC in the saliva of marijuana smokers (23-27). A quantitative fluorometric technique has been reported very recently, in which a known amount of tritiated THC is added to plasma as an internal standard prior to extraction with methyl acetate:petroleum ether:ethanol (66: 33:1.5 v/v, efficiency 70 percent). THC and other phenols are selectively removed from the organic extract by extraction with Claisen's alkali reagent. The alkaline solution is then acidified and reextracted with hexane. The THC is derivatized by treatment with ^{14}C-labeled dansyl chloride before purification by thin-layer chromatography (TLC) on silanized silica gel plates (cyclohexane:ethyl acetate, 95:5 v/v). The dansylated THC spot is located with UV radiation and eluted into a scintillation vial for counting of ^{3}H and ^{14}C. The detection level for this method was found to be 2.5 ng/tube, implying a sensitivity of a few ng/ml of plasma (28).

For periods up to 2 hours after smoking, the saliva of cannabis users was reported to contain concentrations of THC sufficient for detection by two-dimensional TLC with mass spectrometric confirmation. In a study which compared plasma and saliva concentrations of THC in the same subjects, the drug was detected in saliva less than 30 min after smoking and continued to be detectable in saliva even after it could no longer be detected in plasma (29).

A new derivatizing reagent (2-p-chlorosulfophenyl-3-phenylindone) which is reported to be more selective than dansyl chloride has been used in combination with TLC to detect THC and several of its metabolites at serum concentrations as low as 0.2 ng/ml (30). However, the fluorescent THC derivative is unstable and must be measured within a few minutes after it is formed on the TLC plate.

Some of the first methods employed for THC quantitation in pharmacological and metabolic studies involved radiolabeled tracers administered to volunteers. Even minute amounts of cannabinoid products of the metabolism of radiolabeled THC were detectable by scintillation counting following TLC. However, the administration of radioactive substances to human subjects has potential health hazards which limit the use of the technique (31).

Radioimmunoassays (RIA) have been developed for THC. They have the advantages of comparative speed and convenience for clinical uses. This is because they are suited to the simultaneous analysis of multiple samples, often without prior purification. Under favorable conditions, sensitivities of about 2 ng/ml can be achieved. However, because of the variables inherent in the preparation of reagents by serological procedures, RIA lacks the precision of other methods. In attempts to maximize specificity and affinity of the antibodies, various derivatives of THC have been used as haptens to be conjugated to carrier protein. These have included the hemisuccinate ester (32), an azobenzoic acid derivative (33),

an N-oxysuccinimidoyl ester (34), a 10-iodo-9-uriedo linked THC (35), and an 0-carboxymethyl derivative (36). Although the antibodies elicited by these conjugates are highly specific for the 3-ring cannabinoid nucleus, cross-reactions with other cannabinoids, both natural and metabolic, have not been eliminated. This can be an advantage in the case of urine studies, where unmetabolized THC is virtually absent. The high degree of nonspecific binding of THC to lipoprotein is a technical problem in RIA, but 0.1 percent Triton X-405 has been found to effectively solubilize THC in the aqueous assay medium, and an ethanol extraction step has been shown to permit > 92 percent recovery of THC from plasma while removing protein material by precipitation (32). Other RIA's have been developed for the measurement of THC and the major metabolite II (Carboxy-THC) and used to determine the relative THC and Carboxy-THC concentrations in urine and plasma of cannabis smokers (37).

In order to improve the specificity of an RIA method, high performance liquid chromatography (HPLC) has been used to separate THC from other cannabinoids that may be present in a plasma sample (38). The HPLC-RIA method is reported to measure THC plasma concentrations as low as 0.1 ng/ml.

Another immunological technique, the EMIT assay (based on the inhibition of enzymes by antihapten antibodies when the enzymes are covalently linked to the corresponding hapten) has been adapted for THC and Carboxy-THC in urine, using relate dehydrogenase. The hapten synthesized for use in the EMIT system elicited an antibody that reacted strongly with Carboxy-THC, unlike antibodies previously produced with other THC haptens. The antisera were therefore suitable for general screening for THC and its metabolites (39). The recommended cut-off for reporting detection of the cannabinoids is 20 ng/ml.

Another approach involves the use of gas chromatography (GC) to separate THC from other components in plasma. The normal sensitivity of GC using flame ionization detectors (FID) is too low to be of use for in vivo plasma analysis. However, electron capture detectors (ECD) the necessary sensitivity provided the THC is converted to a suitable derivative. The pentafluoropropionate derivative is satisfactory for this purpose; it allows detection at concentrations as low as 2 ng/ml. However, the derivatizing reactions are relatively unspecific; therefore biochemical interferences compromise selectivity. A tandem-column GC technique can be used to achieve greater specificity; for example, THC was detected in blood as the heptafluorobutyrate on a tandem column GC system using hexahydrocannabinol as the internal standard. The first column (5 percent SE-30) provided internal cleanup and the second, a capillary column coated with OV-17, achieved the required resolution. The technique had the advantage of allowing temperature programming in the first column which increased speed and efficiency but did not disturb the base-line response of the electron capture detector. The sensitivity reported for nonbiological samples was 1 pg injected on column (40).

In addition to improved detector response, derivatization of THC can also improve chromatographic behavior by blocking the polar phenolic group. THC derivatives which have been prepared for this purpose include the trimethylsilyl, trifluoroacetyl, acetyl, chloroacetyl , and t-butyldimethylsilyl derivatives. The last named has been shown to exhibit a high degree of stability to moisture and bases (41).

Cannabinoids have also been converted to their diethyl phosphate esters and analyzed by gas chromatography using a nitrogen-phosphorus detector (GC-NPD). The developers of this assay recommend it for routine quantitative analysis of cannabinoids in all toxicological laboratories (42). The procedure utilizes the n-heptyl homolog of Δ^{8}-THC as the internal standard and is capable of detection limits of 0.5 ng/ml in blood and 10 ng/g in brain tissue. For analysis of brain tissue a back extraction into methanolic base serves to separate the cannabinoids from interfering endogenous lipids.

HPLC has been used for separation of THC from other cannabinoids and lipids in biological fluids before analysis by GC-ECD. Reverse phase HPLC elution with 47 percent acetonitrile in water, as well as normal phase HPLC with 25 percent chloroform in heptane, was found to separate THC from its active 11-hydroxy metabolite (III). The resolution of Δ^{8}- and Δ^{9}-THC was achieved with 5 percent tetrahydrofuran in hexane (43). The GC-ECD analysis of pentafluorobenzylated Δ^{9}-THC using HPLC purification had a sensitivity of 1 ng/ml of plasma, while radiochemical analysis of the HPLC separated fraction had a sensitivity of 0.2 ng/ml plasma. For quantitation of THC in plasma in phannacokinetic studies, a known amount of radiolabeled THC was added as an internal standard either to the plasma or to the heptane extract. This permitted calculation of extraction and/or HPLC collection efficiencies for the particular biological sample (44).

The technique of plasma chromatography has been applied to the detection of ultratrace amounts of THC in GC effluents. The positive mobility spectrum for THC shows a strong, single peak with a K_o = 1.06. The peak is attributed to the protonated molecule ion of THC (45).

GC/MS analysis using the technique of selected ion monitoring currently is the most reliable method for measuring the very low concentrations of THC in plasma after smoking. The first GC/MS method for quantitation of THC in blood was reported in 1973 (46). The method involved addition of a deuterated THC analog as an internal standard and purification of the extract by liquid chromatography on a Sephadex LH-20 column. The original procedure was later refined to the point that plasma concentrations as low as 0.3 ng/ml could be measured. Sensitivity was found to be limited by a small amount of undeuterated THC contaminating the internal standard, so the best results have been achieved with a $^{2}H_{7}$-analog (47).

Because of the very small amounts of THC and 11-hydroxy-THC which appear in human plasma and urine, the major metabolite (Carboxy-THC) is a candidate for analytical studies as a method to identify cannabis users. The concentration of Carboxy-THC in plasma exceeds that of THC within 50 min after smoking, and then remains higher for more than 24 hr. A modification of the above GC/MS assay for THC has been applied to identification and estimation of the carboxy metabolite. Extraction from plasma or urine was with diethyl ether (85 percent recovery from plasma, 90 percent from urine), followed by a two-step derivatization which involves esterification of the carboxyl group with diazomethane and trimethylsilation of the phenolic hydroxyl group. The silylated methyl ester of the Δ^8-isomer of the Carboxy-THC was used as an external standard because both isomers have similar retention times but different ion intensities in their mass spectra. In this preliminary study, a sensitivity in the range of a few ng/ml was indicated (48).

A GC/MS assay for THC in the blood of marijuana smokers has been reported which is based on the chemistry of the phenolic group common to all cannabinoids and their metabolites; the basis of the purification technique is the selective extractability of lipid-soluble phenols from hexane by Claisen's alkali. After the back extraction into the aqueous caustic, trimethylanilinium hydroxide was used for on-column methylation of the phenolic group. The trideuteromethyl ether of THC was easily synthesized for use as an internal standard, but the fact that it was, of necessity, added only after the extraction constitutes a disadvantage of the method as a quantitative technique (49).

The Claisen's alkali extraction procedure was also used in an analysis of the active metabolite, 11-hydroxy-THC, in plasma (50). In this case, tetrahexylammonium hydroxide and ethyl iodide were used to alkylate the phenolic group of the metabolite. Subsequent trimethylsilylation of the aliphatic hydroxyl group provided a derivative with satisfactory chromatographic and mass spectral properties. When the corresponding pentadeuteroethyl ether was used as the internal standard, a linear calibration graph was achieved over the range 7 to 120 ng/ml of plasma. The procedure can be used for simultaneous determination of both THC and the 11-hydroxy metabolite in plasma (51).

GC/MS was adapted to an automated system using a computer-managed Olfax II spectrometer and a "probability based matching" algorithm, to measure THC as its trimethylsilyl derivative in extracts of hydrolyzed human urine, and to identify and measure metabolites. The procedure showed promise as a means of increasing the speed and convenience of THC assays while reportedly retaining the specificity of selective ion monitoring (52).

In a pharmacokinetic study of THC and 11-hydroxy-THC in human plasma, the results of GC/MS (both electron impact and chemical ionization) were compared with those of GC-ECD and TLC-radiolabel procedures on the same samples. In the CI procedure THC was derivatized with pentafluoropropionic anhydride, and the pentafluoro-

propionate ester of hexahydrocannabinol was used as the internal standard. Endogenous plasma constituents did not interfere, so preliminary chromatographic purification was not required. In the EI procedure Sephadex LH-20 chromatography was used before GC/MS, but THC was not derivatized. Pharmacokinetic data for human volunteer subjects were in reasonable agreement for the three techniques (53).

Further refinements of the GC/EI-MS technique for THC have included use of THC-$^2H_3$ as internal standard and extraction of THC from plasma with pure hexane to avoid the need for preliminary chromatographic cleanup. The more polar cannabinoids remain with the plasma; therefore hexane extraction would not be suitable when determination of metabolites is necessary. Both the CI and EI methods had detection limits of about 0.5 ng/ml in clinical samples, although the EI mode (focusing exclusively on m/z 314 and 317 and requiring no derivatization step) was reported to have a better defined linearity on calibration curves than the methane CI method (54).

The combination of HPLC with MS has also been reported for analysis of THC in body fluids. A silica gel column was programmed for gradient elution using heptane and methylene chloride. Fractions of the eluent were collected and introduced by direct probe into the mass spectrometer. THC- $^2H_3$ was added to the body fluid and served as an internal standard, a marker for effluent collection from the HPLC (detected by an ultraviolet spectrophotometer) , and as a carrier for the THC. The technique was used to assay THC concentrations in human blood, breath, saliva, bile, and postmortem brain tissue. It was shown to be reproducible, accurate, and linear in the range of 2.5-100 ng/ml of plasma. A positive, statistically significant concentration of THC was detected in the saliva of marijuana smokers in the first hour after smoking (55).

A paper chromatographic system which employs methanol as the mobile phase conveniently separates cannabinoids from the large amounts of endogenous lipid material present in chloroform extracts of blood samples. The region of the paper near the solvent front is eluted with methylene chloride and the cannabinoids are identified by GC/MS. If 15 to 25 ml of whole blood are available, concentrations of THC as low as 0.5 ng/ml can be detected. For quantitative analysis 2,3,4,6-tetrachlorophenyl hexyl ether is suggested as an internal standard. Under the GC/MS conditions used (3 percent OV-1, EI) the tetrachloro ether has a retention time of 0.45 relative to THC and produces fragment ions at the same nominal masses as several of THC's prominent fragment ions. The same report points out the potential for pyrolytic conversion of THC to cannabinol in GC and GC/MS analyses. Thermal decomposition of THC is minimized by keeping all GC and MS temperatures to a minimum consistent with satisfactory chromatography (56).

Solvents used for extraction of THC from biological samples include hexane at neutral pH (52, 53); hexane + 5 percent isoamyl alcohol (49); petroleum ether, sometimes containing 0.5 percent

isoamyl ether or 1.5 percent isopentanol (47,53,55); heptane containing 1.5 percent isoamyl alcohol (21,40,57); and petroleum ether: Ethyl acetate (2:1) (29). Recoveries in excess of 90 percent from plasma have been reported for heptane:isoamyl alcohol (40); chloroform extraction of whole blood gave a THC recovery of 86 percent (56).

The proceedings of a symposium on the analysis of cannabinoids in physiological fluids held in 1977 have been published and provide a good review of recent research in this area (58). Also, the National Institute on Drug Abuse has sponsored two conferences on the analysis of cannabinoids in physiological specimens. The proceedings of the first conference, held in 1976, were published as a NIDA Research Monograph (59). The proceedings of the second conference, held in January, 1980, will be published later in the year (60).

EXPERIMENTAL PROCEDURE

Two methods are described, one for analysis of THC in whole blood or plasma and another for analysis of the metabolites, 11-hydroxy-THC and 9-carboxy-THC, in blood, plasma and urine. The first method involves extraction of the body fluid with hexane, a nonpolar, relatively selective extraction solvent. The very lipophilic THC is efficiently extracted by hexane, but the polar metabolites are poorly extracted. The second method employs a more polar extraction solvent (hexane:ethyl acetate, 7:1 v/v) which removes the metabolites from the body fluid, but unfortunately also extracts endogenous components which interfere with measurement of low concentrations (< 10 ng/ml) of THC. A glass capillary GC column has been used to resolve trimethylsilylated THC from endogenous components (61), but efforts to resolve the interferences using packed GC columns have so far been unsuccessful. This is the reason why two similar but separate procedures are used for quantitation of THC and the two metabolites.

Standards and Reagents

All of the cannabinoid standards used in the development of the method described here were obtained from the Research Triangle Institute, Research Triangle, NC, 27709, through the National Institute on Drug Abuse. Purity and isotopic composition data are given below:

Δ^{9}-Tetrahydrocannabinol (THC)
Purity: 98.7 percent by GC

5'-$^{2}H_3$-Δ^{9}-Tetrahydrocannabinol (THC-$^{2}H_3$)
Purity: > 95 percent by GC, 100 percent by TLC
Isotopic composition: $^{2}H_3$, 96.6 percent; $^{2}H_2$, 2.6 percent; $^{2}H_1$, 0.77 percent; $^{2}H_0$, 0 percent.

11-Hydroxy-Δ^{9}-tetrahydrocannabinol (11-OH-THC)
Purity: >98 percent by GC as the bis-(trimethylsilyl) ether, 100 percent by TLC

5'-$^2H_3$-11-Hydroxy- Δ^9-tetrahydrocannabinol (11-OH-THC-$^2H_3$)
Purity: >95 percent by GC as the bis-(trimethylsilyl) ether, 100 percent by TLC
Isotopic composition: $^2H_3$ 95.8 percent; $^2H_2$, 2.4 percent; $^2H_1$ 1.1 percent; $^2H_0$, 0 percent

11-Nor-9-car-boxy- Δ^9-tetrahydrocannabinol (9-Carboxy-THC)
Purity: 97 percent by GC as the bis-(trimethylsilyl) ether, 100 percent by TLC

5'-$^2H_3$-11-Nor-9-carboxy- Δ^9-tetrahydrocannabinol (9-Carboxy-THC-$^2H_3$)
Purity: 100 percent by GC as the bis-(trimethylsilyl) derivative, 100 percent by TLC
Isotopic composition: $^2H_3$, 95.8 percent; $^2H_2$, 2.4 percent; $^2H_1$, 1.1 percent; $^2H_0$, 0 percent.

The pH 2.0 and pH 7.0 buffer solutions were purchased from Fischer Scientific Company.

N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1 percent trimethylsilyl chloride was purchased from the Pierce Chemical Company, Rockford, IL, 61105. The reagent was stored at -10°C, but allowed to warm to room temperature before aliquots were removed for derivatization.

Pure Δ^9-THC is a colorless, viscous syrup. It is therefore difficult to transfer and weigh small quantities accurately. The Δ^9-THC standards described above were obtained as ethanolic solutions of predetermined concentrations, and were subsequently diluted with methanol to give stock solutions with THC concentrations of 0.10 mg/ml. Of course, the concentrations do not have to be precisely 0.10 mg/ml, but they must be accurately known. A THC stock solution concentration of 0.10 mg/ml is used in this experimental description merely as an example.

The 11-OH-THC and 9-carboxy-THC are solids at room temperature. Because these two metabolites are measured at the same time in biological specimens, a single stock solution containing 0.10 mg/ml of each of the metabolites in methanol can be prepared, as well as a single stock solution containing 0.10 mg/ml of each of the deuterated analogs (11-OH-THC-$^2H_3$ and 9-car-boxy-THC-$^2H_3$) in methanol. These can be prepared by weighing into a 100-ml volumetric flask 10 mg of 11-OH-THC and 10 mg of 9-carboxy-THC, and weighing into a second 100-ml volumetric flask 10 mg of each of the deuterated analogs. Add about 75 ml of methanol to each flask. After the solids have dissolved add additional methanol to the flasks to bring each solution to exactly 100 ml. A series of working standard solutions of the undeuterated cannabinoids can be prepared by appropriate dilution of the stock solutions as described in Chapter 2.

For the measurement of THC or 11-OH-THC and 9-carboxy-THC in body fluids within the concentration range 1 to 500 ng/ml, addition of approximately 40 ng/ml of each of the appropriate deuterated in-

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ternal standards to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml methanolic solution of THC-$^{2}H_{3}$ or 11-CM-THC-$^{2}H_{3}$ and 9-carboxy-THC-$^{2}H_{3}$ to each ml of body fluid. Prepare the 400-ng/ml internal standard solutions by diluting 1 ml of the 0.10-mg/ml stock solutions to 250 ml with methanol.

Cannabinoids, particularly THC, are unstable when exposed to light (4,62), oxygen (4), acid (21), and/or elevated temperatures (62). Consequently, store stock and standard solutions in full, stoppered or capped glass vessels in the dark at below 0°C. The concentrations of the stock solutions should be checked at least every three months. This can be done by addition of a known quantity of an internal standard such as Δ^{4}-androsten-3,17-dione to an aliquot of the stock solution, and then analyzing the solution by GC with a flame ionization detector. If the concentration of the cannabinoid solution has changed by a detectable amount, a new calibration graph should be established for that stock solution.

THC has a strong tendency to adhere to glass surfaces (21). This adsorption can cause poor sensitivity and precision, but can be minimized by using clean, silanized glassware for the sample extraction (63).

Extraction of THC from Plasma or Whole Blood

Transfer a measured quantity (normally 1 ml) of plasma or whole blood to a 20-ml culture tube equipped with a Teflon-lined screw cap. Add 100 µl of a 400-ng/ml methanol solution of the THC-$^{2}H_{3}$ internal standard and vortex for 10 sec. Allow the sample to equilibrate for 15 min. Add 1 ml of pH 7.0 buffer solution and 6 ml of hexane. Cap the tube tightly and gently mix the contents for 30 min using an automated tube rocker or rotator. Centrifuge for 5 min and then carefully transfer just the hexane (top) layer to a second culture tube using a disposable Pasteur pipette. The remaining plasma residue can be discarded. The hexane solution is now washed successively with dilute alkali and dilute acid to remove strongly acidic and basic compounds. Add 2.5 ml of 0.1 N NaOH solution to the hexane extract. After capping the tube, rotate or rock for 30 min and then centrifuge. Using another disposable pipette, remove the aqueous (bottom) layer and discard. Add 2.5 ml of 0.1 N HCl solution to the hexane extract. Tap the tube gently to allow gas bubbles formed by the acid-base reaction to escape. Again, cap the tube tightly, rock or rotate it for 30 min, and centrifuge. Carefully transfer just the hexane (top) layer to a 15-ml glass tube with a tapered or cone-shaped bottom. Concentrate the extract to near dryness at a temperature of 35-40°C under a gentle stream of nitrogen. The extract residue is now transferred to a conical 1-ml glass vial equipped with a Teflon-lined screw cap in the following manner. Add 0.5 ml of hexane to the tube containing the residue and vortex the tube for 10 sec. Using a disposable pipette, transfer the hexane solution to the 1-ml glass vial. Repeat the last step using an additional 0.5 ml of hexane to transfer any remaining extract residue to the 1-ml

vial. Add 10 µ1 of dimethylformamide to the hexane solution to act as a "keeper" to minimize evaporative loss of the THC during the final evaporation and to serve as a solvent for the trimethylsilylation. Slowly concentrate the hexane solution by heating the glass vial in a heating block maintained at 40°C. When all of the hexane has evaporated (approximately 20 min), add 15 µl of bis-(trimethylsilyl) trifluoroacetamide containing 1 percent trimethylsilyl chloride to the vial and cap tightly. Vortex the vial and then heat in an oven at 75°C for 1 hr. The sample. is now ready for GC/MS analysis, but it can be stored at -10°C indefinitely.

GC/MS Analysis of THC-TMS

The experimental conditions for the GC/MS analysis of THC are as follows:

GC column:	1.8 m x 2 mm I.D. glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Reagent gas:	Ammonia introduced into the ion chamber as described in Chapter 2
Temperatures:	Injector, 280°C Column, 190 to 310°C at 20 °/min GC/MS transfer line, 280°C Ion source, 160°C

Under these conditions the THC-TMS should elute from the gas chromatograph at between 2 and 5 min.

Prior to beginning the GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored are the protonated molecule ions for THC-TMS (m/z 387) and THC-$^2H_3$-TMS (m/z 390). With the divert valve in the divert position, inject the organic extract into the gas chromatograph (2 to 8 µ1). After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the THC-TMS peaks have eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of THC in the biological specimen sample is determined by measuring the heights (or areas) of the THC-TMS and THC-$^2H_3$-TMS peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the body fluid concentration of THC by dividing the measured quantity of THC by the exact volume of specimen analyzed.

Extraction of Metabolites from Blood, Plasma, or Urine

To 1 ml of specimen (blood, plasma, or urine) in a 20-ml culture tube equipped with a Teflon-lined screw cap, add 100 µl of a methanol solution containing 40 ng each of the internal standards, 11-OH-THC-$^2H_3$ and 9-carboxy-THC-$^2H_3$ (concentration, 400 ng/ml). Vortex the tube for 10 sec and allow the sample to equilibrate for 15 min. Add 1 ml of pH 2 buffer and 4 ml of a hexane:ethyl acetate solvent (7:1 v/v). Screw the cap on the tube tightly and check for leaks. Slowly rotate or rock the tube for 15 min. Loosen the cap and then centrifuge. Using a disposable Pasteur pipette, transfer the organic layer (top) to a second culture tube and discard the aqueous plasma layer. Add 2 ml of 0.2 N H_2SO_4 to the organic extract. Screw on the cap, tighten, and again check for leaks. Repeat the rocking (15 min) and centrifugation (5 min). Transfer the organic layer (top) to a centrifuge tube, at least 10 ml in volume. Be careful not to transfer any of the aqueous (lower) layer to the centrifuge tube. Discard the aqueous layer. Concentrate the extract to near dryness by heating to 35-40°C under a gentle stream of nitrogen or filtered air. Wash down the sides of the tube with 0.5 ml of methylene chloride. Vortex the tube and transfer the extract to a 1-ml glass vial which has a cone-shaped bottom and a Teflon-lined screw cap. Again wash down the sides of the centrifuge tube with another 0.5 ml of methylene chloride and transfer the solvent wash to the same 1-ml glass vial. Add 10 µl of dimethylformamide to the hexane solution to act as a "keeper" to minimize evaporative loss of the metabolites during the final evaporation and to serve as a solvent for the trimethylsilylation. Evaporate the combined solvents by heating to 30-40°C under a gentle stream of nitrogen or filtered air until all of the methylene chloride is gone (about 20 min). Add 50 µl of bis-(trimethylsilyl) trifluoroacetamide containing 1 percent trimethylsilyl chloride to the vial; cap tightly and heat at 90°C for 1 hr. The sample is now ready for GC/MS analysis. If necessary it can be stored in a freezer until it is to be analyzed; however, be sure to allow the vial to warm to room temperature before removing the cap to withdraw material for injection into the GC/MS. The trimethylsilylated 9-carboxy-THC is particularly vulnerable to hydrolysis. Consequently, exposure of the derivatized extract to air should be kept to a minimum, and prolonged storage should be avoided.

GC/MS Analysis of the 11-OH-THC-TMS and 9-Carboxy-THC-TMS

The experimental conditions for GC/MS analysis of the derivatized THC metabolites are as follows:

GC column:	1.8 m x 2 mm I.D. glass column packed with 3 percent OV-17 or 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Reagent gas:	Ammonia, introduced into the ion chamber as described in Chapter 2

Temperatures: Injector, 280°C
Column, 190 to 310°C at 20°/min
GC/MS transfer line, 280°C
Ion source, 160°C

Under these conditions the 11-OH-THC-TMS should elute from the GC at about 3 min and the 9-carboxy-THC-TMS at about 4.5 min.

The ions to be monitored are the protonated molecule ions for 11-OH-THC-TMS (m/z 475), 11-OH-THC-$^2H_3$-TMS (m/z 478), 9-carboxy-THC-TMS (m/z 489), and 9-carboxy-THC-$^2H_3$-TMS (m/z 491). With the divert valve in the divert position inject 2 to 8 µl of the trimethylsilylated extract into the GC/MS. After approximately 1 min switch the divert valve so that the entire carrier gas flow enters the ion source. The best sensitivity is achieved by first monitoring just the ion currents at m/z 475 and 478 until the 11-OH-THC-TMS has eluted from the GC; then monitor the ion currents at m/z 489 and 491 until the 9-carboxy-THC-TMS has eluted. If the GC/MS data system does not permit rapid changes of the monitored ion masses, all four ion masses must be monitored for the entire time required for elution of both the metabolites. When the 9-carboxy metabolite has eluted, cease data acquisition and return the divert valve to the divert position.

The quantities of 11-hydroxy and 9-carboxy metabolites in the biological sample are determined by measuring the heights (or areas) of the derivatized metabolites and their internal standards in the selected ion current profiles, and relating the ratios of peak heights to calibration graphs (Chapter 2). Calculate the concentrations of the 11-hydroxy and 9-carboxy metabolites in the specimen by dividing the measured quantities of these compounds by the exact volume of specimen extracted.

Discussion of the Experimental Procedures

The method described above for quantitation of THC in blood and plasma was developed and refined over a period of four years. During this time over 2500 plasma samples, submitted by various cannabinoid researchers, were analyzed. One out of every six analytical samples corresponded to a control plasma to which a known amount of THC was added. The measured concentrations of THC in the spiked plasma samples are summarized in Table 1.

TABLE 1. ACCURACY DATA FOR MEASUREMENT OF THC ADDED TO PLASMA.

Prepared THC Concentration (ng/ml)	Mean Measured Concentration ± S.D. (ng/ml)	Number of Samples Analyzed
0.92	0.88 ± 0.39	15
1.84	1.73 ± 0.51	18
4.60	4.75 ± 0.89	24
9.20	9.32 ± 0.72	22
18.4	18.85 ± 0.45	22
46.0	47.21 ± 1.83	21
92.0	91.97 ± 1.92	23
184.0	176.71 ± 1.74	13

Figure 1 shows a representative calibration graph covering the concentration range 0.5 to 184 ng/ml. The correlation coefficient for the graph is 0.9999.

Most of the THC analyses done at Battelle between September 1977 and September 1979 were performed on a Finnigan 4000 GC/MS coupled to an INCOS data system. With the system operating normally, as little as 20 pg of derivatized THC injected into the GC/MS gave a response sufficient to permit measurement with good precision. Usually, only 10 to 20 percent of a derivatized extract was injected at one time. Consequently, for a 1-ml plasma sample, concentrations of THC as low as 0.2 ng/ml could be measured. One technician was able to extract and prepare a set of 36 plasma samples for analysis in a period of 8 hr. An additional 8 hr was required to perform the GC/MS analyses.

The advantages of using CI mass spectrometry with methane and ammonia as reagent gases are evident in Figure 2 and Table 2. Figure 2 compares the EI mass spectrum of the trimethylsilyl derivative of THC with the corresponding CI mass spectra using either methane or the methane-ammonia combination as reagent gas. In the methane-ammonia CI mass spectrum almost all of the THC ion current is concentrated at a single ion mass, corresponding to the protonated molecule ion (m/z 387). The sensitivities achieved by selected ion monitoring analysis of specific quantities of THC are compared in the fourth column in Table 2. Use of methane and am-

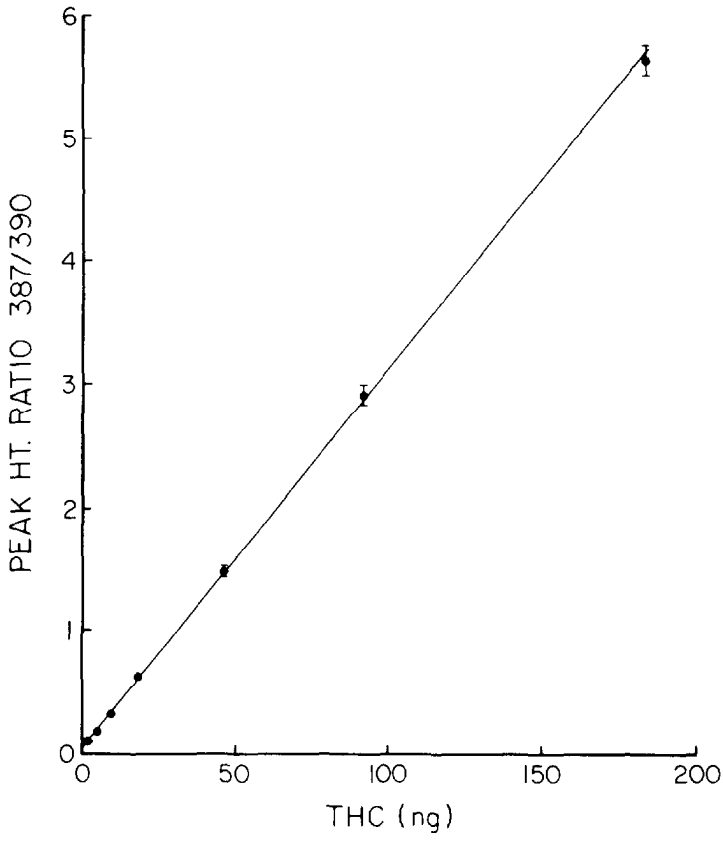


FIGURE 1. CALIBRATION GRAPH FOR ANALYSIS OF THC IN PLASMA

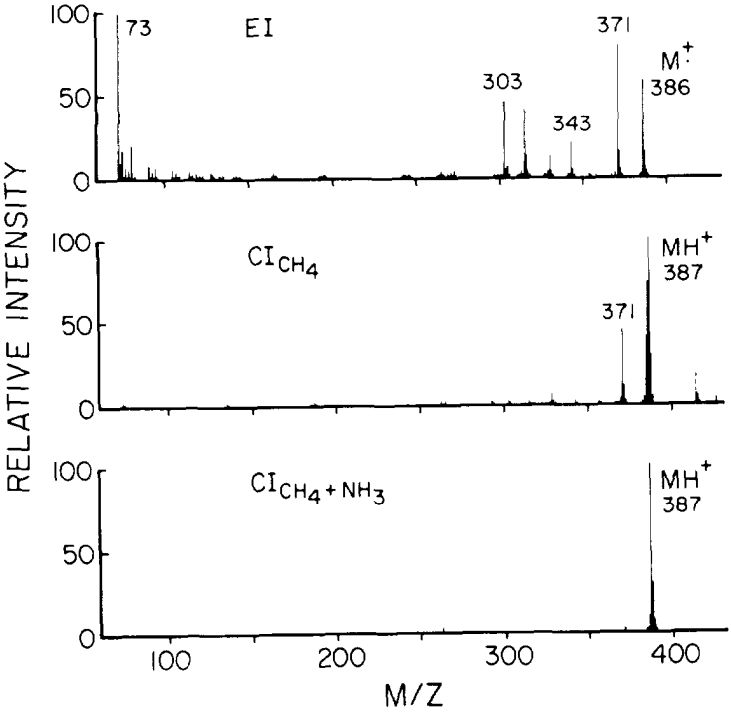


FIGURE 2. MASS SPECTRA OF TRIMETHYLSILYLATED THC

monia gives the largest signal per unit weight of THC. Furthermore, the virtual absence of fragment ions under methane-ammonia chemical ionization means that there is less chance of interference by ions from other components of the extract.

TABLE 2. RELATIVE RESPONSES FOR MAJOR IONS IN THE EI AND CI MASS SPECTRA OF THE TRIMETHYLSILYL ETHER OF THC

Ionization Method	m/z Monitored	Percent of Samples Ion Current	Relative Response Per Unit Weight of THC
EI	386 (M^+)	7	50
CI (CH_4)	387 (MH^+)	24	20
CI (CH_4 + NH_3)	387 (MH^+)	67	100

The efficient protonation of THC-TMS under methane-ammonia chemical ionization conditions was unexpected, since the THC molecule does not contain a basic nitrogen atom. However, apparently in the gas phase THC and its TMS derivative have high proton affinities. The same is true of the TMS derivatives of the two metabolites, as indicated by the abundant protonated molecule ions observed in their methane-ammonia CI mass spectra (Figure 3).

The efficiency of ionization and the selectivity of methane-ammonia chemical ionization are major reasons why plasma concentrations of THC as low as 0.5 ng/ml can be measured without employing, a more extensive extraction and sample purification procedure. However, it is necessary to heat the GC column to temperatures well above the elution temperature of THC-TMS in order to clear the column of endogenous compounds, such as cholesterol. If this is not done, interfering peaks will appear in the ion current profiles generated by subsequent injections. Maintenance of the column at 280-300°C overnight with normal carrier gas flow will also help to retain good column performance.

If the procedures outlined above are followed, a GC column should give satisfactory performance for at least several hundred analyses. However, the performance of the column should be evaluated each day before the analysis of specimens is begun. The resolution of the GC column can be evaluated by injecting a solution which contains an equal mixture of Δ^8-THC-TMS and Δ^9-THC-TMS (~50 µg/ml in hexane). Under the stated GC conditions a good quality 1.8-m x 2-mm packed OV-17 column should give a chromatogram showing at least a detectable valley between the peaks corresponding to the two cannabinoid derivatives. The sensitivity of the GC/MS can be evaluated by injecting 1 µl of a standard solution contain-

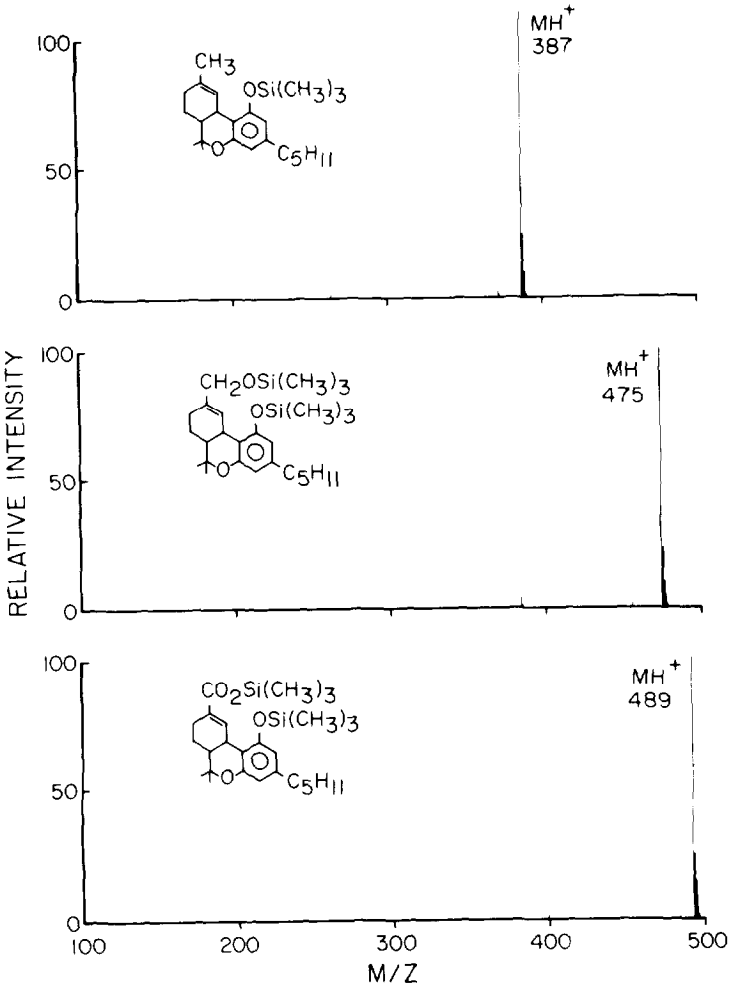


FIGURE 3. METHANE-AMMONIA CI MASS SPECTRA OF TRIMETHYLSILYL DERIVATIVES OF THC (TOP), 11-OH-THC (MIDDLE), AND 9-CARBOXY-THC (BOTTOM)

ing 1 ng of THC-TMS. The peak in the m/z 387 ion current profile which corresponds to the THC-TMS should be symmetrical and have a signal-to-noise ratio of greater than 50:1.

The procedure described above for analysis of the THC metabolites has not been used as extensively as the THC method, and therefore it is not as thoroughly validated. A similar procedure, in which the 9-carboxy-THC was first converted to its methyl ester by treatment with ethereal diazomethane and then trimethylsilylated, was employed at Battelle for the analysis of approximately 300 plasma samples. Figure 4 shows representative calibration graphs acquired at the time these assays were conducted. Table 3 lists the assay precision for the analysis of plasma samples to which a known concentration of each of the metabolites was added.

TABLE 3. PRECISION DATA FOR MEASUREMENT OF THC METABOLITES ADDED TO PLASMA

Metabolite Concentration (ng/ml)	11-OH-THC RSD (%)	9-Carboxy-THC RSD (%)
1	7	31
5	4	7
10	2	2
50	1	3
100	3	2

It has been shown recently that up to 80 percent of the 9-carboxy-THC in urine from marijuana smokers is present in the form of a glucuronide conjugate (64). The glucuronic acid residue appears to be attached to the metabolite by an ester linkage with the 9-carboxyl group of the metabolite. The conjugate is not cleaved by conventional treatment with ß-glucuronidase and sulphatase, but can be cleaved by alkaline hydrolysis (65). The recommended conditions for hydrolysis are to add the biological specimen to an equal volume of 1 M sodium hydroxide in methanol and heat on a steam bath (100°C) for 12 minutes. Acidify the mixture to pH 2 by addition of 1 ml of 1 N sulfuric acid and 1 ml of pH 2 buffer. The total 9-carboxy-THC concentration can then be determined by extraction with hexane:ethyl acetate, derivatization, and GC/MS analysis as previously described.

The analysis of THC in decomposed blood or tissues presents special problems. The main difficulty is that the efficiency of extraction of THC from decomposed blood with hexane is poor and erratic. Even deuterated THC added to decomposed blood as an inter-

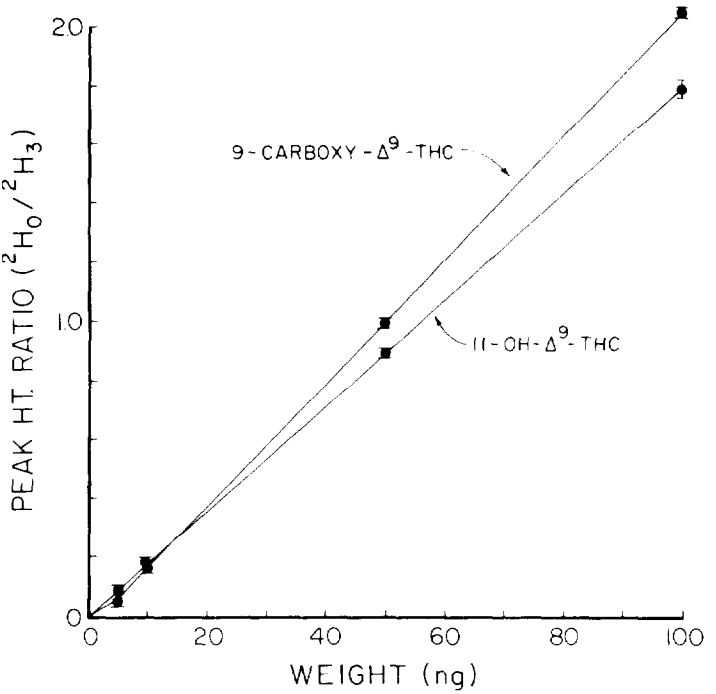


FIGURE 4. CALIBRATION GRAPH FOR ANALYSIS OF 11-OH-THC AND 9-CARBOXY-THC AS THEIR TRIMETHYLSILYL DERIVATIVES

nal standard is difficult to extract efficiently with hexane. Apparently the THC binds to biochemical components in the decomposed blood in a manner that inhibits its removal by conventional extraction procedures. A procedure was recently reported for the quantitative determination of THC in cadaver blood (66). The method involves extraction of 3 ml of blood with a total of 50 ml of acetone, evaporation of the acetone, extraction of the residue with petroleum ether, and then chromatography on Sephadex LH-20 followed by GC/MS analysis. A simpler procedure was developed at Battelle for extraction of THC from hemolyzed and decomposed blood (67); it gave acceptable quantitative results for concentrations of THC down to 1 ng/ml for most batches of hemolyzed blood. However, occasionally large variations in extraction efficiency did occur, indicating that the method needs further refinement. The procedure consists of addition of the internal standard to 1 ml of hemolyzed blood followed by 1 ml of 3 N H_2SO_4 and 1 ml of acetonitrile. The mixture is sonicated and the resulting protein precipitate removed after centrifugation. The supernatant is then neutralized with 3 N NaOH, and 1 ml of pH 7.0 buffer is added. The aqueous solution is extracted twice with 6 ml of hexane. The remaining steps, which include successive washes of the hexane extract with dilute alkali and dilute acid followed by concentration and derivatization, are identical to the procedure for extraction of THC from normal plasma.

PHYSICAL, CHEMICAL AND SPECTROMETEIC DATA FOR Δ^9-TETRAHYDROCANNABINOL

Nomenclature

Chemical Name: 6aR-trans-6a,7,8,10a-Tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol

Empirical Formula: $C_{21}H_{30}O_2$

Chemical Registry Number: 1972-08-3

Physical Constants

Appearance: Colorless viscous syrup

Boiling Point: 200°C at 0.02 mm

Specific Rotation: $\alpha]_D^{20}$ = -150.5° (c = 0.53 in $CHCl_3$)

Solubility: Very soluble in hexane, chloroform and ethanol. Solubility in water, 3 mg/liter

pKa: 10.6

UV Absorption: λ_{max} (ethanol) = 283, 276 nm (log E 3.21, 3.20)

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR 11-HYDROXY-Δ^9-TETRAHYDROCANNABINOL

Nomenclature

Chemical Name: 6aR-trans-6a,7,8,10a-Tetrahydro-1-hydroxy-6,6-dimethyl-3-pentyl-6H-dibenzo[b,d]pyran-9-methanol

Empirical Formula: $C_{21}H_{30}O_3$

Chemical Registry Number: 36557-05-8

Physical Constants

Appearance: White, amorphous solid

Melting Point: 139-140°C

Solubility: Soluble in ethanol, acetone. Sparingly soluble in benzene, chloroform. Nearly insoluble in water, petroleum ether

UV Absorption: λ_{max} (ethanol) = 276, 282 nm

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR 11-NOR-9-CARBOXY-Δ^9-TETRAHYDROCANNABINOL

Nomenclature

Chemical Name: 6aR-trans-6a,7,8,10a-Tetrahydro-1-hydroxy-6,6-dimethyl-3-pentyl-6H-dibenzo[b,d]pyran-9-carboxylic acid

Empirical Formula: $C_{21}H_{28}O_4$

Chemical Registry Number: 56354-06-4

Physical Constants

Appearance: White, crystalline solid

Melting Point: 210-213°C

Solubility: Soluble in ethanol, acetone. Sparingly soluble in chloroform, ethyl ether. Nearly insoluble in water, benzene, petroleum ether

REFERENCES

1. S. Cohen in R.C. Petersen, ed., Marihuana Research Findings: 1976. National Institute on Drug Abuse Research Monograph 14, DHEW Pub. (ADM) 77-501. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., ch. 9 (July 1977).

2. A. Sperling. J. Chromatogr. Sci., 10, 268 (1972).

3. R. Karler in R.C. Petersen, ed., Marihuana Research Findings: 1976, ch. 2. See reference 1.

4. J.W. Fairbairn, J.A. Liebmann, and M.G. Rowan. J. Pharm. Pharmacol., 28, 1 (1976).

5. R. Mechoulam and Y. Gaoni. J. Am. Chem. Soc., 87, 3273 (1965).

6. A.J. Singer, ed. "Marihuana: Chemistry, Pharmacology, and Patterns of Social Use", Ann. N.Y. Acad. Sci., 191, 1-269 (1971).

7. R. Mechoulam, ed. Marijuana, Academic Press, New York and London (1973).

8. W.D.M. Paton and J. Crown. Cannabis and its Derivatives, Oxford Univ. Press, London (1972).

9. R.E. Willette, ed., Drugs and Driving. National Institute on Drug Abuse Research Monograph 11, DHEW Pub. (AIM) 77-432. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C. (March 1977).

10. G. Nahas. U.N. Bull. Narcotics, 29, 13 (1977).

11. M.L. Lee, M. Novotny, and K.D. Bartle. Anal. Chem., 48, 405 (1976).

12. R. Karler in R.C. Petersen, ed., Marihuana Research Findings: 1976, ch. 3. See reference 1.

13. E.G. Leighty, A.F. Fentiman, Jr., and R.L. Foltz. Res. Commun. Chem. Pathol. Pharmacol., 14, 13 (1976).

14. R. Mechoulam, W.K. McCallum, and S. Burstein. Chem. Rev., 76, 75 (1976).

15. G.G. Nahas, W.D.M. Paton, and J.E. Idanpaan-Heikkila, eds., Marihuana: Chemistry, Biochemistry, and Cellular Effects. Springer-Verlag, New York (1976).

16. M.C. Braude and S. Szara, eds., Pharmacology of Marihuana, Vol. 1 & 2. Raven Press, New York (1976).

17. M.E. Wall. Ann. N.Y. Acad. Sci., 191, 23 (1971).

18. D.J. Harvey, B.R. Martin, and W.D.M. Paton. J. Pharm. Pharmacol., 29, 495 (1977).

19. J. Schou, L.D. Prockop, G. Dahlstrom, and C. Rohde. Acta Pharmacol. et Toxicol., 41, 33 (1977).

20. S.L. Kanter, L.E. Hollister, F. Moore, and D.E. Green. Clin. Chem., 20, 860 (1974).

21. E.R. Garrett and C.A. Hunt. J. Pharm. Sci., 63, 1056 (1974).

22. R. Mechoulam. Science, 168, 1159 (1970).

23. R. Boudon. Euro. J. Toxicol., 9, 11 (1976).

24. M. Frei-Hausler and R.W. Frei. J. Chromatogr., 79, 209 (1973).

25. M. Frei-Hausler and R.W. Frei. J. Chromatogr., 84, 214 (1973).

26. A.P. Melikian and I.S. Forrest. J. Pharm. Sci., 62, 1025 (1973).

27. H. Bowd, D.H. Swann, B. Towell, and J.H. Turnbull, in J.B. Birks, ed., Excited States Biol. Mol. Proc. Int. Conf. 1974. Wiley, Chichester, England, p. 128 (1976).

28. J.M. Schermann, H. Hoellinger, N.-H.-Nam, R. Bourdon, and E. Fournier. Clin. Chem. Acta, 79, 401 (1977).

29. W.W. Just, N. Filipovic, and G. Werner. J. Chromatogr., 96, 189 (1974).

30. J.A. Vinson, D.D. Patel, and A.H. Patel. Anal. Chem., 49, 163 (1977).

31. L. Lemberger, in Garattini F. Hawking, A. Goldin, and I.J. Kopin, eds., Advances in Pharmacology and Chemotherapy. Academic Press, New York and London, 10, p. 221 (1972).

32. J.D. Teale, E.J. Forman, L.J. King, E.M. Piall, and V. Marks. J. Pharm. Pharmacol., 27, 465 (1975).

33. J.D. Grant, S.J. Gross, P. Lomax, and R. Wong. Nature New Biol., 236, 216 (1972).

34. C.E. Cook, M.L. Hawes, E.W. Amerson, C.G. Pitt, and D. Williams in R.E. Willette, ed., Cannabinoid Assays in Humans. National Institute on Drug Abuse Research Monograph 7, DHEW Pub. (ADM) 76-339. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., p. 15 (May 1976).

35. P.T. Tsui, K.A. Kelly, M.M. Ponpipom, M. Strahilevitz, and A.H. Sehon. Can. J. Biochem., 52, 252 (1974).

36. A.R. Chase, P.R. Kelley, A. Taunton-Rigby, R.T. Jones, and T. Harwood in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 1. See reference 34.

37. S.J. Gross and J.R. Soares in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 10. See reference 34.

38. P.L. Williams, A.C. Moffat, and L.J. King. J. Chromatogr., 155, 273 (1978).

39. G.L. Rowley, T.H. Armstrong, C.P. Crowl, W.M. Eimstad, W.M. Hu, J.K. Kam, R. Rogers, R.C. Ronald, K.E. Rubenstein, B.G. Sheldon, and E.F. Ullman in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 28. See reference 34.

40. D.C. Fenimore, R.R. Freeman, and P.R. Loy. Anal. Chem., 45, 2331 (1973).

41. E.E. Knaus, R.T. Coutts, and C.W. Kazakoff. J. Chromatogr. Sci., 14, 525 (1976).

42. N.K. McCallum and E.R. Cairns. J. Anal. Toxicol., 2, 89 (1978).

43. E.R. Garrett and C.A. Hunt in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 28. See reference 34.

44. E.R. Garrett and C.A. Hunt. J. Pharm. Sci., 66, 20 (1977).

45. F.W. Karasek, D.E. Karasek, and S.H. Kim. J. Chromatogr., 105, 345 (1975).

46. S. Agurell, B. Gustafsson, B. Holmstedt, K. Leander, J.-E. Lindgren, I. Nilsson, F. Sandberg, and M. Asberg. J. Pharm. Pharmacol., 25, 554 (1973).

47. A. Ohlsson, J.-E. Lindgren, K. Leander, and S. Agurell in R. E. Willette, ed., Cannabinoid Assays in Humans, p. 48. See reference 34.

48. M. Nordqvist, J.-E. Lindgren, and S. Agurell in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 64. See reference 34.

49. J.M. Rosenfeld, B. Bowins, J. Roberts, J. Perkins, and A.S. Macpherson. Anal. Chem., 46, 2232 (1974).

50. J.M. Rosenfeld and V.Y. Taguchi. Anal. Chem., 48, 726 (1976).

51. J.M. Rosenfeld. Anal. Letters, 10, 917 (1977).

52. D.E. Green in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 70. See reference 34.

53. M.E. Wall, T.M. Harvey, J.T. Bursey, D.R. Brine, and D. Rosenthal in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 107. See reference 34.

54. D. Rosenthal, T.M. Harvey, J.T. Bursey, D.R. Brine, and M.E. Wall. Biomed. Mass Spectr., 5, 312 (1978).

55. J.L. Valentine, P.J. Bryant, P.L. Gutshall, O.H.M. Gan, E.D. Thompson, and H.C. Hiu in R.E. Willette, ed., Cannabinoid Assays in Humans, p. 96. See reference 34. Also, J. Pharm. Sci., 66, 1263 (1977).

56. J.N. Pirl, V.M. Papa, and J.J. Spikes. J. Anal. Toxicol., 3, 129 (1979).

57. L. Lemberger, S.D. Silberstein, J. Axelrod, and I.J. Kopin. Science, 170, 1320 (1970).

58. J.A. Vinson, ed., Cannabinoid Analysis in Physiological Fluids, ACS Symposium Series 98. American Chemical Society, Washington, D.C. (1979).

59. R.E. Willette, ed., Cannabinoid Assays in Humans. National Institute on Drug Abuse Research Monograph 7, DHEW Pub. (ADM) 76-339. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C. (May 1976).

60. R.L. Hawks, ed., The Analysis of Cannabinoids in Biological Fluids. National Institute on Drug Abuse Resarch Monograph Series. In preparation.

61. R.L. Foltz and B.J. Hidy in R.L. Hawks, ed., The Analysis of Cannabinoids in Biological Fluids. See reference 60.

62. C.M. Bonuccelli. J. Pharm. Sci., 68, 262 (1979).

63. D.C. Fenimore, C.M. Davis, J.H. Whitford, and C.A. Harrington. Anal. Chem., 48, 2289 (1976).

64. A.C. Moffat in R.L. Hawks, ed., The Analysis of Cannabinoids in Biological Fluids. See reference 60.

65. P.L. Williams, A.C. Moffat, and L.J. King. J. Chromatogr., 186, 595 (1979).

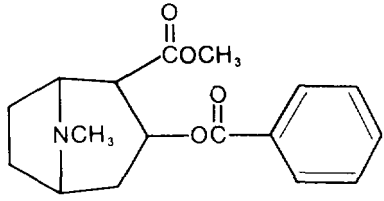
66. D. Rosenthal and D. Brine. J. Forensic Sci., 24, 282 (1979).

67. B.J. Hidy and R.L. Foltz. Final Report on Quantification of Cannabinoids in Body Fluids. National Institute on Drug Abuse, Contract No. HSM-42-72-183, January 1980.

Chapter 7

Cocaine and Its Major Metabolite, Benzoylecgonine

Cocaine (3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester, I) is an alkaloid obtained from the leaves of Erythroxylon coca or by synthesis from ecgonine. Although cocaine hydrochloride has limited medical use as a topical anesthetic, it is widely used as a stimulant by drug abusers. The major geographical sources of illicit cocaine are Peru, Bolivia, and Ecuador. The plant from which it comes is largely restricted to the Andes mountain regions of these countries. In these areas the plant leaves have been chewed by the native population for many centuries for



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religious purposes and as an adjunct to work, much as coffee is consumed as a stimulant in our culture (1) . However, when the psychoactive ingredients of the leaf are isolated and injected or inhaled intranasally in concentrated form, the pharmacological effects can be quite different from those which occur in the traditional pattern of cocaine use among Andean Indians. The leaves contain only about 1 percent cocaine, and the oral route appears to delay absorption and decrease toxicity.

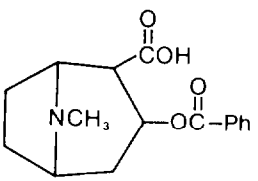
Cocaine was first isolated from coca leaves in 1860, and its therapeutic possibilities were quickly recognized by some physicians, especially in Europe. By 1890 it was incorporated into a variety of patent nostrums and "tonics" such as Coca Cola, although since 1910 the latter has not contained any cocaine (2). The dangerous qualities of cocaine soon became apparent, and following its inclusion in a U.S. narcotics law in 1914, cocaine went "underground," to be used primarily in the Bohemian subculture of artists and musicians. It reemerged about 1970 as the "status" drug among middle-class drug abusers in this country, who commonly refer to it as "coke" or "snow" (3). The reason for its popularity is the nature of the stimulation it produces; i.e., feelings of heightened sexuality, psychic energy, and self-confidence which are of short duration (a few minutes) but very intense.

Cocaine is one of the most powerful natural central nervous system stimulants known (4). It blocks reuptake of norepinephrine into nerve endings, acting first on the brain cortex to increase motor activity. Overdose causes anxiety, depression, and confusion, and can eventually lead to convulsions and respiratory arrest. Direct cardiotoxicity can cause cardiac arrest, and even small doses increase pulse rate and blood pressure. Ventricular fibrillation and sudden death may result, which sets cocaine apart from other local anesthetics, such as lidocaine and procaine, which tend to desensitize the myocardium to fatal arrhythmia. The fatal oral dose of cocaine is stated to be 1.2 g, but as little as 20 mg applied to mucous membranes can cause death, and fatal anaphylactic shock has been reported with use of even lower doses (3). A recent dose-response study of cocaine in human volunteers showed that 100 mg administered intranasally or 10 mg given intravenously were sufficient to induce significant subjective and cardiovascular effects (5). Continued nasal inhalation, commonly called "snorting," can lead to necrosis of the nasal mucosa.

Although true physical dependence and tolerance are not proven for cocaine, a very strong psychological dependence can develop. The cocaine habit is said to be the most seductive, rapidly injurious, and difficult to eradicate of all drug habits (4). Frequent use of large doses may lead to weight loss, insomnia, and anxiety (2) With continued high-dose use, a true toxic psychosis may result as with amphetamine, causing symptoms of paranoia and violent behavior (3).

PHARMACOKINETICS AND METABOLISM

Cocaine is metabolized rapidly, principally by de-esterification [hydrolysis by plasma esterases (6) and liver enzymes (7)] and N-demethylation. These processes result in the formation of benzoylecgonine (II), ecgonine (III), ecgonine methyl ester, and several N-demethylated products. Of these metabolites, only norcocaine (IV) has shown biological activity equivalent to cocaine in the inhibition of ^{3}H-norepinephrine uptake by rat brain synaptosomes (8). A radioisotope study with two human volunteers demonstrated that N-demethylation does take place in man after oral administration of cocaine (9).



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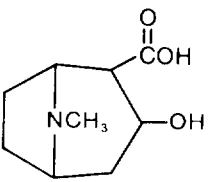

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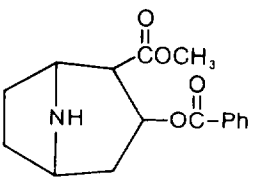

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Benzoylecgonine and ecgonine are highly polar and therefore they do not cross the blood-brain barrier in pharmacologically-significant concentrations. However, norcocaine has been found in the brains of monkeys given repeated doses of cocaine (8), and the N-demethylation of cocaine has been shown to occur in the brain of rats (10). The lipophilic nature of cocaine and norcocaine helps to account for their penetration across the blood-brain barrier. In animal studies which compared the disposition and pharmacokinetics of radiolabeled cocaine in acute versus chronically treated rats, chronic treatment led to consistently higher concentrations of cocaine being sequestered in fat depots. Although cocaine disappeared rapidly from brain in acute treatment, measurable amounts persisted in brain and other tissues of chronically treated rats long after the disappearance of cocaine from plasma (11). In dogs, however, brain-to-plasma ratios of cocaine were lower in chronically than in acutely treated animals 2 to 4 hr after injection, and unmetabolized cocaine did not persist in the CNS (12). Radiolabel studies in rats have also shown localization of cocaine or its metabolites in other tissues, including kidney, lung, spleen, testes, intestine, muscle, liver, and heart (13).

In man less than 20 percent, and often only 1 percent, of the parent drug is excreted in urine; the amount excreted is related to dose and urine acidity. Cocaine is also found in the bile. The principal urinary metabolites, II (25-54 percent of dose) and III, are highly water soluble and are largely excreted within 24-36 hr after administration of cocaine (14). A time-course study of the urinary excretion of benzoylecgonine following intranasal administration of cocaine in human subjects showed the appearance of the metabolite in 1-4 hr and a peak urine concentration at 10-12 hr (15). In another study, peak urine concentrations of benzoylecgonine (up to 75 μg/ml) occurred 1-12 hr after nasal inhalation of clinical doses of cocaine (1.5 mg/kg) and diminished slowly over several days; it was still detectable by radioimmunoassay at 144 hr in one patient. Maximum urine concentrations of unchanged cocaine (up to 24 μg/ml) occurred within 2 hr of intranasal administration and diminished rapidly. Benzoylecgonine/cocaine ratios in urine were too varied among individuals to be of use in predicting cocaine concentrations from benzoylecgonine data or vice versa (16).

Following an intravenous dose of 100 mg of cocaine hydrochloride to three human subjects, cocaine concentrations in plasma peaked at 5 min and then declined over 5-6 hr, with a distributional half-life in the plasma of 20-40 min, corresponding to the time-course of psychologic effects. The mean biologic half-life of cocaine in plasma was reported to be 2.8 hr. In the same study, benzoylecgonine was detected in plasma 5 min after intravenous administration of cocaine. Plasma concentrations of the metabolite increased rapidly in the first hour, remained stable for 2-4 hr, and then declined slowly. After 24 hr, the benzoylecgonine was still detectable. The persistence of benzoylecgonine in plasma suggested that it was strongly bound to plasma proteins (17). The pharmacokinetic measurements were comparable to those reported for patients receiving cocaine in the form of a topical anesthetic, and indicated rapid

absorption of cocaine through mucous membranes. Plasma concentrations of cocaine in another human study increased rapidly for 15-20 min after intranasal application of 1.5 mg/kg, peaked at 15-60 min, and then decreased gradually for 3-5 hr. Maximum plasma concentrations ranged from 120 ng/ml to 474 ng/ml (18,19). In cases of acute intoxication, blood concentrations of cocaine as low as 200 ng/ml have been associated with fatalities, although concentrations up to 9.0 µg/ml are more commonly reported in cocaine deaths when no other drugs were present (20).

ANALYTICAL METHODOLOGY

Because of its rapid metabolism, detection and quantitation of cocaine in body fluids is difficult. In addition to the low concentrations likely to be encountered in blood and in urine (21), the assay of cocaine presents technical problems caused by hydrolysis of the drug by plasma enzymes and under conditions of storage and laboratory manipulation. Even when stored in a frozen state, 40 percent of the cocaine in plasma was shown to be lost within 5 days, and 60 percent within a month; at room temperature 45 percent of the cocaine in plasma was lost within 1 hr. However, addition of sodium fluoride (2.5 mg/ml plasma) to the plasma to inhibit enzymatic hydrolysis prevented any appreciable loss of cocaine in plasma maintained at room temperature for 2 hr (22). Under strongly basic (pH > 10) or acidic (pH< 5) conditions, chemical hydrolysis of cocaine to benzoylecgonine and ecgonine is accelerated. For these reasons, measurement of the cocaine metabolites, particularly benzoylecgonine, has been emphasized in the development of assay procedures. However, the metabolites of cocaine are very polar and also amphoteric; they are not readily extractable into the organic solvents normally used for drug screening, and therefore specialized methods must be employed for their recovery from biological materials,

Colorimetric techniques, which are at best semiquantitative, have been used to determine cocaine in urine (23) and other biological fluids. An early study on the distribution and metabolism of cocaine in dogs and rabbits was based on such a method; it involved extraction with chloroform, passage of the extract through a sucrose column, and addition of bromcresol purple. Optical densities of the resulting yellow solutions were determined on a spectrophotometer at 410 nm, and a sensitivity of 0.5-1.0 µg/ml in urine, blood, and tissue homogenates was reported (24,25). Spectrophotometric methods, however, are neither specific nor sufficiently sensitive for use in most cases of cocaine analysis.

Thin-layer chromatography (TLC), commonly used for routine identification of abused drugs in urine, requires special solvent systems for separation as well as for extraction of cocaine metabolites. In one TLC method, cocaine and its metabolites were extracted from urine with 25 percent ethanol in chloroform at pH 10-11 and chromatographed on silica gel plates developed in methanol:ammonia (99:1) and sprayed with an iodoplatinate reagent. The solvent system separated ecgonine from benzoylecgonine and cocaine, but to

separate the latter two compounds from each other it was necessary to use ethyl acetate:methanol:ammonia (85:10:10) (26). Another TLC assay involved extraction with a mixture of chloroform: isopropanol:dichloroethane (8:1:3) at pH 8.5. The sensitivity for benzoylecgonine analyzed by this procedure was 3-5 μg/ml (27).

More recently, a TLC method using a double solvent system on silica gel sheets involved a butylation step after extraction of cocaine and its metabolites from urine with chloroform:ethanol (3:2) at pH 8-9; it provided a sensitivity of 3 μg/ml (28). The sensitivity limits for TLC of cocaine and benzoylecgonine in urine were subsequently lowered to 0.1 and 0.25 μg/ml, respectively, by spraying the developed plates with Dragendorff's reagent followed by an overspray with dilute H_2SO_4 and exposure to iodine vapors (29). Another refinement of TLC detection procedures involved preliminary absorption of the compounds onto paper loaded with SA-2 cation exchange resin. Extraction from the resin was made with chloroform: isopropanol: dichloroethane (4.5:0.9:4.5); sensitivity for cocaine and its metabolites was 1 μg/ml. in 20 ml of urine. If other drugs were to be assayed in the same urine sample, cocaine and the other drugs were first extracted from the paper at pH 10 and chromatographed in a two-stage solvent system. Benzoylecgonine was extracted from the aqueous buffer phase after acidification and readjustment of the pH to 8.1-8.4. The sensitivity limit for benzoylecgonine was 2 μg/ml. Alternatively, benzoylecgonine alone was directly extracted from 5 ml of urine with the chloroform: isopropanol: dichloroethane solvent and was detected at a sensitivity limit of 0.5 μg/ml by TLC using Dragendorff's reagent and an iodine-potassium iodide reagent (30).

The enzyme-multipled immunoassay technique (EMIT) is widely used in urine screening for cocaine abuse, and has a detection limit of 1 μg/ml of benzoylecgonine. No extraction is required, and the assay takes less than 2 min to perform. It does not detect cocaine nor is it applicable to plasma (31). A commercially available radioimmunoassay has been developed based on a ^{125}I-labeled benzoylecgonine derivative (32). The RIA is now widely used for screening urine samples for benzoylecgonine and can also be used for quantitative measurement of "benzoylecgonine equivalents" which include cocaine and other tropane alkaloids which cross-react with the antibody. The assay will detect benzoylecgonine at urine concentrations as low as 2 ng/ml, but 100 ng/ml has been recommended as the "cutoff" concentration for positives in routine screening. An evaluation of the RIA method has been published in which it is compared with GC, TLC and EMIT methods for analysis of benzoylecgonine in urine (33).

Quantitative analysis of cocaine in biological fluids has relied primarily on the development of gas chromatographic methods (GC). Cocaine can be gas chromatographed without derivatization, but its metabolites cannot. The carboxylic acid groups of II and III must be esterified with some reagent such as diazomethane, dimethylformamide dimethylacetal, N,N-dicyclohexyl carbodimide, or n-butanol-boron trifluoride. Esterification with 2-chloroethanol in the pre-

sence of boron trichloride has the advantage of introducing a halogenated substituent which renders the molecule suitable for electron-capture detection (34). A solvent extraction procedure can be used to separate benzoylecgonine and ecgonine from basic and neutral components of biological extracts prior to esterification and GC analysis of the metabolites (35).

Gas Chromatography (GC) with flame-ionization detection (FID) has often been used for detection of cocaine and metabolites, especially in urine. In one of the first studies of human cocaine metabolism, GC-FID with benzhexol as an internal standard was used for measurement of cocaine in urine. Chromatography was on a 3 percent OV-17 column at 185°C, and a linear response was obtained over a range of 0.5-15.0 μg/ml. Benzoylecgonine was assayed in this system by continuous extraction with chloroform containing 5-α-cholestane as an internal standard; the extracted benzoylecgonine was methylated to cocaine for gas chromatography (36,37). GC-FID on a 2.5 percent SE- 30 column at 200°C was reported as a method capable of detecting 1 μg/ml of benzoylecgonine in urine. The benzoylecgonine was salted out of the urine into 95 percent ethanol, purified by TLC, eluted in water, and methylated before gas chromatography . The overall recovery was only 30-40 percent, but an efficiency of 90-100 percent was reported for the initial ethanol extraction (38). Cocaine can be mistaken for pentazocine, levorphenol, or methaqualone on the OV-17 columns commonly used for drug screening; for this reason, an on-column reaction of cocaine with trimethyl anilinium hydroxide, which yields an unidentified product with a different retention time, has been suggested as a confirmatory step in the detection of cocaine in urine (39).

For concurrent determination of 9 CNS-active drugs in human plasma, including cocaine, GC-FID analysis on a 3 percent OV-1 column followed extraction with benzene-isopropanol at pH 10. Recovery of cocaine was about 90 percent, and sensitivity limits corresponded to plasma drug concentrations of 0.05 μg/ml. The technique had the advantage of comparative simplicity for routine purposes (40). Another procedure designed to determine both cocaine and benzoylecgonine in urine involved methylation of the metabolite, converting it to cocaine in 72 percent yield before GC-FID analysis on a 3 percent OV-17 column. Butylanthraquinone was used as an internal standard. The extraction technique (chloroform:ethanol, 4:1) gave a recovery of 93 percent for cocaine and 65 percent for benzoylecgonine at pH 5.5-9.5. The sensitivity reported was 0.20 μg/ml in a 5-ml specimen, which was adequate for the measurement of urinary drug and metabolite concentrations of postsurgical patients up to 24 hr after topical cocaine anesthesia (250 mg applied to nasal mucosa) (41).

Similarly, simultaneous determination of cocaine and its metabolites in urine, using gas chromatography on columns coated with 3 percent SP-2250-DA or 3 percent SE-30, has been reported. In this case extraction was into chloroform:ethanol (3:1), followed by propylation of benzoylecgonine using an organic base and 1-iodopropane. Recovery of 99 percent of the cocaine and 80 percent of the benzoylecgonine, combined with 98 percent conversion of the metabolite to its

n-propyl ester, provided detection limits of 0.2 µg/ml in 5 ml of urine. Back extraction and purification were required, and also synthesis of the n-pentyl ester of benzoylecgonine for use as an internal standard. The procedure measured concentrations ranging from 0.2 to 18 µg/ml of benzoylecgonine in samples of urine from drug screening programs, and demonstrated concentrations of less than 0.3 µg/ml of cocaine and 8-136 µg/ml of benzoylecgonine in pooled 8-hour urine samples from postsurgical patients following cocaine anesthesia (42).

A method reported for simultaneous analysis of cocaine and benzoylecgonine in blood, bile, urine or tissue involved an extractive alkylation step to convert benzoylecgonine to its ethyl ester which was then readily separated from cocaine by gas chromatography (3 percent OV-17, 225°C). n-Propyl benzoylecgonine was used as the internal standard (43).

The sensitivity of GC analysis for cocaine can be increased up to 100-fold by use of a nitrogen-phosphorus detector. With this type of detector sub-nanogram quantities of the drug can be measured according to a reported procedure which involved the use of the propyl ester of benzoylecgonine as an internal standard, back-extraction into 0.1 H_2SO_4, and chromatography on an OV-17 column at 255°C (21). Another application of GC with nitrogen-phosphorus detection for the measurement of cocaine in blood involved an ethylmorphine internal standard, extraction with benzene:isopropanol (9:1) , and chromatography on a 3 percent SP-2100-DB column. Following topical application of cocaine to nasal mucosa, as little as 20 ng of cocaine was detected in 1 ml of whole blood by this method (44).

An electron-capture detector has also been used for the measurement of benzoylecgonine in blood. The procedure involved extraction of the metabolite and an internal standard (chlorproethazine) from plasma with chloroform: ethanol (4:1) at pH 9.5. The dried extract was alkylated with pentafluorobenzyl bromide and the derivative partitioned into benzene to eliminate more polar interfering substances, then reextracted into acid. Sensitivity for the method was 5 ng of benzoylecgonine per ml of plasma. However, the presence of a background contaminant peak in urine decreased the sensitivity of the procedure to 50 ng/ml for urine analysis (17).

Measurement of 20-30 ng/ml of cocaine in urine, without the necessity of evaporative concentration procedures, was achieved in an adaptation of the GC-ECD technique which used an 0-acylated derivative of reduced cocaine (heptafluorobutyric anhydride or pentafluoropropionic anhydride added to the cyclohexane extract following reduction with $LiA1H_4$) (45). A very similar procedure was subsequently used to measure cocaine in plasma as well as urine (46).

High performance liquid chromatography (HPLC) on a reverse phase column was used for separation and measurement of cocaine and benzoylecgonine extracted from urine. A detection limit of 0.1 µg/ml of urine was achieved by monitoring the ultraviolet absorption of the HPLC effluent at 200 nm. The chromatography procedure also per-

mitted the separation and detection of norcocaine and benzoylecgonine; however, debenzoylated metabolites such as ecgonine could not be detected because of insufficient UV absorption, even at 200 nm (47).

The high specificity and sensitivity of gas chromatography/mass spectrometry (GC/MS) has been applied to the analysis of cocaine and its metabolites in biological fluids (48-50). Concentrations of cocaine and norcocaine in human urine were determined by a procedure which involved initial extraction at pH 8.5 into cyclohexane followed by back-extraction into 0.1 N HCl, and after adjustment to basic pH with 1 N NH_4OH, reextraction with cyclohexane. Before GC/MS analysis the norcocaine was converted to its N-trifluoroacetamide derivative. Deuterium labeled analogs were used as internal standards. Cocaine-$^2H_3$ was prepared by treatment of benzoylecgonine with dimethylformamide dimethyl-$^2H_6$ acetal (Pierce, Rockford, Illinois), and norcocaine-$^2H_3$ was obtained by N-demethylation of the deuterated cocaine, The assay measured 2 ng/ml of cocaine and norcocaine with a precision of 5 percent (48). A similar assay was developed by the same research group for quantitation of cocaine and benzoylecgonine. Deuterium-labeled internal standards were again used, except the deuterated cocaine was prepared by alkylation of norcocaine with C^2H_31 and benzoylecgonine-$^2H_3$ was generated from the labeled cocaine. Chloroform: isopropanol (4:1) was used to extract the benzoylecgonine from urine after adjustment of the pH to 9 with 0.1 N NH_4OH. The extracted benzoylecgonine was then converted to its ethyl ester by treatment with diazoethane generated by addition of N-ethyl-N-nitro-N-nitrosoguanidine to a biphasic solution of KOH and ether. Electron impact ionization gave molecular ions of moderate abundance for cocaine (m/z 303) and benzoylecgonine ethyl ester (m/z 317) which were monitored together with the molecular ions for the deuterated internal standards. When all systems were working well, an assay sensitivity of approximately 2 ng/ml of cocaine and 5 ng/ml of benzoylecgonine could be achieved (49)

In another application of GC/MS to the assay of cocaine metabolites in urine, benzoylecgonine was simultaneously extracted from urine and converted to its ethyl ester by a 10-min extractive-alkylation procedure. The butyl ester of benzoylecgonine served as the internal standard. Quantitation was accomplished by GC/MS using probability-based matching (SO).

HPLC is promising as a means of purifying polar metabolites extracted from body fluids; advantages are extraction selectivity and adaptability to automation (51). Other reported extraction procedures vary with the analytical method and the purpose of the assay. Cocaine is easily extracted from alkaline aqueous media into organic solvents, including cyclonexane (12,45), ether (36,37), 2 percent isoamyl alcohol in n-heptane (17,18,21), benzene:isopropanol 9:1 (recovery 85-90 percent) (40,44), and chloroform (recovery 85-100 percent) (24). The recovery of cocaine is best at pH 9.5 (93 percent from chloroform:ethanol). It drops to 76 percent at pH 10.5 because of hydrolysis (52).

Benzoylecgonine can be removed from the aqueous phase by exhaustive continuous extraction with chloroform; by single extraction with chloroform:isopropanol:1,2-dichloroethane (4.5:0.9:4.5) (30) or chlorofornnethanol (4:1, recovery 65-80 percent) (17,41,42); salted out together with other polar metabolites into alcohol from a K_2CO_3 solution or a KH_2PO_4-K_2HPO_4 solution (38); or extracted into methanol from lyophilized urine (90-100 percent recovery) (38). The lower pH possible with salting out procedures avoids saponification of benzoylecgonine. Adsorption on nonionic (e.g., XAD-2) resins, followed by elution with chloroform: isopropanol (3:1), is recommended for extraction of some cocaine metabolites from tissue homogenates, but ecgonine and norecgonine are not adsorbed by the resin (35).

Two recently-published books review information on the history, chemistry, pharmacology, analysis, and sociological aspects of cocaine (1,53).

EXPERIMENTAL PROCEDURE

Because cocaine is rapidly metabolized it is often desirable to analyze for both the parent drug and its major metabolite, benzoylecgonine. Therefore, two procedures are described; both are modifications of published methods developed at the Center for Human Toxicology, University of Utah (54). Procedure 1 consists of a simple, direct extraction which permits rapid measurement of cocaine concentrations only. Procedure 2 takes longer, but permits simultaneous measurement of both cocaine and benzoylecgonine concentrations. The latter procedure consists of extraction with chloroform: isopropanol from a sodium chloride-saturated solution of the body fluid, derivatization of the extracted benzoylecgonine by heating with N,N-dimethylformide di-n-propyl acetal, sample purification by back-extraction of the basic drug and the derivatized metabolite into acid, and finally adjustment to basic pH and reextraction into an organic solvent prior to quantitation by (GC/MS.

Cocaine is rapidly hydrolyzed by plasma esterases to ecgonine methyl ester (55), so it is important to inhibit these enzymes as soon as blood samples are collected for subsequent cocaine analysis (56). This can be done by collecting the sample in a tube containing sodium fluoride, such as the grey stoppered 7-ml Venoject tubes (Kimble-Terumo, Inc., Elkton, MD 21921) which are supplied with 17.5 mg of solid sodium fluoride and 14 mg of potassium oxalate. An additional 50 µl of a saturated sodium fluoride solution is added for each ml of blood. The sample is then centrifuged and the plasma transferred to a Teflon-lined, screw cap culture tube and frozen until analysis.

Standards and Reagents

Cocaine hydrochloride and benzoylecgonine of high purity can be purchased from Applied Science laboratories, State College, PA 16801. Cocaine hydrochloride labeled with 3 deuterium atoms on the N-methyl group (cocaine-$^2H_3$ HCl), and benzoylecgonine labeled with deuterium

on the benzoyl group (benzoylecgonine-$^2H_3$) were obtained from the National Institute on Drug Abuse. On the basis of mass spectral analysis the cocaine-$^2H_3$ had an isotopic composition of: $^2H_3$, 96 percent; $^2H_2$, 1 percent; $^2H_1$, 1 percent; and $^2H_0$, 0 percent. The isotopic composition of the benzoylecgonine-$^2H_3$ was: $^2H_5$, 7 percent; $^2H_4$, 31 percent; $^2H_3$, 40 percent; $^2H_2$, 21 percent; $^2H_1$ and $^1H_1$, 0 percent.

Prepare a pH 9.6 buffer by dissolving 500 grams of potassium monohydrogen phosphate (K_2HP0_4) in about 500 ml of distilled water. Allow the solution to cool to room temperature and then add sufficient distilled water to make exactly one liter.

N,N-Dimethylformamide di-n-propyl acetal was purchased from the Aldrich Chemical Company, Milwaukee, WI 53233.

Stock solutions of cocaine and benzoylecgonine are prepared as follows for the purpose of determining calibration graphs and preparing working standards. Weigh into a 100-ml volumetric flask about 11.2 mg of cocaine hydrochloride (equivalent to 10 mg of free base) and 10 mg of benzoylecgonine, and record the weights to the nearest 0.1 mg. Dissolve the measured drug and metabolite in about 75 ml of methanol and then add additional methanol to give exactly 100 ml of solution. This solution will be referred to as the "0.10 mg/ml stock solution" although it should be labeled with its actual concentration based upon the exact measured weights of cocaine and benzoylecgonine. A series of working standard solutions can then be prepared by appropriate dilution of the stock solution as described in Chapter 2.

The stock solution of cocaine-$^2H_3$ and benzoylecgonine-$^2H_3$ is prepared in the same manner. For the measurement of drug and metabolite in body fluids within the concentration range 1 to 1000 ng/ml, addition of approximately 40 ng of the internal standards to each ml of body fluid is satisfactory. This can be conveniently done by adding 100 µl of a methanolic solution containing 400 ng/ml of both cocaine-$^2H_3$ and benzoylecgonine-$^2H_3$ to each ml of body fluid. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of cocaine-$^2H_3$ and benzoylecgonine-$^2H_3$ to 250 ml with methanol.

Store stock and working standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction - Method 1 (Cocaine only)

Transfer 1 ml of whole blood, plasma or urine to a 5-ml glass-stoppered, conical centrifuge tube. Add 100 µl of the internal standard solution containing cocaine-$^2H_3$ and benzoylecgonine-$^2H_3$ at concentrations of 400 ng/ml. Vortex the mixture for 10 sec and then allow it to equilibrate for 15 min. Add 1 ml of K_2HPO_4 buffer (pH 9.6) followed by 100 µl of a solvent consisting of toluene:heptane:isoamyl alcohol in a volume ratio of 70:20:10. Immediately stopper the tube and vortex for at least 30 sec. Centrifuge the mixture at 2000 x G for 15 min. The organic solvent should form a narrow upper

layer from which an aliquot can be removed with a syringe for injection into the GC/MS. If a distinct solvent layer is not obtained, add an additional 100 µl of organic solvent and recentrifuge.

Extraction - Method 2 (Cocaine and Benzoylecgonine)

Transfer 1 ml of whole blood, plasma or urine to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of sample can be used, but it must be measured accurately,). Add 100 µl of the internal standard solution containing cocaine-$^2H_3$ and benzoylecgonine-$^2H_3$ at concentrations of 400 ng/ml. Vortex the mixture for 10 sec and allow it to equilibrate for 15 min. Check the pH. If it is not 7.0 ± 0.5, add 1 ml of a pH 7 buffer. Saturate the mixture with sodium chloride (about 0.5 g) to improve the extraction efficiency of benzoylecgonine. Add 8 ml of a solvent mixture of chloroform:isopropanol (9:1 v/v). Cap the tube and check for a leak around the cap. Gently mix the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min, remove the upper aqueous layer by aspiration, and transfer the lower organic layer into a 12-ml conical, glass-stoppered centrifuge tube. If particulate matter is present in the organic extract, it should be removed by filtering the organic extract through silylated glass wool. It is important that the transferred organic extract be absolutely dry and devoid of any of the lipid interface that forms between the phases. Remove the solvent by evaporation with a gentle stream of nitrogen or filtered air and heat at not greater than 70°C. All of the volatile solvents must be removed! Add 50 µl of dimethylformamide and 50 µl of N,N-dimethylformamide di-n-propyl acetal. Reflux the mixture for 30 sec over an open flame (see Discussion of the Experimental Procedure). Allow the mixture to cool to room temperature and then add 1 ml of 0.5 N H_2SO_4. Vortex mix for about 10 sec. Add 3 ml of a solvent system consisting of toluene:heptane:isoamyl alcohol in a volume ratio of 70:20:10. Vortex for 30 sec and then centrifuge. Remove the upper organic phase by aspiration. Neutralize the aqueous acid phase by adding 1 ml of 0.5 N NaOH and 1 ml of the K_2HPO_4 buffer. Vortex mix and check that the pH is between 9 and 10. If it is not, adjust by adding a few drops of 0.5 N NaOH or 0.5 N H_2SO_4, as appropriate. Immediately extract the cocaine and benzoylecgonine n-propyl ester from the basic aqueous solution by adding 3 ml of the toluene:heptane:isoamyl alcohol (70:20:10) solvent, vortex for 30 sec, and centrifuge. Transfer by means of a disposable Pasteur pipette the upper organic layer to a silylated, 12-ml concentrator tube having a conical or nipple-shaped bottom. Remove the solvent by evaporation under a gentle stream of nitrogen or filtered air and at a temperature not greater than 70°C. Reconstitute the extract residue in 30 µl of chloroform for GC/MS analysis.

GC/MS Analysis

The experimental conditions for the GC/MS analysis of cocaine and benzoylecgonine n-propyl ester are as follows:

GC column:	1.8 m x 2mm (I.D.) glass column packed with 3 percent OV-1 on Gas Chrom Q 100/120 mesh (Applied Science Laboratory, 'State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Reagent gas:	Ammonia, introduced into the ion chamber as described in Chapter 2
Temperatures	Injector, 280°C Column, 230°C GC/MS transfer line, 280°C Ion source, 160°C

Under these conditions the cocaine should elute from the gas chromatograph at between 2 and 4 min, and the benzoylecgonine n-propyl ester should elute at between 3 and 5 min.

Before beginning the GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored are the protonated molecule ions for cocaine (m/z 304), cocaine-$^2H_3$ (m/z 307), benzoylecgonine n-propyl ester (m/z 332), and benzoylecgonine-$^2H_3$ n-propyl ester (m/z 335). With the divert valve in the divert position, inject into the GC/MS 2 to 6 µl of the organic extract from either procedure 1 or 2. After approximately 1 min switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer and begin data acquisition. If the extract from procedure 1 is being analyzed, only the cocaine ion masses (m/z 304 and 307) are monitored. For analysis of extracts from procedure 2 the best sensitivity is achieved by first monitoring only the cocaine ion masses until the cocaine has eluted; then the benzoylecgonine n-propyl ester ion masses are monitored. If the GC/MS data system does not permit rapid changes of the monitored ion masses, all four ion masses must be monitored during the elution of the drug and the derivatized metabolite. Normally, the latter procedure will result in only a small sacrifice in sensitivity. When the benzoylecgonine n-propyl ester has eluted, cease data acquisition and return the divert valve to the divert position. The quantities of cocaine and benzoylecgonine in the body fluid are determined by measuring the peak heights (or areas) of the drug, the derivatized metabolite, and their internal standards in the selected ion current profiles, and relating the ratios of peak heights to calibration graphs (Chapter 2). Calculate the body fluid concentrations of cocaine and benzoylecgonine by dividing the measured quantities of these compounds by the exact volume of body fluid extracted.

Discussion of the Experimental Procedure

Benzoylecgonine must be converted to a volatile derivative before it can be gas chromatographed. Various methods of derivatization have been used, including methylation to cocaine (36,37,41), propylation by treatment with 1-iodopropane (42), alkylation with pentafluoro-

benzyl bromide (17), and ethylation by treatment with diazoethane (38) or by extractive alkylation with iodoethane (43,49). The method of derivatization described here is simple and efficient if the procedure is followed precisely. The reaction of N,N-dimethylformamide di-n-propyl acetal with benzoylecgonine is rapid and quantitative if the reaction mixture is heated to a sufficiently high temperature (~150°C). Temperatures of this magnitude are conveniently achieved by repeatedly passing the bottom of the reaction tube slowly through the tip of a small, open flame until the reaction mixture just begins to boil. Evolution of a white vapor will occur. If all of the volatile solvents (chloroform and isopropanol) have not been removed prior to the addition of the derivatizing agent, the reaction mixture will boil at a lower temperature and the reaction may not go to completion.

Other N,N-dimethylformamide acetals have been tried, but the di-n-propyl acetal was chosen because it gives a benzoylecgonine ester which has a particularly suitable retention time under the gas chromatographic conditions specified. The N,N-dimethylformamide di-n-propyl acetal was purchased from the Aldrich Chemical Company, Milwaukee, WI 53233. Some difficulty was encountered with impurities in samples of this reagent purchased from a different supplier.

Figure 1 compares the electron impact (EI) and the chemical ionization (CI) mass spectra of cocaine. The corresponding mass spectra of the propyl ester of benzoylecgonine are similar except that the abundant ions in the CI mass spectra are shifted 28 daltons higher. The CI mass spectrum of cocaine obtained using methane and ammonia as reagent gases shows only one abundant ion, which corresponds to the protonated molecule ion (m/z 304). The protonated molecule ion peak in the methane CI mass spectrum has a relative intensity of about 50 percent of the base peak at m/z 182, which corresponds to a fragment ion resulting from loss of benzoic acid from the protonated molecule ion. The EI mass spectrum has a moderately abundant ion at m/z 182 which could be used for selected ion monitoring analysis of cocaine, However the analogous ion (m/z 210) in the EI mass spectrum of the propyl ester of benzoylecgonine would not be a satisfactory ion to monitor if the deuterium atoms in the internal standard are located on the benzoyl group because they would be lost during formation of this fragment ion.

If just the protonated molecule ions are monitored, the methane-ammonia CI gives about a threefold improvement in sensitivity over methane CI. The methane-ammonia CI also gives the highest selectivity. On the other hand, when methane alone is used as the reagent gas, the ion masses corresponding to both the protonated molecule ion and the MH^+-$PhCO_2H$ fragment ion can be monitored. This will provide additional confidence in the quantitative results because any co-eluting extract component is unlikely to contribute equally to the ion currents at both of the ion masses monitored.

Figures 2 and 3 show representative calibration graphs acquired using extraction procedures 1 and 2, respectively. Table 1 gives accuracy and precision data obtained using both procedures.

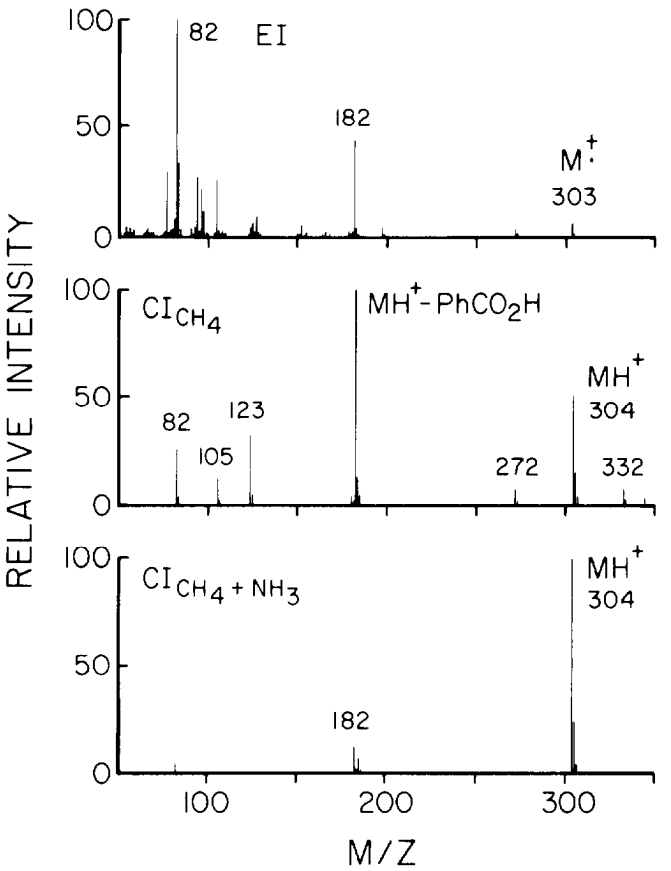


FIGURE 1. MASS SPECTRA OF COCAINE

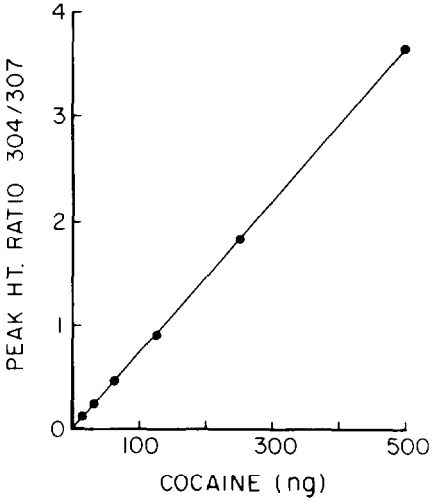


FIGURE 1. CALIBRATION GRAPH FOR ANALYSIS OF COCAINE IN PLASMA BY PROCEDURE 1

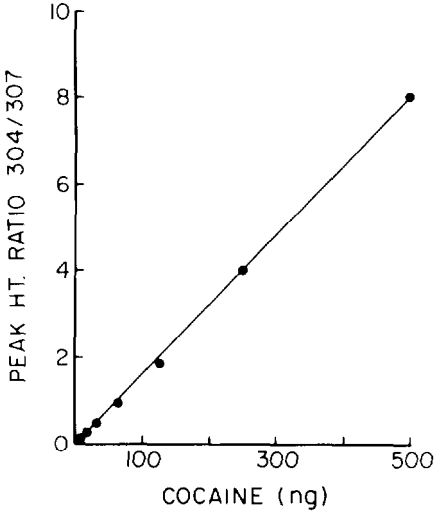


FIGURE 3. CALIBRATION GRAPH FOR ANALYSIS OF COCAINE IN PLASMA BY PROCEDURE 2

TABLE 1. ACCURACY AND PRECISION DATA

	Drug	Spiked Value (ng/ml)	N	Mean Measured Conc. (ng/ml)	Coefficient Of Variation %
	Within-Run				
Proc. 1	Cocaine	75	10	74.6	2.1
Proc. 2	Cocaine	290	10	296	2.2
	Benzoyl-ecgonine	660	10	657	2.3
	Run-to-Run				
Proc. 1	Cocaine	75	10	78.2	4.6
Proc. 2	Cocaine	290	10	285	5.4
	Benzoyl-ecgonine	660	10	675	3.9

Body fluid concentrations of cocaine and benzoylecgonine sometimes exceed 1 µg/ml. When this occurs the sample should be diluted by a measured amount with distilled water so that the resulting concentration falls within the range of 0 to 1000 ng/ml. The diluted sample can then be analyzed as described.

Cocaine, and to a lesser extent benzoylecgonine, can undergo enzymatic and/or chemical hydrolysis (54). The rate of chemical hydrolysis is greatly accelerated at alkaline pH. For example, at pH 9.8, 40 percent of the cocaine in a 3 µM solution was converted to benzoylecgonine in 1 hr at 37°C (54). Also, benzoylecgonine in methanol can undergo reestcrification to cocaine, particularly if acid is present. For these reasons, all body fluid samples to be analyzed for cocaine should be stabilized with sodium fluoride and stored in a frozen state. Aqueous solutions of cocaine and benzoylecgonine and methanolic solutions of benzoylecgonine should be stored under refrigeration and for no longer than a few days.

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR COCAINE

Nomenclature

Chemical Name: 3-Benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester

Empirical Formula: $C_{17}H_{21}N\ O_4$

Chemical Registry Number: 50-36-2

Physical Constants

Appearance: White crystalline powder

Melting Point: 98°C

Boiling Point: 187-188°C @ 0.1 mm

Specific Rotation: $\alpha]_D^{20}$ = -16° (c = 4 in chloroform)

Solubility: 1 g of cocaine dissolves in 600 ml of water, 6.5 ml of ethanol, 0.7 ml of chloroform, and 3.5 ml of diethyl ether.

pKa: 8.70

UV Absorption: λ max = 231 nm (logε = 4.2) and 274 nm (log ε = 3.0)

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR BENZOYLECGONINE

Nomenclature

Chemical Name: 3-Benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid

Empirical Formula: $C_{16}H_{19}NO_4$

Chemical Registry Number: 519-09-5

Physical Constants

Appearance: White crystalline powder

Melting Points: 195°C with decomposition, the tetrahydrate melts at 86°C

Specific Rotation: $\alpha]_D^{15}$ = -45° (c = 3 in ethanol)

Solubility: Very soluble in hot water, soluble in ethanol, insoluble in ether

UV Absorption: λ max = 234 nm and 275 nm (in 0.1 N H_2S0_4)

REFERENCES

1. R.C. Petersen and R.C. Stillman, eds., Cocaine: 1977. National Institute on Drug Abuse Research Monograph 13, DHEW Pub. (ADM) 77-432. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C. (May 1977).

2. S. Cohen. J. Amer. Med. Assoc., 231, 74 (1975).

3. G.R. Gay, C.W. Sheppard, D.S. Inaba, and J.A. Newmeyer. Int. J. Addictions, 8, 1027 (1973).

4. T. Harwood. Drug Enforcement, 1, 20 (Spring 1974).

5. R.B. Resnick, R.S. Kestenbaum, and L.K. Schwartz. Science, 195, 696 (1977).

6. D. Taylor, V.S. Estevez, L.F. Englert, and B.T. Ho. Res. Commun. Chem. Pathol. Pharmacol., 14, 249 (1976).

7. E.G. Leighty and A.F. Fentiman, Jr. Res. Commun. Chem. Pathol. Pharmacol., 8, 65 (1974).

8. R.L. Hawks, I.J. Kopin, R.W. Colburn, and N.B. Thoa. Life Sciences, 15, 2189 (1974).

9. T. Inaba, D.J. Stewart, and W. Kalow. Clin. Pharmacol. Ther., 23, 547 (1978).

10. A.L. Misra, P.K. Nayak, M.N. Patel, N.L. Vadlamani, and S.J. Mulé. Experientia, 30, 1312 (1974).

11. P.K. Nayak, A.L. Misra, and S.J. Mulé. J. Pharmacol. Exp. Ther., 196, 556 (1976).

12. A.L. Misra, M.N. Patel, V.R. Alluri, S.J. Mulé, and P.K. Nayak. Xenobiotica, 6, 537 (1976).

13. A.L. Misra in S.J. Mulé, ed., Cocaine: Chemical, Biological, Clinical, Social, and Treatment Aspects. CRC Press, Cleveland, OH, p. 73 (1976).

14. R. Byck and C. Van Dyke in R.C. Petersen and R.C. Stillman, eds., Cocaine: 1977, ch. V. See reference 1.

15. C.Van Dyke, R. Byck, P.G. Barash, and P. Jatlow. Clin. Chem:, 23, 241 (1977).

16. H.E. Hamilton, J.E. Wallace, E.L. Shimek, Jr., P.Land, S.C. Harris, and J.G. Christensen. J. Forensic Sci., 22, 697 (1977).

17. M.J. Kogan, K.G. Verebey, A.C. DePace, R.B. Resnick, and S. J. Mulé. Anal. Chem., 49, 1965 (1977).

18. R. Byck, P. Jatlow, P. Barash, and C. Van Dyke. Psychopharmacol. Bull., 12, 47 (1976).

19. C. Van Dyke, P. Barash, P. Jatlow, and R. Byck. Science, 191, 859 (1976).

20. B.S. Finkle and K.L. McCloskey in R.C. Petersen and R.C. Stillman, eds., Cocaine: 1977, ch. VIII. See reference 1.

21. P.I. Jatlow and D.N. Bailey. Clin. Chem., 21, 946 (1975).

22. J.I. Javaid, H. Dekirmenjian, J.M. Davis, and C.R. Schuster J. Chromatogr., 152, 105 (1978).

23. H. Bronisz, B. Wysocka, and W. Rusiecki. Disserationes Pharm., 15, 343 (1963).

24. L.A. Woods, J. Cochin, E.J. Fornefeld, F.G. McMahon, and M.H. Seevers. J. Pharmacol. Exp. Ther., 101, 188 (1951).

25. L.A. Woods, F.G. McMahon, and M.H. Seevers. J. Pharmacol. Exp. Ther., 101, 200 (1951).

26. S.J. Mulé. J. Chromatogr., 55, 255 (1971).

27. N.N. Valanju, M.M. Baden, S.N. Valanju, D. Mulligan, and S.K. Verma. J. Chromatogr., 81, 170 (1973).

28. M.L. Bastos, D. Jukofsky, and S.J. Mulé. J. Chromatogr., 89, 335 (1974).

29. J.E. Wallace, H.E. Hamilton, H. Schwertner, D.E. King, J.L. McNay, and K. Blum. J. Chromatogr., 114, 433 (1975).

30. K.K. Kaistha and R. Tadrus. J. Chromatogr., 135, 385 (1977).

31. Syva Corp. Palo Alto, CA 94304.

32. Roche Diagnostics. Nutley, NJ 07110.

33. S.J. Mulé, D. Jukofsky, M. Kogan, A. DePace, and K. Verebey. Clin. Chem., 23, 796 (1977).

34. J.M. Moore. Clin. Chem., 21, 1538 (1975).

35. M.L. Bastos and D.B. Hoffman in S.J. Mulé, ed., Cocaine: Chemical, Biological, Clinical, Social, and Treament Aspects, p. 35. See reference 13.

36. F. Fish and W.D.C. Wilson. J. Chromatogr., 40, 164 (1969).

37. F. Fish and W.D.C. Wilson. J. Pharm. Pharmacol., 21 135S (1969).

38. S. Koontz, D. Besemer, N. Mackey, and R. Phillips. J. Chromatogr., 85, 75 (1973).

39. R.H. Hammer, J.L. Templeton, and H.L. Panzik. J. Pharm. Sci., 63, 1963 (1974).

40. F. Medzihradsky and P.J. Dahlstrom. Pharmacol. Res. Commun., 7, 55 (1975).

41. J.E. Wallace, H.E. Hamilton, D.E. King, D.J. Bason, H.A. Schwertner, and S.C. Harris. Anal. Chem., 48, 34 (1976).

42. D.L. von Minden and N.A. D'Amato. Anal. Chem., 49, 1974 (1977).

43. J.C. Valentour, V. Aggarwal, M.P. McGee, and S.W. Goza. J. Anal. Toxicol., 2, 134 (1978).

44. B.H. Dvorchik, S.H. Miller, and W.P. Graham. J. Chromatogr., 135, 141 (1977).

45. J.W. Blake, R.S. Ray, J.S. Noonan, and P.W. Murdick. Anal. Chem., 46, 288 (1974).

46. J.I. Javaid, H. Dekirmenjian, E.G. Brunngraber, and J.M. Davis. J. Chromatogr., 110, 141 (1975).

47. P.I. Jatlow, C. Van Dyke, P. Bar-ash, and R. Byck. J. Chromatogr., 152, 115 (1978).

48. S.P. Jindal, T. Lutz, and P. Vestergaard. Biomed. Mass Spectr., 5, 658 (1978).

49. S.P. Jindal and P. Vestergaard. J. Pharm. Sci., 67, 811 (1978).

50. J.E. Graas and E. Watson. J. Anal. Toxicol., 2, 80 (1978).

51. A.P. Graffeo, D.C.K. Lin, and R.L. Foltz. J. Chromatogr., 126, 717 (1976).

52. S.J. Mulé, M.L. Bastos, D. Jukofsky, and E. Saffer. J. Chromatogr., 63, 289 (1971).

53. S.J. Mulé, ed., Cocaine: Chemical, Biological, Clinical, Social, and Treatment Aspects. See reference 13.

54. D.M. Chinn, D.J. Crouch, M.A. Peat, B.S. Finkle, and T.A. Jennison. J. Anal. Toxicol., 4, 37 (1980).

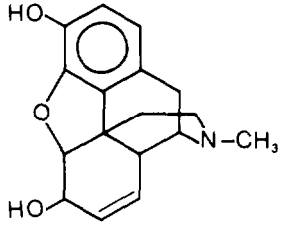
55. D.J. Stewart, T. Inaba, B.K. Tang and W. Kalow. Life Sciences, 20, 1557 (1977).

56. P. Jatlow in S.J. Mul, ed., Cocaine: Chemical, Biological, Clinical, Social, and Treatment Aspects, p. 61. See reference 13.

Chapter 8

Morphine

Archaeological and historical evidence indicates that opium has been used as an analgesic since at least the third century B.C. and probably as long ago as the late Bronze Age in Europe. Morphine, a pentacyclic alkaloid and the chief active component of opium, was isolated in 1805 by a German pharmacist, although the correct chemical structure (I) was first proposed in 1923. Total synthesis was achieved in 1952 (1). In addition to analgesia, morphine causes side effects such as constipation, lethargy and nausea, and a great many derivatives have been synthesized in efforts to alter or antagonize some of morphine's pharmacological effects (2).



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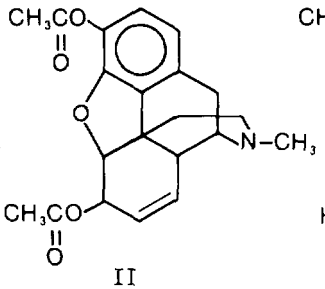

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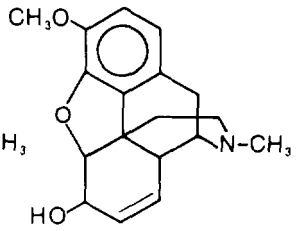

III

Heroin (3,6-diacetylmorphine, II) was introduced in 1898. It was a more effective analgesic than morphine, but it soon became apparent that heroin was more toxic and produced physical dependence and tolerance more rapidly than morphine. Heroin abuse increased in the period between the World Wars (1920 to 1935) and again after 1968. By 1974 the U.S. Bureau of Narcotics estimated that there were over 400,000 heroin addicts in the United States (1). In spite of voluminous published data reflecting thousands of animal and human experiments over the past 40 years, the mechanism of heroin and morphine dependence is still not understood.

Codeine (III), a widely used methyl ether of morphine, has milder analgesic and respiratory depressant effects, and is an effective cough suppressant. It is demethylated in vivo and as much as 17 percent of a given dose may appear in the urine as morphine (3).

The tertiary amino group accounts for the basic properties of the morphine molecule. Natural morphine and its salts are strongly levorotary. The d-isomer has been synthesized but has no significant pharmacological activity (1).

PHARMACOKINETICS AND METABOLISM

Because of the comparative absence of cardiovascular effects, morphine has been revived as a major adjunct to anesthesia, stimulating kinetic studies in human subjects (4). The maximum pharmacological effect of morphine occurs within minutes after intravenous injection, corresponding to the maximum measured concentration of the free drug in plasma (5) . The clearance of morphine from blood is rapid, with about 80 percent of an administered dose excreted in urine within 8 hr although traces can be detected 72-100 hr after administration, especially in addicts. The long retention time is usually attributed to protein binding, but there is also the likelihood of reabsorption of morphine from the intestinal mucosa after hydrolysis of conjugates transported from the liver in the bile (1). After intravenous injection, a plasma half-life of 2 to 3 hr and a clearance time of 10 to 44 hr have been determined by radioimmunoassay (RIA), but the RIA technique does not distinguish between the free and conjugated forms of the drug (4,5). Following intravenous administration of 10-mg doses to adult volunteers, the serum concentration of morphine ranged from 0.04 to 0.10 μg/ml after 2 hr and fell to 0.002 to 0.007 μg/ml after 12 hr (5). Morphine is rapidly absorbed after intramuscular or subcutaneous injection with a maximum pharmacological effect 60 to 90 min after administration, again corresponding to the highest plasma drug concentrations (6).

Probably because of its high pKa (9.85), morphine is poorly absorbed from the stomach. The maximum plasma concentration of free morphine after oral administration in comparison experiments was less than 20 percent of that obtained by intravenous administration of the same dose. Furthermore, the concentration of conjugated drug in serum 1 hr after oral administration was 16 times higher than the free morphine concentration, suggesting a high hepatic extraction ratio (5). In cases of drug overdose, the minimum lethal concentration of morphine has been reported as 0.2 μg/ml in blood or 0.2-0.4 μg/g in muscle. For addicts, the figures are higher. In one case, an addict who had died of other causes had a blood morphine concentration of 2.8 μg/ml (7). Urine concentrations show no correlation with lethality (7). It has been suggested, but is unsubstantiated, that in some acute deaths from heroin overdose, toxic adulterants such as quinine may be primary agents in the lethal reaction (8). Acute respiratory depression has also been suggested as a likely mechanism of heroin overdose deaths, especially in cases where the drug is present in combination with other CNS depressants such as alcohol (9).

Morphine possesses no functional groups which readily undergo hydrolysis. Heroin, however, is rapidly hydrolyzed. Less polar than morphine, it has high lipid and membrane solubility which could account for rapid absorption and passage through the blood-brain barrier. The major pharmacologic effects of heroin, however, are due to 6-monoacetyl-morphine (6-MAM) and morphine; the half-life of heroin itself in blood is less than 20 min, and the principal product clearing the blood is 6-MAM. Its presence in plasma can be considered presumptive evidence for heroin use (10). Because 6-MAM

is converted to morphine in the liver, the analysis of human urine after heroin administration yields primarily morphine in the same ratio of free to conjugated form as that seen after parenteral administration of morphine, i.e., about 1.9 (1). However, the presence of 6-MAM and its 3-glucuronide in human urine has been reported following intravenous heroin administration, in amounts representing less than 5 percent of the dose (11). In a study of heroin kinetics in man, the urinary excretion half-life of free morphine was found to be 1.3 hr; of 6-MAM, 1.3 hr; of conjugates, 2.8 hr; and of total normorphine, 2.7 hr (12).

The metabolism of morphine in man is summarized in Table 1. Synthetic reactions are responsible for detoxification by converting morphine or its metabolites, including normorphine, to readily excreted water-soluble compounds such as 3- and 6-glucuronides and 3-sulfate, by means of microsomal enzyme systems in liver, brain, kidney, and intestine. Quantitatively minor synthetic reactions include methylation of normorphine and 3-0-methylation of morphine to codeine (1). The 0-methyl transferase activity which produces codeine increases in addicts, a fact which suggests altered metabolism in prolonged opiate use and a possible marker for abuse (13). Norcodeine, a major metabolite of codeine in man, has also been identified in the urine of some heroin addicts, in quantities proportional to the formation of codeine from morphine (14).

The maximal reported recovery of morphine and biotransformation products is less than 85 percent of the dose, with excretion linearly related to urine output (15). Although small amounts of morphine and metabolites are eliminated in feces and saliva, the metabolic fate of 15 to 20 percent of administered drug remains unknown.

ANALYTICAL METHODOLOGY

Thin-layer chromatography (TLC) has been the most commonly employed method for qualitative (screening) detection of morphine; it is rapid, sensitive, and economical. Technical improvements in solvent systems, plates, sheets, and sprays have appeared regularly in the recent literature (16,17). For example, picogram quantities of morphine in urine or brain homogenates were detected on polyamide plates by prior treatment of the n-butanol extracts with dansyl chloride to fonn a highly fluorescent morphine derivative which could be visualized on the dried plates under ultraviolet light (18).

Spectrofluorometric methods are based on the conversion of morphine to a fluorophore, excitation and measurement of emission at specific wavelengths. Most methods employ the oxidation of morphine to pseudomorphine by potassium ferricyanide in weakly alkaline solution, and achieve detection levels as low as 0.1 µg/ml (19) . Fluorescence can also be achieved without potassium ferricyanide by treating the extract (chloroform: isopropanol, 3:1) with concentrated H_2SO_4, then concentrated NH_40H, and autoclaving for 15 min at 120°C. The latter procedure can be automated for application to large numbers of samples with detection levels of 0.2 µg/ml (20). As little as 10 ng of morphine in plasma was detected in a modified method which precipitated plasma proteins before extraction, used only siliconized

glassware, and decreased the reaction volume to 40 µl in order to increase sensitivity. Readings were made on a filter fluorometer (21). Because the decay of pseudomorphine fluorescence is rapid, fluorometry has largely been limited to qualitative use. However, coupling the photofluorimeter to a computer system, which reads reaction kinetics within 3 milliseconds of mixing, has shown promise as a quantitative technique (22).

A quantitative spectrofluorometric method, using high performance liquid chromatography of the oxidized urine extract with an internal standard of dihydromorphine, has been developed (23). The mixture of hydrolyzed urine and dihydromorphine was extracted with chloroform: isopropanol (9:1) and mixed with potassium ferricyanide immediately before injection onto a porous silica column coupled to a fluorimeter set at λ_{ex} 320 nm and λ_{em} 436 nm. The solvent system contained methanol: 2 N NH_4OH:1 N NH_4NO_3 (30: 20:10). There was a linear relationship between the amount of morphine in the original solution and the ratio of the emission peak height of the mixed dimer (pseudomorphine + pseudodihydromorphine) formed during oxidation to that of the fluorescent dihydromorphine standard. This allowed calibration suitable for quantitative analysis with a sensitivity of 0.01 µg/ml. The technique has the advantage of being highly specific for morphine. Interference by other drugs, particularly normorphine, N-allyl normorphine, dihydromorphine, and 6-monoacetyl morphine is a potential problem in other fluorometric procedures because of emission spectra identical to pseudomorphine. In these instances fluorometry must be combined with a chromatographic separation (19).

Immunoassays developed for morphine include the free radical assay technique (FRAT) which employs spin labeling with nitroxide-labeled drugs and is sensitive to 0.5 µg/ml of morphine; the enzyme multiplied immunoassay technique (EMIT) in which morphine is conjugated to lysozyme and the combination of the labeled drug with antibodies to morphine renders the enzyme inactive; radioimmunoassay (RIA); and hemagglutination inhibition (HI) in which the presence of morphine in test material prevents agglutination of morphine-coated red blood cells by an antimorphine antibody. A major advantage of immunoassays is that biofluids can be tested directly, without sample extraction or concentration. They can also be extremely sensitive, detecting as little as 0.03 µg/ml morphine (RIA and HI), and fast (2 to 4 min for EMIT and FRAT) (17). However, immunoassays are subject to cross-reactivity with drugs of similar molecular structure. Morphine antibodies have been shown to react with codeine, dextromethorphan, pethidine, dihydromorphinone, propoxyphene, diamorphine, and 6-MAM (24,25). Attempts to reduce cross- reactions have produced antiserums raised against various haptens: an antiserum to a conjugate of 6-succinyl morphine with bovine serum albumin reacted only slightly with morphine glucuronide and was used to measure free morphine in serum of patients receiving oral doses, at a detection level of 0.3 ng/ml (24). Other haptens tried have included morphine-3-glucuronide and oxymorphone-6-carboxy-methoxime (26). Haptens are usually attached to the carrier at one of the carbon atoms of morphine, but an antiserum capable of distinguishing morphine from codeine was obtained by conjugating carrier pro-

tein to the nitrogen atom of nomorphine. The antibody produced did not react with codeine or with the 3-0-monoglucuronide of morphine, and detected 15 pg of morphine in the presence of codeine (27). An antiserum specific for codeine has also been reported, prepared from a conjugate using N-butyroylnorcodeine as hapten (28). While immunoassays, especially RIA, can be adapted to quantitative studies by preparation of calibration graphs, the lack of absolute specificity remains a problem. Nevertheless, a solid-phase RIA, which involves the absorption of antibody to polymeric surfaces, has been adapted for morphine assay using an antibody to morphine-6-hemisuccinate purified by affinity chromatography. The assay is reported to respond to as little as 0.5 ng of morphine per ml of serum (29).

Gas chromatography (GC) can be used for screening biofluids, but because of the relatively long time required to complete a GC analysis, it is more often used for confirmation of TLC or immunoassay results, and for quantitative studies in pharmacokinetics and metabolism. GC systems equipped with flame ionization detectors (GC-FID) are sensitive enough to detect 15 ng of morphine/ml. Conversion of the morphine extracted from bile, urine, blood, or liver to the less polar diacetyl derivative (heroin) can increase sensitivity of GC-FID to 1 ng (17). The polarity of morphine, which causes adsorption in the GC column, can also be altered by silylation. A derivatization procedure using bis-(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1 percent trimethylchlorosilane as both solvent and silylating agent is reported to give 100 percent conversion of morphine to its bis-(trimethylsilyl) ether within 20 min at room temperature (30). Bis-(0-trimethylsilyl) morphine can also be formed quantitatively and reproducibly by simultaneous injection of trimethylsilylimidazole and the drug solution onto the gas chromatographic column (31).

A quantitative GC method developed for heroin and 6-monoacetyl morphine in plasma utilized an alkali-flame detector (nitrogen/phosphorus detector). Plasma samples at pH 9.0 containing ethylmorphine as internal standard were extracted with benzene, and the dried extracts were chromatographed as their acetylated derivatives after treatment with trifluoroacetic anhydride-benzene (1:5). The alkali-flame detector permitted quantitation of drug concentrations as low as 100 ng/ml, with detection of 20 ng/ml. Extraction was done quickly after venipuncture to avoid in vitro enzymatic hydrolysis of the compounds (32).

Electron-capture detection (GC-ECD) has been used to quantitate morphine after conversion to halogen-containing derivatives (33-37). In one procedure nalorphine was added as an internal standard, and the morphine and nalorphine were trifluoroacetylated following extraction from 1-2 ml of serum or plasma with 10 percent isobutanol in chloroform, back extraction with 0.5 N HCl, and reextraction with 10 percent isopropanol in ethyl acetate. With a ^{63}Ni detector a sensitivity limit of 25 ng/ml in serum was reported (33). A refinement of the ECD technique, using a ^{3}H-ECD, detected 0.5 ng/ml morphine in plasma extracts and 0.1 ng morphine in 30 mg of brain tissue from rats. The samples were extracted with toluene:butanol

(9:1) at pH 8.9, back extracted into 0.1 N H_2SO_4, and reextracted with toluene:butanol. Morphine and the internal standard (nalorphine) were derivatized with pentafluoropropionic anhydride (34). Both morphine and codeine have been quantitated by derivatization with pentafluoropropionic anhydride and analysis by GC-ECD using a ^{63}Ni detector. Detection limits were 2 pg for morphine and 20 pg for codeine in test solutions (35).

A GC-ECD method which can assay as little as 0.1 ng/ml of morphine in human urine has been reported (36). Morphine was converted to its heptafluorobutyryl derivative for GC separation on 3 percent OV-17 after extraction with ethyl acetate of 2 ml of alkalinized urine. Codeine was used as an internal standard. The sensitivity limit reported for this procedure was 100 pg morphine/ml of urine. Fluorinated derivatives were also prepared for GC-ECD quantitation studies of morphine, codeine, and 6-MAM present as contaminants in illicit heroin (37). Heptafluorobutyric anhydride (HFBA) yielded suitable derivatives within a 5-min reaction time, and the derivatives were extracted from an acetonitrile-sodium bicarbonate solution into petroleum ether before chromatography on 3 percent OV-17 on Gas Chrom Q. Morphine, codeine, and 6-MAM in heroin were readily measured at levels as low as 0.001, 0.01, 0.01 percent, respectively. The minimum detectable quantity for each compound was ~20 pg for morphine, 80 pg for codeine, and 100 pg for 6-MAM. However, when procaine was present as an adulterant, it was highly reactive with HFBA and caused significant interference with the chromatograms of the heptafluorobutyryl derivatives.

GC coupled with mass spectrometry (GC/MS) offers the best method available for the unequivocal identification of morphine and its metabolites in biological materials. It can serve as a check on results of less sensitive and specific assays, as well as being a tool for positive confirmation of structures. The technique of selected ion monitoring has been applied to quantitation of morphine and related compounds in a variety of body fluids (10,38-43).

Morphine was measured in urine at concentrations as low as 5 ng/ml using GC/MS with chemical ionization (42). Morphine labeled with three deuterium atoms on the N-methyl group served as the internal standard. After adjustment of the urine to pH 8.5 it was extracted with chloroform: isopropanol (4:1) and the extract was treated with bis-(trimethylsilyl) acetamide. The resulting bis-(trimethylsilyl) morphine was separated and measured by chromatography on a 3 percent OV-17 column maintained at 230°C; monitored ions corresponded to two prominent fragment ions [$(M\text{-}CH_3)^+$ and $MH^+\text{-}HOSi(CH_3)_3$] in the methane chemical ionization mass spectra of the derivatized drug and the deuterated internal standard.

Morphine and codeine were measured simultaneously in blood by a GC/MS procedure involving addition of a deuterated internal standard to the blood, acid hydrolysis of conjugates, a series of extractions to separate the bases from other components of the extract, derivatization with trifluoroacetic anhydride, and measurement of the bis-(trifluoroacetyl) morphine and mono-(trifluoroacetyl) codeine using gas chromatography (3 percent OV-17, 230°C) and electron impact mass spectrometry (43). The assay was applied

to the analysis of 916 blood samples from individuals arrested for suspicion of opiate intoxication. The average concentration of morphine was 0.29 µg/ml. For most samples the codeine concentration was approximately 5 to 10 percent of the morphine concentration. The lower limit for acceptable quantitative measurement was considered to be 20 ng/ml.

In an application of GC/MS to a study of the relationship between brain levels of morphine and analgesia in rats, the internal standard was again trideuterated morphine and the drug was chromatographed as its trifluoroacetyl derivative (40). Sensitivity was such that the low concentrations of morphine present in brain following therapeutic doses could be measured easily, with a lower quantitation limit of 8 ng/g of tissue.

Free and bound morphine, as well as free 6-MAM have been quantitated in rabbit blood by GC/MS (10). Trideuterated morphine and 6-MAM-$^2H_3$ were added to 0.2 ml plasma before precipitation of proteins by acetone. The supernatant was evaporated to dryness under nitrogen and the residue taken up in 2.5 ml of 0.1 N HCl and purified by two extractions with benzene. The aqueous phase was adjusted to pH 8.5, extracted with chloroform:isoamyl alcohol (3:1), and evaporated to dryness before derivatization with trifluoroacetic anhydride. The derivatives were taken up in chloroform for GC/MS analysis on a 6 ft x 2 mm silanized column packed with 1 percent OV-1 on Gas Chrom Q, using helium as carrier gas and a column temperature of 230°C. With electron-impact ionization the highest ion intensities were associated with fragments at m/z 364 for the protio compound and 367 for the deuterio compound. As an assay for free and bound heroin metabolites, the method represented a 100-fold improvement over GC-FID methods for these compounds, with a sensitivity of 1 ng/ml.

Extractive alkylation with pentafluorobenzyl (PFB) bromide, using tetrabutylammonium (TBA) as counter ion and ethyl acetate as solvent, has been used to quantitate morphine in plasma at concentrations as low as 5 ng/ml (41). With this procedure morphine was extracted from aqueous solutions with 98 percent efficiency. Alkylation of the phenolic hydroxyl group occurred simultaneously. The PFB derivative of morphine could then be analyzed directly by GC/MS. However, better results were obtained if the PFB morphine was further derivatized by treatment with trifluoroacetic anhydride. The resulting PFB-TFA derivative was chromatographed on a 2 percent OV-17 column maintained at 245°C. Quantitation was achieved by measuring the ion currents at m/z 380 and 383, corresponding to prominent fragment ions in the electron impact mass spectra of the PFB-TFA-morphine and its trideuterated analog.

Morphine concentrations determined by spectrofluorometry in blood and brain were approximately one-half those obtained by GC/MS (44). The difference may result from quenching interferences in the fluorometric assay and the greater sensitivity of the GC/MS method. Hydrolysis of blood samples increased the apparent concentration of morphine by about 260 percent, while the corresponding increase in brain samples was only about 25 percent. Presumably, the increase is caused by hydrolysis of the glucuronide conjugate of morphine.

Charge-exchange ionization using 10 percent nitric oxide in nitrogen as reagent gas can offer an advantage over CI or EI ionization for the analysis of morphine and heroin in that the charge-exchange mass spectra of these compounds show less fragmentation (45). Field ionization also causes little or no fragmentation of morphine-type molecules and can be used to determine trace amounts of drugs in biological media by isotope dilution analysis with multilabeled diluents such as morphine-$^{2}H_{5}$ and codeine-$^{2}H_{8}$ (46,47).

Quantitation of total morphine in biological fluids requires hydrolysis to free the drug from conjugates. Enzymatic hydrolysis has generally proven to be less efficient than acid hydrolysis of morphine glucuronides because of variations in potency among enzyme preparations. The most efficient enzymatic recovery found in a comparative study was 64 percent. Critical parameters included choice of buffer system, pH, temperature, and enzyme concentration. High-temperature (autoclaving or refluxing) acid hydrolysis does not require such critical control of conditions. The same study reported a yield of 93 percent of available morphine when the conjugate solution was autoclaved at 125°C for 30 min using 8-15 percent hydrochloric acid (48). However, hydrolysis conditions vary widely in the literature, and some authors have reported complete hydrolysis of morphine-3-glucuronide in 30 min at 100°C by 4 N HCl (10).

Extraction procedures for morphine also vary, but because of its phenolic hydroxyl and tertiary amine the optimum pH for extraction is 8.5-10 (49). Chloroform is most often used as the organic solvent, usually with the addition of isopropanol (7,15,23), n-butanol (19,21), or isoamyl alcohol (10) in a ratio of either 9:1 or 3:1. Recoveries of 60-70 percent have been reported for chloroform: isopropanol (3:1) (7). However, toluene:butanol (9:1) (34), ethyl acetate:isobutanol (9:1) (50), ethyl acetate + 10 percent isopropanol (40), benzene (32), ether (51), and 1,2-dichloroethane + 30 percent isopropanol (12) are among other solvents reported. A morphine recovery of 85-100 percent from urine has been claimed for a system involving adsorption on a XAD-2 resin column and elution with chloroform: isopropanol (17).

A study (52) of methods of recovering morphine from biological samples prior to analysis resulted in the following conclusions: (1) acid hydrolysis of morphine conjugates is preferable to enzymatic hydrolysis unless other acid-labile drugs are to be analyzed, (2) chloroform:isopropyl alcohol (4:1) is the extraction solvent of choice because it removes most of the morphine from the biological sample in a single extraction and it is readily volatile, (3) sodium bicarbonate and ammonium carbonate are the preferred "salting out" agents, and (4) the ratio of biological sample to solvent should be about 1:4.

A review of the analysis of morphine by gas phase methods, including procedures for extraction from urine and blood for various biomedical applications and quantitation, was published in 1977 (53).

EXPERIMENTAL PROCEDURE

The experimental procedure involves a single extraction of a buffered and salt-saturated solution of the biological fluid with a solvent system consisting of toluene:heptane: isoamyl alcohol (70: 20:10 v/v). 'The extract is then concentrated and derivatized by treatment with trifluoroacetic anhydride. Quantitation is achieved by selected ion monitoring using chemical ionization with methane and ammonia as reagent gases.

Standards and Reagents

The morphine used in the development of this procedure was purchased from Applied Science Laboratories, State College, PA 16801. No impurities were detected by either gas chromatography or mass spectrometry. Morphine labeled with 3 deuterium atoms on the N-methyl group (morphine-$^2H_3$) was prepared by a published procedure (54,55). Mass spectral analysis of the morphine-$^2H_3$ showed that 94 percent of the molecules contained 3 deuterium atoms, while 0.5 percent contained no deuterium atoms. Gas chromatography indicated a chemical purity of greater than 99 percent.

Prepare a pH 9.6 buffer by dissolving 500 grams of potassium monohydrogen phosphate (K_2HPO_4) in about 500 ml of distilled water. Allow the solution to cool to room temperature and then add sufficient distilled water to make exactly one liter.

Stock solutions of morphine and morphine-$^2H_3$ used to determine calibration graphs and prepare working standards are prepared as follows. Weigh into a 100-ml volumetric flask about 10 mg of morphine and record its weight to the nearest 0.1 mg. Dissolve the measured morphine in about 75 ml of methanol and then add additional methanol to make exactly 100 ml of solution. In the following paragraphs this solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled with its actual concentration based on the exact measured weight of morphine. A series of standard solutions can be prepared by appropriate dilution of this stock solution as described in Chapter 2.

The stock solution of morphine-$^2H_3$ is prepared in the same manner. For the measurement of morphine in body fluids within the concentration range 1 to 500 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml morphine-$^2H_3$ methanolic solution to each ml of specimen. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of morphine-$^2H_3$ to 250 ml with methanol.

Store stock and standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

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Extraction

Transfer 1 ml of the specimen (plasma, serum, whole blood, or urine) into a 5-ml glass stoppered, conical centrifuge tube. Add 100 µl of the 400-ng/ml morphine-$^2H_3$ internal standard solution and vortex for about 10 sec. Allow the sample to equilibrate for 15 min, and then add about 0.5 g of solid sodium chloride followed by 1 ml of the K_2HPO_4 buffer (pH 9.6). Vortex the mixture for about 10 sec. Add 1 ml of a solvent system consisting of toluene:heptane:isoamyl alcohol in a volume ratio of 70:20:10. Stopper the tube and vortex mix for at least 30 sec. Separate the phases by centrifugation for 10 min. Transfer most of the organic layer (top) to a 12-ml glass stoppered, conical centrifuge tube. Be careful not to transfer any of the aqueous (bottom) layer. Evaporate the organic extract just to dryness by heating to 70°C under a stream of filtered air. Stopper the tube and store under refrigeration until shortly before the GC/MS analysis is ready to be performed. For derivatization, allow the tube to warm to room temperature and then add 200 µl of chloroform plus 100 µl of trifluoroacetic anhydride. Cap the tube and heat at 70°C for 10 min. Allow the tube to cool at room temperature for 10 min and then evaporate to dryness by heating at 40°C under a gentle stream of dry nitrogen or dry air. The derivatized extract is subject to hydrolytic decomposition upon prolonged exposure to laboratory air, so it is important to reconstitute the residue immediately in about 30 µl of chloroform and make the injection into the GC/MS.

GC/MS Analysis

Recommended experimental conditions for the GC/MS analysis of the trifluoroacetyl derivative of morphine are as follows:

GC column	1.8 m x 2 nun (I.D.) glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier gas:	Methane, 15-20 ml/min
Reagent gas:	Ammonia, introduced into the ion chamber as described. in Chapter 2.
Temperatures:	Injector, 270°C Column, 225°C isothermal GC/MS transfer iine, 260ºC Ion source, 160°C

Under these conditions the bis-(trifluoroacetyl) morphine should elute at between 2 and 4 min.

Before beginning the GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 478 and 481. They correspond to the protonated molecule ions for bis-(trifluoro-

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acetyl) morphine and the $^2H_3$-analog, respectively. With the divert valve in the divert position, inject 2 to 6 µl of the chloroform solution of the extract into the gas chromatograph. After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer and begin data acquisition. When the morphine derivative has eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of free morphine in the biological sample is determined by measuring the heights (or areas) of the bis-(trifluoroacetyl) morphine peak and the $^2H_3$-analog peak in the ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the specimen concentration of free morphine by dividing the measured quantity of morphine by the exact volume of specimen extracted.

Discussion of the Experimental Procedure

The procedure described here is relatively rapid, yet it results in excellent sensitivity and specificity. Morphine concentrations as low as 1 ng/ml can be measured accurately and interference from other components of the extract is seldom a problem. If interferences are encountered it may be possible to remove them by incorporating a back-extraction into the method. When a back-extraction is desirable, follow the previously described procedure to the point where the toluene:heptane:isoamyl alcohol extract (approximately 1 ml) is transferred into a 12-ml glass stoppered, conical centrifuge tube. Then add 1 ml of 0.2 N H_2SO_4. Stopper the tube and shake or vortex for at least 30 sec. Discard the organic layer (top) and neutralize the aqueous layer (bottom) by addition of 1 ml of 0.2 N NaOH. Saturate the solution with sodium chloride (~1 g) and add 2.0 ml of the K_2HPO_4 buffer. Confirm that the pH of the solution is between 8.5 and 9.0, and then extract the morphine with 2 ml of the toluene:heptane:isoamyl alcohol solvent. Finally, proceed with the derivatization and GC/MS analysis as previously described.

Preparation of the bis- (trimethylsilyl) derivative of morphine by heating the extract with bis-(trimethylsilyl) trifluorocetamide (BSTFA) at 60°C for 1 hr, is an alternative to formation of the trifluoroacetyl derivative. Both derivatives have been used with satisfactory results. Trifluoroacetylation occurs somewhat more rapidly than the trimethylsilyation. However, the excess trifluoroacetic anhydride should be removed by evaporation prior to GC/MS analysis to avoid degradation of the GC column. (Injection of excess BSTFA onto the GC column does not appear to degrade column performance). Figures 1 and 2 show the methane-ammonia CI mass spectra of bis-(trifluoroacetyl) morphine and bis-(trimethylsilyl) morphine, respectively. In both spectra the only abundant ions correspond to the protonated molecule ions. A direct comparison has not been made of the stability of these two morphine derivatives, but no major differences have become evident so far.

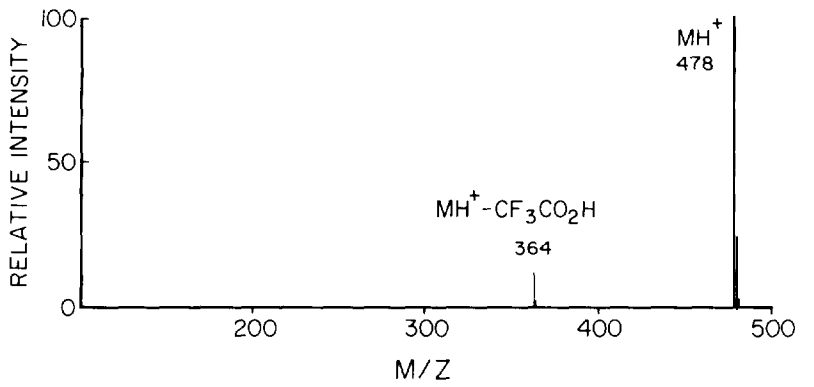


FIGURE 1. METHANE-AMMONIA CI MASS SPECTRUM OF BIS-(TRIFLUOROACETYL) MORPHINE

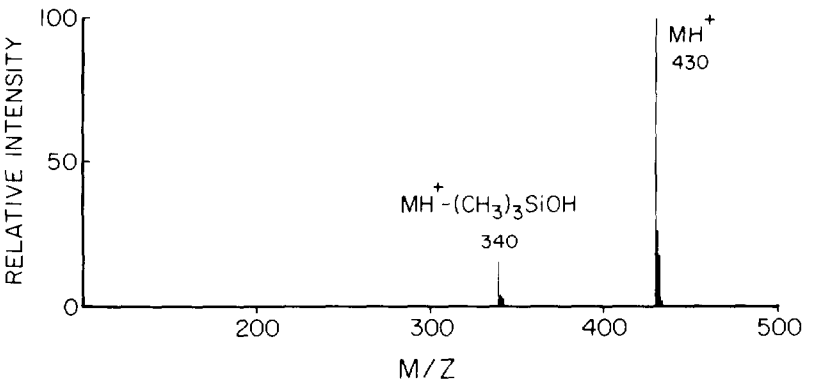


FIGURE 2. METHANE-AMMONIA CI MASS SPECTRUM OF BIS-(TRIMETHYLSILYL) MORPHINE

The procedure described here measures only free morphine in the body fluid. In urine, most of the excreted morphine is present as glucuronide and sulfate conjugates. Consequently, in order to measure the total morphine it is necessary to hydrolyze the conjugated morphine before extraction. Acid hydrolysis can be performed as follows (43):

Transfer 1 ml of specimen into a 12-ml culture tube. Add 100 µl of the 400-ng/ml morphine-$^2H_3$ internal standard solution and vortex for about 10 sec. Allow the sample to equilibrate for 15 min. Add 1 ml of 6 N HC1 (prepared by a 1 to 1 dilution of concentrated hydrochloric acid with distilled water). Vortex the mixture and then heat in a boiling water bath for 1 hr. After cooling to room temperature neutralize the mixture by slowly adding 1 ml of 6 N NaOH. Saturate the solution with sodium chloride to facilitate extraction of the free morphine. Add 2 ml of the K_2HPO_4 buffer and check to see that the pH of the mixture is between 8.5 and 9.0. Extract the morphine with 1 ml of the toluene:heptane:isoamyl alcohol solvent, and proceed with the derivatization and GC/MS analysis as previously described.

Other opiate narcotics such as codeine and 6-monoacetyl morphine can be measured simultaneously by the described method if the corresponding deuterium-labeled analogs are available for use as internal standards. The protonated molecule ions for trifluoroacetyl codeine and its $^2H_3$-analog occur at m/z 396 and 399, respectively; in the case of the trifluoroacetyl derivative of 6-monoacetyl morphine and its $^2H_3$- analog, these ions occur at m/z 4.24 and 427. Figures 3 and 4 show representative calibration graphs for morphine and codeine in plasma. The graphs are linear for drug concentrations from 1 to 500 ng/ml. If higher concentrations are encountered or anticipated, the specimen should be diluted with distilled water in order to reduce the drug concentration so that it falls within the linear range of the calibration graph.

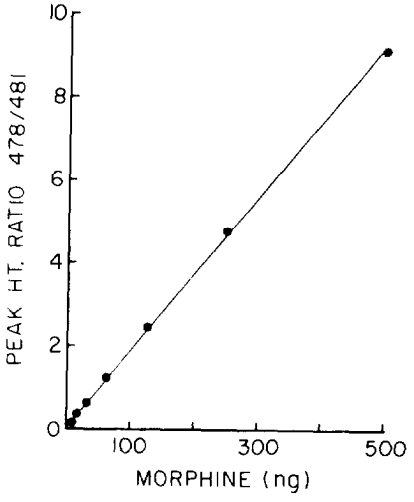


FIGURE 3. CALIBRATION GRAPH FOR ANALYSIS OF MORPHINE IN PLASMA

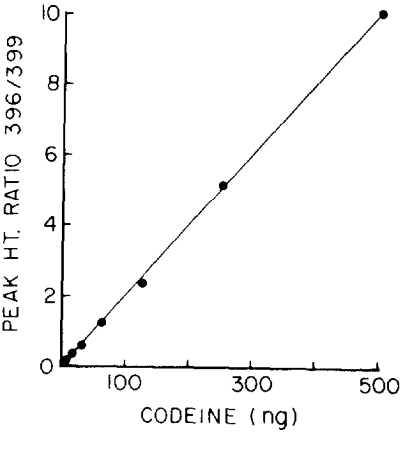


FIGURE 4. CALIBRATION GRAPH FOR ANALYSIS OF CODEINE IN PLASMA

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR MORPHINE

Nomenclature

Chemical Name: 7,8-Dihydro-4,5-epoxy-17-methyl-morphinan-3,6-diol

Empirical Formula: $C_{17}H_{19}NO_3$

Chemical Abstracts Registry Number: 57-27-2

Physical Constants

Melting Points: 254°C with decomposition

Specific Rotation: $\alpha]_D^{25}$ = -132° (methanol)

Solubility: 1g of morphine dissolves in 5000 ml of water at room temperature, 1100 ml of boiling water, 210 ml of ethanol, 1220 ml of chloroform, 6250 ml of diethyl-ether, or 10 ml of boiling methanol.

pKa: 9.85

UV Absorption: λ max 287 nm (ethanol)

REFERENCES

1. U. Boerner, S. Abbott, and R.L. Roe. Drug Metabolism Reviews, 4, 39 (1975).
2. A.F. Casy in D. Clouet, ed., Narcotic Drugs: Biochemical Pharmacology. Plenum, New York, ch. 1 (1971).
3. G.J. Mannering, A.C. Dixon, E.M. Baker III, and T. Asami. J. Pharmacol. Exp. Ther., 111, 142 (1954).
4. P.A. Mitenko and P.C. Duke. Clin. Research, 22, 726A (1974).
5. S. Spector and L.S. Vesell. Science, 174, 421 (1971).
6. S.F. Brunk and M. Delle. Clin. Research, 21, 467 (1973).
7. S. Felby, H. Christensen, and A. Lund. Forensic Sci., 3, 77 (1974).
8. D.C. Perry. Clin. Toxicol, 8, 239 (1975).
9. J.R. Monforte. J. Forensic Sci., 22, 718 (1977).
10. W.O.R. Ebbighausen, J.H. Mowat, H. Stearns, and P. Vestergaard. Biomed. Mass Spectr., 1, 305 (1974).

11. S.Y. Yeh, R.L. McQuinn, and C.W. Gorodetzky. J. Pharm. Sci., 66, 201 (1977).

12. S.Y. Yeh, C.W. Gorodetzky and R.L. McQuinn. J. Pharmacol. Exp. Ther., 196, 249 (1976).

13. U. Boerner and S. Abbott. Experientia, 29, 180 (1973).

14. U. Boerner, K.L. Roe, and C.E. Becker. J. Pharm. Pharmacol., 26, 393 (1974).

15. S.Y. Yeh. J. Pharmacol. Exp. Ther., 192, 201 (1975).

16. J.F. Taylor in D.Clouet, ed., Narcotic Drugs: Biochemical Pharmacology. Plenum, New York, ch. 2 (1971).

17. S.J. Mulé. J. Chromatogr. Sci., 12, 245 (1974).

18. H.H. Loh, I.K. Ho, T.M. Cho, and W. Lipscomb. J. Chromatogr., 76, 505 (1973).

19. H. Kupferberg, A. Burkhalter, and E.L. Way. J. Pharmacol. Exp. Ther., 145, 247 (1964).

20. S.J. Mulé and P.L. Hushin. Anal. Chem., 43, 708 (1971).

21. A.E. Takemori. Biochem. Pharmacol., 11, 1627 (1968).

22. J.M. Hicks, R.A. Sonoff, and K.J. Koch. Clin. Chem., 20, 901 (1974).

23. I. Jane and J.F. Taylor. J. Chromatogr., 109, 37 (1975).

24. G.W. Aherne, V. Marks, B.A. Morris, E.M. Piall, J.D. Robinson, and R.G. Twycross. Brit. J. Pharmacol., 54, 228P (1975).

25. D. Sohn, J. Simon, M.A. Hanna, G.V. Ghali, R.A. Tolba, and V. Melkonian. Anal. Chem., 45, 1498 (1973).

26. M. Koida, M. Takahashi, and H. Kaneto. Japan. J. Pharmacol., 24, 707 (1974).

27. G. Gintzler, E. Mohacsi, and S. Spector. Europ. J. Pharmacol., 38, 149 (1976).

28. J.W.A. Findlay, R.F. Butz, and K.M. Welch. Life Sci., 19, 389 (1976).

29. M. Steiner and J.L. Spratt. Clin. Chem., 24, 339 (1978).

30. G.R. Wilkinson and E.L. Way. Biochem. Pharmacol., 18, 1435 (1969).

31. K.E. Rasmussen. J. Chromatogr., 120, 491 (1976).

32. D.A. Smith and W.J. Cole. J. Chromatogr., 105, 377 (1975).

33. J.E. Wallace, H.E. Hamilton, K. Blum, and C. Petty. Anal. Chem., 46, 2107 (1974).

34. B. Dahlström and L. Paalzow. J. Pharm. Pharmacol., 27, 172 (1975).

35. M.J. Kogan and M.A. Chedekel. J. Pharm. Pharmacol., 28, 261 (1976).

36. G. Nicolau, G. Van Lear, B. Kaul, and B. Davidow. Clin. Chem., 23, 1640 (1977).

37. J.M. Moore. J. Chromatogr., 147, 327 (1978).

38. W.O.R. Ebbighausen, J.H. Mowat, and P. Vestergaard. J. Pharm. Sci., 62, 146 (1973).

39. W.O.R. Ebbighausen, J.H. Mowat, P. Vestergaard, and N.S. Kline. Adv. in Biochem. Psychopharmacol., 7, 135 (1973).

40. P.P. Hipps, M.R. Eveland, E.R. Meyer, W.R. Sherman, and T.J. Cicero. J. Pharmacol. Exp. Ther., 196, 642 (1976).

41. W.J. Cole, J. Parkhouse and Y.Y. Yousef. J. Chromatogr. 136, 409 (1977).

42. P.A. Clarke and R.L. Foltz. Clin. Chem., 20, 465 (1974).

43. D. Pearce, S. Wiersema, M. Kuo, and C. Emery. Clin. Toxicol., 14, 161 (1979).

44. D. Reed. Clin. Toxicol., 14, 169 (1979).

45. I. Jardine and C. Fenselau. J. Forensic Sci., 20, 373 (1975).

46 . M. .Anbar and W.H. Aberth. Anal. Chem., 46, 59A (1974).

47. J.A. Lawson, J.I. Degraw, and M. Anbar. J. Heterocyclic Chem., 13, 593 (1976).

48. F. Fish and T.S. Hayes. J. Forensic Sci., 19, 676 (1974).

49. T.N. Higgins and J.D. Taylor. Clin. Biochem., 7, 280 (1974).

50. G.R. Nakamura and E.L. Way. Anal. Chem., 47, 775 (1975).

51. F. Fish and W.D.C. Wilson. J. Chromatogr., 40, 164 (1969).

52. D.B. Predmore, G.D. Christian, and T.A. Loomis. J. Forensic Sci., 23, 481 (1976).

53. J.P.G. Tehnot and K.D. Haegele in D. Glick, ed., Methods of Biochemical Analysis, Vol. 24. Interscience, John Wiley and Sons, New York, p. 101 (1977).

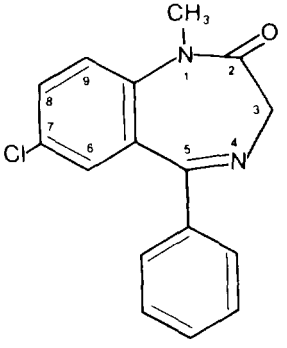
54. C. Elison, H.W. Elliott, M. Look, and H. Rapoport. J. Med. Chem., 6, 237 (1963).

55. H. Rapoport and M. Look. U.S. Patent 2,890,221; Chem. Abstr., 54, 612f (1960).

Chapter 9

Diazepam and its Major Metabolite, N-Desmethyldiazepam

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, I) was first synthesized in 1959 and introduced clinically in the early 1960's as an antianxiety agent and a muscle relaxant (1). It can be synthesized by the reaction of 2-methylamino-5-chlorobenzophenone and ethyl glycinate, or by a variety of other routes (2) . Diazepam has been marketed since 1963 as Valium and has become the most commonly used prescription drug in the Western world (1).



I

The most important clinical effects of the drug are mediated through the central nervous system (CNS); the limbic system of the brain, which controls emotions, has been implicated in attempts to locate the site of antianxiety activity. Diazepam has muscle relaxant and strong anticonvulsant properties also, but the sites of action for these activities have not been identified. Unlike barbiturates, drugs of the benzodiazepine class do not appear to produce clinically important hepatic microsomal induction and are more benign than other sedative-hypnotic drugs with respect to cardiovascular and respiratory depression (1). Tolerance does develop, however, so that larger doses become necessary to achieve the desired effects. Recent clinical studies have indicated that sensitivity to the CNS-depressant effects of diazepam increases with age (3). Side effects of therapeutic doses (usually 20 mg daily or less) can include drowsiness, ataxia, disorientation, and vertigo (4). There is evidence for an association between the ingestion of diazepam in the first trimester of pregnancy and the incidence of oral clefts in infants (5). However, because of its relatively wide margin of safety, death due to diazepam overdose is rare. Significant impairment of psychomotor skills at doses of 10-20 mg was demonstrated in a study related to driving ability, and this impairment combined with the sleep-inducing effect of the drug makes its possible contribution to traffic accidents an impor-

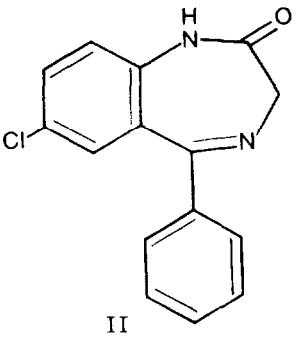
tant consideration (6). Diazepam potentiates the depressant effect of methadone, adding to problems of addict management. Abuse of diazepam (40-80 mg daily), leading to severe withdrawal symptoms, has been reported in drug treatment programs (7). These dangers associated with the misuse of diazepam caused the Food and Drug Administration to reclassify it as a Schedule IV drug.

METABOLISM AND PHARMACOKINETICS

In man, diazepam undergoes two major metabolic transformations: N-demethylation and 3-hydroxylation. In the first of these routes, diazepam is metabolized by liver microsomal enzymes to N-desmethyldiazepam (II) which is then hydroxylated to form oxazepam (III). The diazepam hydroxylation pathway yields IV, which is subsequently demethylated to III (3). The major metabolite present in blood is II, although III and IV are also found, and with chronic administration II will equal or exceed diazepam blood concentrations. The contribution of N-desmethyldiazepam (II) to the clinical effects of diazepam in man is not clear (3,8), although the pharmacological properties of the metabolite are generally considered to be similar to its precursor; and the anticonvulsant effect, in fact, seems to require dealkylation (9). Oxazepam exerts anticonvulsant activity in mice comparable to that of N-desmethyldiazepam. In man, 60 to 75 percent of a dose of diazepam administered orally is excreted in the urine. The most abundant metabolite in human urine is the oxazepam-glucuronide, but N-desmethyldiazepam, IV-glucuronide, and p-hydroxy-diazepam (V) are also found (10). Intact diazepam has not been reported in more than trace amounts in urine (11).

The increase in plasma concentrations of N-desmethyldiazepam relative to the parent drug following repeated doses of diazepam has suggested that diazepam might induce its own metabolism (12). However, there is evidence for an alternative explanation; i.e., the tissues may be saturated with the metabolite following the first pass hepatic metabolism so that repeated doses result in elevated plasma concentrations (13). N-desmethyldiazepam has also been reported in human plasma as a metabolite of chlorodiazepoxide (Librium) (14).

Diazepam is highly bound (> 97 percent) to plasma proteins (15) and is metabolized completely but slowly in man, with a terminal elimination half-life usually reported to be between 20 and 50 hr, although it can range up to 90 hr in normal individuals (1,8,16). The half-life is age-dependent; i.e., up to 5 times longer in elderly persons than in young adults (16). The half-life of desmethyldiazepam is longer, about 50-100 hr (8,17), and it too is about 97 percent protein-bound. Diazepam is lipid soluble but only sparingly soluble in water at physiological pH, so that intramuscular absorption is slow and erratic, and oral or intravenous routes of administration are preferred (15). Because of its long half-life, diazepam will accumulate in plasma. For example, if administered three times per day, the concentration may increase 3-fold over that following a single dose. Desmethyldiazepam accumulates more slowly, usually appearing in plasma 24-36 hr after



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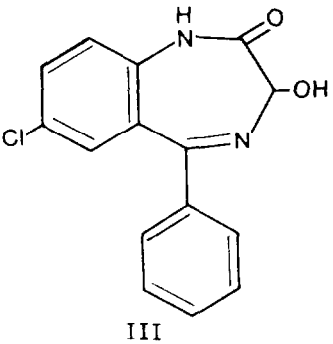

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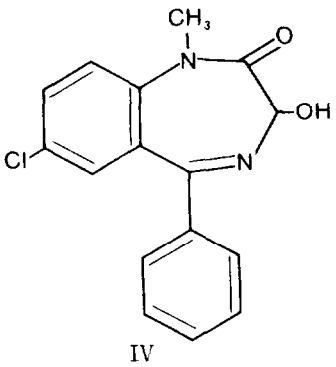

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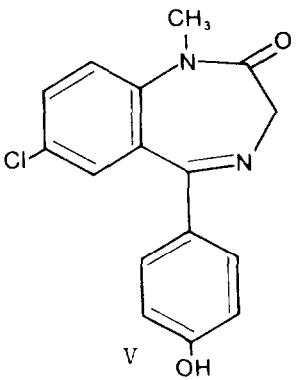


V

the first dose, and both the parent drug and the metabolite reach steady state after 5-7 days (18,19). Both diazepam and N-desmethyldiazepam can be present in pharmacologically active concentrations a week after medication is stopped (19). However, wide variations in pharmacokinetic profiles are seen between individuals, and no simple correlation exists between clinical response and plasma levels of diazepam and N-desmethyldiazepam. Nevertheless, a minimum steady state plasma concentration of diazepam of 400 ng/ml has been reported to be necessary for relief of acute anxiety (20).

During chronic therapy in patients given doses of 4-60 mg diazepam per day, plasma concentrations are reported to range from 20-200 ng/ml of diazepam and 32-2750 ng/ml of N-desmethyldiazepam. In the same study, absorption of diazepam and its metabolite by erythrocytes was observed after 11 weeks of administration. Uncon-

jugated forms of diazepam and metabolites appeared in urine following several weeks of medication (21).

After intravenous injection of 20 mg of diazepam, a maximum concentration of 1600 ng/ml was reached in 15 min, while 20 mg taken orally gave a maximum blood concentration of 490 ng/ml in 30 min and the maximum after intramuscular administration was 290 ng/ml in 60 min (22). In other investigations, however, oral administration of 10 mg diazepam resulted in peak plasma concentrations of 100-200 ng/ml after 1.5 hr (23). 'The drug was labeled with tritium for this study, and the decline of radioactivity in the plasma indicated a rapid and extensive uptake by tissues followed by a redistribution into blood of both diazepam and N-desmthyldiazepam.

The rate at which diazepam returns to the central compartment from the deep peripheral compartment has been shown to be the rate-controlling factor in the elimination of diazepam from the body and in formation of dealkylated metabolites; only 18 percent of the drug in the body is present in the central compartment and available for elimination at any given time (17). In vitro displacement of diazepam from human serum albumin by lauric acid demonstrated that protein binding, as well as tissue distribution, could be affected by fluctuations in plasma free fatty acids (24). Because human bile contains virtually no diazepam, N-desmethyldiazepam, or their glucuronide conjugates, as shown by a study using ^{14}C-diazepam their long half-lives in plasma cannot be explained by enterohepatic circulation (10). The presence of liver disease, however, has been found to decrease the protein binding of diazepam and to prolong its plasma elimination half-life (16). Chronic ingestion of alcohol. (25), as well as food intake (26), can also affect the pharmacokinetics of intravenously administered diazepam.

The entry of free, unbound diazepam and N-desmethyldiazepam into the cerebrospinal fluid (CSF) appears to be by passive diffusion, quickly reaching concentrations equivalent to 2-4 percent of the total plasma concentration (11). After long-term diazepam therapy, however, some accumulation of the metabolite in the CSF relative to plasma has been observed which could reflect high concentrations of the metabolite in brain tissue following repeated administration of diazepam (9). Desmethyldiazepam is known to accumulate in fetal brain, heart, liver, and lung tissues when the mother has taken diazepam (27).

The concentration of diazepam in saliva has been shown to be directly proportional to the concentration of unbound drug in plasma (saliva/plasma ratio 0.03). This finding is significant in that it raises the possibility of obtaining a sample by a noninvasive method for the determination of plasma concentrations (28).

An extensive review of the clinical pharmacokinetics of diazepam and N-desmethyldiazepam has been published (29).

ANALYTICAL METHODOLOGY

A quantitative ultraviolet spectrophotometric assay capable of measuring blood concentrations of diazepam and N-desmethyldiazepam resulting from doses greater than 50 mg was reported in 1966. Quantitation was based on measurement of absorbance values at 240 nm. The sensitivity limit of 0.3 μg/ml was sufficient for toxicological purposes (18). A fluorometric screening method capable of detecting benzodiazepines in blood and urine at concentrations of 25 ng/ml has been reported more recently, but it does not distinguish between diazepam and its metabolites. The benzodiazepines present are hydrolyzed to benzophenones and then converted to fluorescent 9-acridanones by heating with lead dioxide (30).

For human metabolic studies using ^{14}C-labeled diazepam, thin-layer chromatography (TLC) of extracts, using chloroform: acetone (9:1) as developing solvent, followed by scintillation counting of the appropriate sections of gel, has been used for measurement of diazepam and its metabolites in blood, urine, and bile (10). Recently, another TLC procedure was developed for use in bioavailability studies. Carbamazepine was added as an internal standard to buffered serum (pH 8) before extraction with n-hexane containing 10 percent isobutanol. The dried residue was dissolved in chloroform and spotted on the TLC plate; the developing solvent was ethyl acetate:benzene:ammonia (35:15:0.15). After drying, the plates were exposed to HCl gas for 30 min and then to radiation at 254 nm. Diazepam, N-desmethyldiazepam, and the internal standard were converted to fluorescent compounds by this procedure, and fluorescence was enhanced by dipping the plates in a 10 percent solution of paraffin wax in light petroleum. Quantitative fluorimetry (λ_{ex} 360 nm, λ_{em} 460 nm) was then possible based on calibration graphs relating peak height ratios to drug concentrations over the range 50-500 ng/ml (31).

Differential pulse polarography based on the reduction peak of the 4,5-azomethine group, while not specific for diazepam among 1,4-benzodiazepines, can measure this class of compounds in blood at a sensitivity of 250 ng/ml. The concentration of drugs and metabolites is calculated on the basis of the resultant current of appropriate internal standards. Resolution of particular benzodiazepines can be achieved by incorporation of a TLC step, which also increases the sensitivity to 50 ng/ml (32).

Currently, the techniques most widely used for analysis of benzodiazepines include high performance liquid chromatography (HPLC), immunoassays, and gas chromatography with electron capture detection (GC-ECD). HPLC has the advantage that it can be used to analyze for any of the benzodiazepines and their major metabolites. The immunoassay techniques (RIA, EMIT) offer greater speed and, when large numbers of samples are involved, lower cost than chromatographic methods; however, they have a very limited dynamic range and are more suitable for qualitative screening than quantitative measurement. GC-ECD offers excellent sensitivity and specificity if a high quality column is used, but is unsatisfactory for the

analysis of some benzodiazepines and their metabolites because of their thermal instability. A more detailed discussion of each of the techniques follows.

Quantitative high performance liquid chromatography on porous silica, using n-heptane:isopropanol:methanol (40:10:1) with detection by UV absorption at 232 nm, was developed for routine forensic determinations of diazepam and N-desmethyldiazepam in human blood. Extraction was made with benzene followed by a back extraction into acid. When concentrations were greater than 100 ng/ml., 1 ml of blood was sufficient for quantitation by HPLC, but for lower concentrations (25-100 ng/ml), 2 ml of blood and repetition of each extraction step was necessary (33).

A reverse-phase HPLC column was used for simultaneous measurement of diazepam and its pharmacologically active metabolites, N-desmethyldiazepam and oxazepam (34). An internal standard (chlordiazepoxide) was added to 250 µl of serum or plasma together with a sodium phosphate buffer (pH 11) and the mixture was extracted with chloroform. The extract was chromatographed on a µ-Bondapak CN column (Waters Association, Milford, MA) using a solvent system of acetonitrile and phosphate buffer (1:2). UV detection at 254 nm gave a linear response from 20 to 2000 ng for each drug and metabolite. Day-to-day precision was reported to be 5 to 10 percent. Two recent reports also describe reverse-phase HPLC procedures for diazepam which were used to confirm measurements obtained by GC-ECD). Both methods employed UV detection at 254 nm (35,36).

An electrochemical detector operated in a reduction mode served as the detector for an HPLC assay for diazepam, nitrazepam, and chlordiazepoxide. A mobile phase of methanol:water (60:40) containing 0.05 M ammonium acetate (pH 7.25) was used with a reverse-phase column. Interference due to reduction of oxygen is a major problem when electrochemical detectors are operated in the reduction mode. Attempts to remove oxygen from the solvent and sample failed to completely eliminate this interference. However, diazepam, which can be measured at a reduction potential of -0.93 v, could be detected at quantities as low as 3 ng (37).

A radioimmunoassay (RIA) procedure for diazepam in blood extracts was developed following production of two different antibodies, one specific for diazepam and the other recognizing both diazepam and its metabolite, N-desmethyldiazepam. The RIA was capable of detecting 1 ng of the drug in dried ether extracts of whole blood, and the response was linear to 100 ng (38). In a separate RIA developed specifically for N-desmethyldiazepam, cross reaction with diazepam and most other benzodiazepines was less than 1 percent; this assay permitted detection of as little as 3 ng/ml of the metabolite in only 10 µl of plasma (39). Another immunoassay procedure, EMIT, has also been reported for benzodiazepines (40), but it is not specific for diazepam and is used only for urine because of interference by enzymes in plasma.

Gas chromatography (GC) has been used extensively in the analysis of benzodiazepines. The high polarity and low volatility of the benzodiazepines require use of a thermally-stable liquid phase and well-deactivated column packings. Because gas chromatography of these compounds, and particularly N-desmethyldiazepam, is difficult, some of the earlier GC methods involved conversion of the benzodiazepines to benzophenones by acid hydrolysis prior to extraction and GC analysis (41,42). However, with recent improvements in GC column technology it is now possible to chromatograph both diazepam and N-desmethyldiazepam without prior chemical transformations (Table 1).

Gas chromatography with flame ionization detection (FID) does not offer sufficient sensitivity to assay blood concentrations of diazepam after therapeutic dosage. However, because of the presence of the electronegative chlorine substituent, diazepam and its metabolites are well suited for electron capture detection (ECD) and many assays using this detector have been published (9,28,35,36, 43-50).

In general, the GC-ECD assays are able to measure diazepam concentrations as low as about 10 ng/ml, although a sensitivity of 1 ng/ml has been reported for an assay of the drug in human cerebrospinal fluid when a ^{63}Ni detector was used and griscofulvin was added as an internal standard to the extraction residue just before injection into the chromograph (9). An equally sensitive (1 ng/ml) method for the assay of N-desmethyldiazepam, suitable for pharmacokinetic studies, uses N-desmethyl tetrazepam as the internal standard and relies on extraction from plasma with diethyl ether and an acid back extraction for additional purification. A recovery of at least 70 percent is reported. The dried ether residue is dissolved in hexane:acetone (4:1) and chromatographed on a column containing 1 percent Poly-A103 on Gas Chrom Q with a ^{63}Ni detector. This degree of sensitivity requires scrupulously clean glassware, extremely pure reagents, and careful extraction/purification procedures (4 7). ECD assays are time-consuming and are usually unsuitable for rapid analysis of large numbers of clinical samples; therefore a simplified procedure was developed in which 2 ml of Na_3PO_4-saturated distilled water was added to 1 ml of whole blood to precipitate proteins (final pH = 12.8). After thorough vortex mixing, the sample was extracted with benzene:methylene chloride (9:1). The extract was evaporated to dryness, dissolved in 100 µl benzene:acetone:methanol (80:15:10) and analyzed by GC-ECD without further purification. The sensitivity reported for this procedure was 5 ng/ml of blood (43).

In another adaptation of GC-ECD for rapid (< 1 hr) quantitation of diazepam in 1-ml samples of human blood, methyl clonazepam was added to serum or plasma as an internal standard prior to extraction with toluene at pH 9, back extraction into acid, and reextraction with toluene. Underivatized diazepam and N-desmethyldiazepam were analyzed on a 3 percent OV-17 column equipped with ^{63}Ni-ECD (44) Similarly, sensitivities of 5 ng diazepam and 10 ng N-desmethyldiazepam per ml of plasma have been reported for automated

TABLE 1. GAS CHROMATOGRAPHIC ASSAYS FOR DIAZEPAM AND/OK N-DESMETHYLDIAZEPAM

Extraction Solvent	GC Column	Internal Standard	Detector	Reference
Toluene:heptane (4:1)	2% OV-17	Griseofulvin	ECD	9
Diethyl ether plus back extraction	1% Poly-A103	Desmethyl-tetrazepam	ECD	47
Benzene: methylene chloride (9:1)	3% OV-17		ECD	43
Toluene plus back extraction	3% OV-17	Methyl-clonazepam	ECD	44
Benzene	3% OV-17	Methyl nitrazepam	ECD	45,48
Toluene:heptane (4:1) plus 1.6% isoamyl alcohol	3% OV-17	Medazepam	ECD	46
Toluene:heptane: isoamyl alcohol (76:20:4)	3% OV-17	Flunitrazepam	ECD	35
Hexane:ethyl acetate (7:3)	3% SP2250-DB	Prazepam	ECD	36
Toluene	3% OV-17	Flurazepam	ECD	28
Toluene:heptane (9:1)	3% OV-17	Prazepam	ECD	49
Diethyl ether plus back extraction	3% OV-17	Prazepam	NPD	51
Benzene:isoamyl alcohol	3% OV-17	Ro 7-9957 and Ro 7-9749	ECD	50
- -	3% OV-17	N-C^2H_7-diazepam	MS	52
Ethyl acetate		N-C^2H_7-diazepam	MS	53

Extraction Solvent	GC Column	Internal Standard	Detector	Reference
Diethyl ether plus back extraction	1% OV-17	Medazepam	FID	54
Butyl chloride	2% OV-17	Prazepam	FID	55

GC-ECD analysis using benzene for extraction and methylnitrazepam as internal standard (45). When a single-step extraction method was combined with GC using a frequency-response ECD, linear sensitivity over the range of 10- 500 ng/ml of serum was achieved (46). The method permitted results of up to 40 clinical samples to be reported on the same day.

Another rapid extraction procedure has been applied to simultaneous quantitation of four benzodiazepines in blood and plasma. One ml of saturated borate buffer was added to 0.05 to 1.0 ml of body fluid and the mixture extracted with 1 ml of a solvent system consisting of toluene:heptane:isoamyl alcohol (76:20:4). After centrifugation, 1.5 µl of the organic phase was removed and injected directly into the GG-ECD. The method gave adequate sensitivity for measurement of therapeutic levels of the benzodiazepine drugs and their metabolites (35).

N-Desmethyldiazepam has been measured in plasma by GC-ECD following administration of dipotassium clorazepate, a drug which undergoes spontaneous decarboxylation to N-desmethyldiazepam at pH < 4 (48). A rapid and convenient extraction procedure was used, consisting of addition of 0.1 ml saturated potassium chloride solution to 0.1 ml of plasma and extraction with 1 ml of benzene containing 25 ng of methylnitrazepam as the internal standard.

Various methods have been reported for improving the chromatographic characteristics of the benzodiazepines, and particularly N-desmethyldiazepam. The need for priming the GC column before beginning each day's analyses is frequently mentioned. Priming agents that have proven beneficial include: 1) blank plasma extracts (35,50,59), 2) lecithin in ethanol (5 g/l) (49), and 3) cholesterol in acetone (49). A new liquid phase (SP 2250-DB, Supelco, Inc.) was found to considerably reduce the GC peak tailing of N-desmethyldiazepam when compared with OV-17 or OV-1 columns (36).

Because the free N-hydrogen in N-desmethyldiazepam can hydrogen bond to active sites in the GC column, conversion of the metabolite to an N-alkyl derivative can result in dramatically improved chromatographic behavior. The N-butyl derivative was formed by treatment of a dimethylacetamide solution of the extracted residue with tetrabutylammonium hydroxide and 1-iodobutane (49). The N-butylation procedure gave more reliable results than formation of the

trimethylsilyl derivative (54). A similar derivatization involving formation of the N-propyl derivative of N-desmethyldiazepam was combined with use of a nitrogen-phosphorus detector for quantitation of diazepam and its major metabolite in whole blood (51).

Although the FID is not as sensitive as the ECD, it is less easily contaminated and because of its wider range of linearity it is often preferred for routine use in pathology and toxicology laboratories. The incorporation of back extraction procedures and internal standards allows diazepam to be measured at serum concentrations as low as 80 ng/ml (54). However, because many neutral and basic drugs may be present in the final extract, confirmation of identity by TLC is desirable (55).

The use of mass spectrometry as the detector system for GC separation of drugs in biological fluids is indicated when the drugs are present in the picogram to low nanogram per ml range. N-C^2H_3 Diazepam was used as the internal standard in two studies concerned with the quantitation of diazepam in plasma (52) and in breast milk (53) by GC/MS.

Both diazepam and N-desmethyldiazepam are readily extracted from biological media by various organic solvents. The extractions are normally done at alkaline pH. Back extraction into acid has been employed to achieve additional sample purification. However, some salts of the drug and its metabolites are difficult to extract from solvents such as chloroform and methylene chloride.

Diethyl ether is commonly used for extraction of diazepam and N-desmethyldiazepam from blood, urine, or bile (16,18,38,54,56); at pH 7 the recovery efficiency is 90 percent. Other solvents reported for diazepam extraction include benzene (> 90 percent from blood, urine hydrolysates, or tissue homogenates) (10,26,43,45,50); benzene:methylene chloride (9:1, > 85 percent from blood) (3,32,43); ethyl benzoate (56); chloroform (90 percent) (58,59); hexane:isoamyl alcohol (98.3:1.7) from blood or gastric fluid (30); 1-chlorobutane followed by chloroform after back extraction into acid (100 percent from blood or tissue homogenates) (55); and toluene: heptane:isoamyl alcohol (78: 20:2, 96 percent at pH 9.5 from blood, homogenates, and urine) (21,35); or toluene containing 1.5 percent amyl alcohol to prevent absorption onto glass (83 percent from blood) (44).

The choice of solvent and pH depends in part on the metabolites to be measured. For example, the hydroxy metabolites have different partition properties from diazepam, and oxazepam is lost at a pH greater than 10 (21). Because diazepam metabolites occur mainly in the form of water-soluble conjugates in urine, Amberlite XAD-2 resin has been recommended for extraction of these compounds from urine made alkaline to pH 8-9. Absorbed compounds are eluted with ethyl acetate:methanol:acetic acid (90:10:0.1). The eluate is evaporated and the residue is taken up in buffer and hydrolyzed with ß-glucuronidase/arylsulfatase before extraction with ethyl acetate. A recovery of 95 percent has been reported for this procedure (60).

Diazepam is remarkably stable; no loss of concentration was found to occur in plasma samples stored for 1 year at 20°C, 8 weeks at 4°C, or 20 days at room temperature (61).

Extensive reviews of the analysis of diazepam and other benzodiazepines in biological samples have been published (4.1,62).

EXPERIMENTAL PROCEDURE

Blood concentrations of the active metabolite, N-desmethyldiazepam, are often comparable to, or greater than that of the parent drug. Therefore it is important to measure the concentrations of both compounds. The following procedure involves addition of deuterated analogs of diazepam and its major metabolite to the body fluid specimen for use as internal standards and a simple direct extraction into a small voulme of organic solvent followed by injection of an aliquot of the organic extract into the GC/MS. The ratios of concentrations of the drug and metabolite to their deuterated analogs are measured by selected ion monitoring using chemical ionization with methane and ammonia as reagent gases. Because the method involves only a single extraction, no concentration of the extract, and no derivatization, it is very rapid; also it should provide sufficient sensitivity to measure drug and metabolite concentrations at least as low as 5 ng/ml.

Standards and Reagents

The diazepam used in the development of this method was purchased from Applied Science Laboratories, State College, PA 16801. The N-desmethyldiazepam was obtained as a gift from Hoffmann-La Roche Inc., Nutley, N.J. 07110. The pentadeuterated analogs of diazepam and N-desmethyldiazepam were synthesized starting from bromobenzene-$^2H_5$ by published procedures (63). 0n the basis of GC/MS analysis, both deuterated analogs were more than 99 percent pure and had the following isotopic composition: $^2H_5$, 96.0 percent; $^2H_4$, 3.4 percent; $^2H_3$, 0.4 percent; and $^2H_2$, $^2H_1$ and $^2H_0$ < 0.2 percent. All of the deuterium atoms were located on the phenyl ring of each structure. The uncorrected melting points for the deuterated compounds were: diazepam-$^2H_5$, 131-132°C; N-desmethyldiazepam-$^2H_5$, 215-216°C.

Prepare a pH 9.6 buffer by dissolving 500 grams of potassium monohydrogen phosphate (K_2HP0_4) in about 500 ml of distilled water. Allow the solution to cool to room temperature and then add sufficient distilled water to make exactly one liter.

For the purpose of preparing calibration graphs and working standards stock solutions of diazepam and N-desmethyldiazepam are prepared as follows. Weigh into a 100-ml volumetric flask about 10 mg of diazepam and 10 mg of N-desmethyldiazepam, and record the weights to the nearest 0.1 mg. Dissolve the measured drug and metabolite in about 75 ml of methanol and then add additional methanol to givo exactly 100 ml of solution. This solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled

with its actual concentration based upon the exact measured weights of diazepam and N-desmethyldiazepam. A series of working standard solutions can then be prepared by appropriate dilution of the stock solution as described in Chapter 2.

The stock solution of diazepam-$^2H_5$ and N-desmethyldiazepam-$^2H_5$ is prepared in the same manner. For the measurement of drug and metabolite in body fluids within the concentration range 5 to 1000 ng/ml, addition of approximately 40 ng of the internal standards to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a methanolic solution containing 400 ng/ml of both diazepam$^2H_5$ and N-desmethyldiazepam-$^2H_5$ to each ml of specimen. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of diazepam-$^2H_5$ and N-desmethyldiazepam-$^2H_5$ to 250 ml with methanol.

Store stock and working standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction Procedure

Transfer 1 ml of body fluid (whole blood, plasma, or urine) to a 5-ml glass-stoppered, conical centrifuge tube. Add 100 µl of the internal standard solution containing 400 ng/ml of diazepam-$^2H_5$ and N-desmethyldiazepam-$^2H_5$ and vortex the mixture for 10 sec. Allow the sample to equilibrate for 15 min and then add 1 ml of the K_2HPO_4 buffer (pH 9.6), followed by 100 µl of a solvent consisting of toluene:heptane:isoamyl alcohol in a volume ratio of 70:20:10. Stopper the tube and vortex mix for at least 30 sec. Separate the phases by centrifugation for 10 min. The organic solvent will have formed a narrow upper layer from which an aliquot can be removed with a syringe for injection into the GC/MS, If there is not a clean separation of layers and a clear upper solvent layer, repeat the centrifugation at a higher speed (≥ 2000 X G).

GC/MS Analysis

The experimental conditions for the simultaneous GC/MS analysis of diazepam and N-desmethyldiazepam are as follows:

GC column:	1 m x 2 mm (I.D.) glass column packed with 3 percent OV-17 on Ultra-bond 20M, 100/120 mesh (RFR Corp., Hope, RI, 02831)
Carrier gas:	Methane, 15-20 ml/min
Reagent gas:	Ammonia, introduced into the ion chamber as described in Chapter 2
Temperatures	Injector, 290°C Column, 270°C isothermal GC/MS transfer line, 280°C Ion source, 160°C

328-011 0 - 80 - 10

Under these conditions the diazepam should elute at 1 to 2 min and the N-desmethyldiazepam at 2 to 4 min.

Prior to beginning the GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 285, 290, 271 and 276. They correspond to the protonated molecule ions for diazepam, diazepam-$^2H_5$, N-desmethyldiazepam, and N-desmethyldiazepam- $^2H_5$, respectively. With the divert valve in the divert position, inject 4 to 10 µl of the organic extract layer into the GC/MS. After approximately 1 min switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer and begin data acquisition. For maximum sensitivity monitor just the diazepam ion masses (m/z 285 and 290) until the dliazepam elutes from the GC column, and then monitor the N- desmethyldiazepam ion masses (m/z 271 and 276) until it elutes. If the GC/MS data system does not permit rapid changes in the monitored ion masses, all four ion masses must be monitored during the elution period for the drug and its metabolite. Normally, the latter procedure will result in only a small sacrifice in sensitivity. When the N-desmethyldiazepam has eluted, cease data acquisition and return the divert valve to the divert position. The quantities of diazepam and N-desmethyldiazepam in the body fluid are determined by measuring the peak heights (or areas) of the drug, the metabolite and the internal standards in the selected ion current profiles and relating the ratios of peak heights to calibration graphs (Chapter 2). Calculate the body fluid concentrations of diazepam and N-desmethyldiazepam by dividing the measured quantities of these compounds by the exact volume of specimen extracted.

Discussion of the Experimental Procedure

Diazepam and some of the other benzodiazepines and their metabolites are most often analyzed by GC-ECD which can provide very high sensitivity and specificity. The GC/MS method described here is capable of comparable sensitivity and even better specificity than the GC-ECD methods. Figures 1 and 2 compare the methane CI and the methane-ammnonia CI mass spectra of diazepam and N-desmethyldiazepam. All of the spectra are dominated by abundant protonated molecule ion peaks. For these two compounds either reagent gas can be used and should provide similar sensitivity, but the methane-ammonia reagent gas combination will result in a more selective ionization. High selectivity is necessary because the single, direct extraction procedure results in a relatively "dirty" extract.

Additional specificity can be provided by also monitoring the ^{37}Cl-isotope peaks (m/z 287, 292, 273, and 278) for each of the protonated molecule ions. The peak height of the ^{37}CI-isotope peak should be exactly one-third the intensity of the corresponding ^{35}Cl-isotope peak height for each compound. Any deviation from a ratio of one-third indicates an interfering compound is contributing to the ion current at one of the monitored masses. Usually, a visual examination of the ion current profiles will disclose

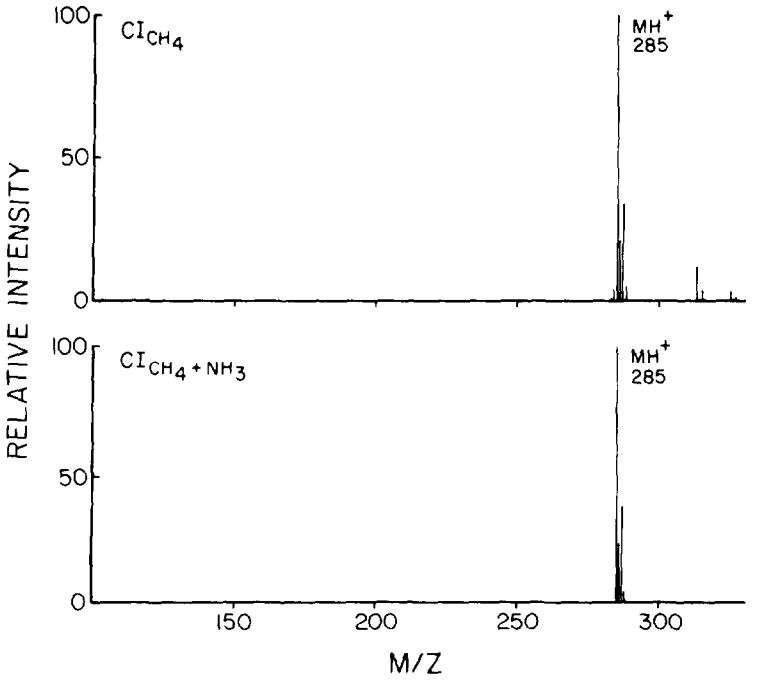


FIGURE 1. CHEMICAL IONIZATION MASS SPECTRA OF DIAZEPAM

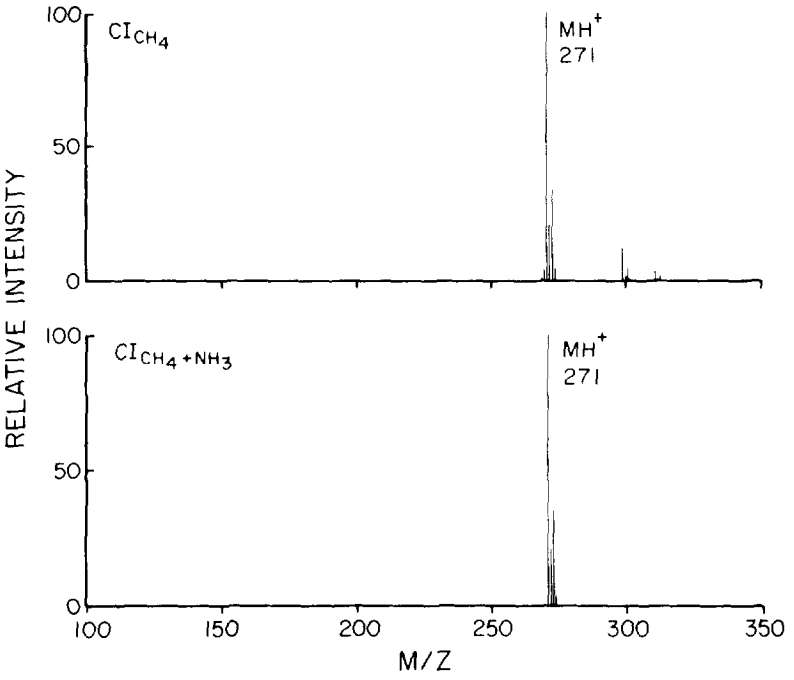


FIGURE 2. CHEMICAL IONIZATION MASS SPECTRA OF N-DESMETHYLDIAZEPAM

which of the peak heights is inaccurate due to the interference; i.e. the peak will have a shoulder, or will be perceptibly broadened, or will have an abnormal retention time.

If even greater sensitivity is needed, negative ion chemical ionization can be used. Diazepam and N-desmethyldiazepam have high electron affinities and therefore are efficiently ionized by electron capture under methane chemical ionization conditions. In unpublished work conducted at Battelle Columbus Laboratories, up to a 30-fold improvement in sensitivity was achieved by switching from positive ion detection to negative ion detection while operating the mass spectrometer under conventional methane CI conditions. Unfortunately, there are significant problems associated with quantitative measurements obtained with electron-capture negative ion chemical ionization. One difficulty is illustrated in Figure 3, which compares the negative ion CI mass spectra of diazepam acquired at ion source temperatures of 80° and 170°C. In the spectrum acquired at the lower ion source temperature the molecular anion at m/z 284 is the only prominent peak observed, and the chloride anion at m/z 35 has a relative intensity of only 10 percent. At an ion source temperature of 170°C a dissociative electron capture process dominates so that the chloride anion becomes the most intense peak in the spectrum, while the molecular anion peak has virtually vanished (about 2 percent relative intensity). Consequently, in order to use electron-capture negative ion chemical ionization for quantitative measurement of diazepam with high sensitivity, it is necessary to maintain a low ion source temperature (< 100°C) even though the ion source tends to become contaminated more rapidly at low temperatures. The large temperature effect experienced with negative ion chemical ionization can also cause poor reproducibility in quantitative measurements, although the use of deuterated analogs as internal standards largely compensates for changes in the ionization process.

The choice of chromatographic column is critical in the GC/MS analysis of diazepam and N-desmethyldiazepam, particularly when low nanogram-quantities are injected onto the column. The Ultra-bond 20M column packing supplied by the RFR Corporation is a very inactive surface, well suited for gas chromatography of polar drugs and metabolites. Both diazepam and N-desmethyldiazepam give narrow, relatively symmetrical peaks when chromatographed under the conditions specified here.

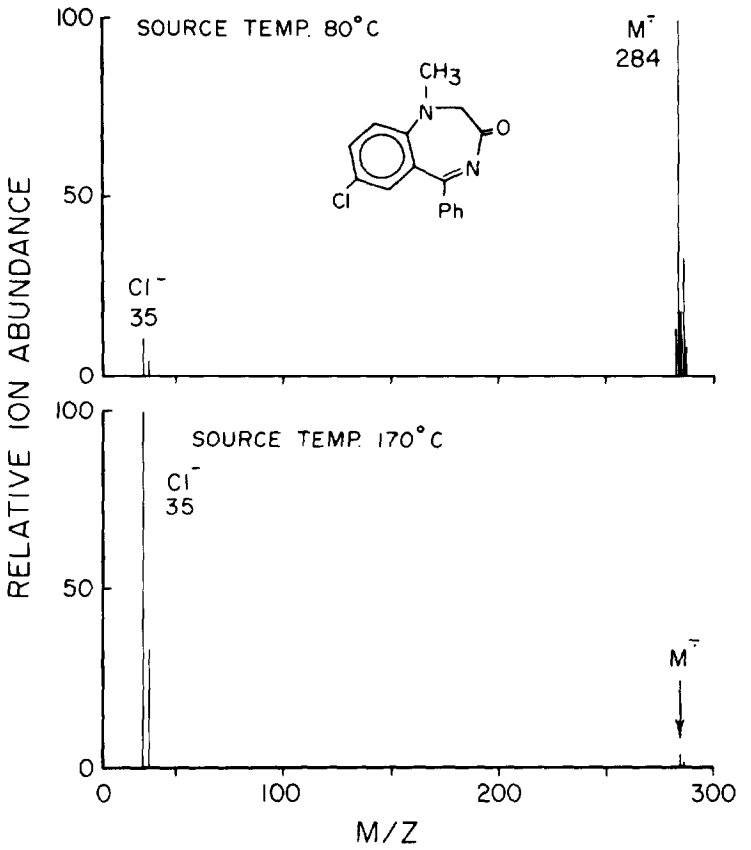


FIGURE 3. NEGATIVE ION METHANE CI MASS SPECTRA OF DIAZAPEM RECORDED AT DIFFERENT ION SOURCE TEMPERATURES

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR DIAZEPAM

Nomenclature

Chemical Name: 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one

Empirical Formula: $C_{16}H_{13}C1N_2O$

Chemical Abstracts Registry Number: 439-14-5

Trade Names: Ansiolin, Apavrin, Apozepam, Atensine, Atilen, Bialzepam, Calmpose, Ceregulart, Dipam, Eridan, Faustan, Lembrol, Levium, Morosan, Noan, Pacitran, Paxate, Paxel, Relanium, Seduxen, Setonil, Stesolid, Stesolin, Tranimul, Valium, Vival, Vivol

Physical Constants

Physical Appearance: Off-white to yellow crystalline powder

Melting Point: 125-126°C

Specific Rotation: optically inactive

pKa: 3.4

Solubility: At room temperature 1 g of diazepam will dissolve in 20 1 of water, 56 ml of diethyl ether, 20 ml of methanol, 4.5 ml of benzene, and less than 2 ml of chloroform.

UV Absorption: λ_{max} at 242, 285 and 368 nm (in 2N HCl)

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR N-DESMETHYLDIAZEPAM

Nomenclature

Chemical Name: 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

Empirical Formula: $C_{15}H_{11}C1N_2O$

Chemical Abstracts Registry Number: 1088-11-5

Physical Constants

Physical Appearance: Off-white to yellow crystalline powder

Melting Point: 216-217°C

REFERENCES

1. D.J. Greenblatt and Richard I. Shader. New England J. Med., 291, 1011 (1974).

2. A. MacDonald, A.F. Michaelis, and B.Z. Senkowski, in K. Florey, ed., Analytical Profiles of Drug Substances, Vol. 1. Academic Press, New York, p. 79 (1972).

3. M.M. Reidenberg, M. Levy, H. Warner, C.B. Coutinho, M.A. Schwartz, G. Yu, and J. Cheripko. Clin. Pharmacol. Ther., 23, 371 (1978).

4. J.J. Tanja, C.R. Clark, and D.E. Griffies. J. Anal. Toxicol., 1, 175 (1977).

5. I. Saxén and L. Saxén. Lancet, 2 (7933), 498 (1975).

6. J.F.W. Haffner, J. Morland, J. Setekleiv, C.E. Stromsaether, A. Danielsen, P.T. Frivik, and F. Dybing. Acta Pharmacol. Toxicol., 32, 161 (1973).

7. J. Wiersum. J. Amer. Med. Assoc., 227, 79 (1974).

8. L. Hillestad, T. Hansen, and H. Melsom. Clin. Pharmcol. Ther., 16, 485 (1974).

9. J. Hendel. Acta Pharmacol. Toxicol., 37, 17 (1975).

10. W.A. Mahon, T. Inaba, T. Umeda, E. Tsutsumi, and R. Stone. Clin. Pharmcol. Ther., 19, 443 (1976).

11. J. Kanto, L. Kangas, and T. Siirtola. Acta Pharmacol. Toxicol., 36, 328 (1975).

12. R. Sellman, J. Xanto, E. Raijola, and A, Pekkarinen. Acta Pharmacol. Toxicol., 37, 34.5 (1975).

13. K. Korttila, M.J. Mattila, and M. Linnoila. Acta Pharmacol. Toxicol., 36, 190 (1975).

14. R. Dixon, M.A. Brooks, E. Postma, M.R. Hackman, S. Spector, J.D. Moore, and M.A. Schwartz. Clin. Pharmacol. Ther., 20, 450 (1976).

15. U. Klotz, K.H. Antonin, and P. Bieck. Arch. Pharmacol., 287, Suppl, R90 (1975).

16. U. Klotz, G.R. Avant, A. Hoyumpa, S. Schenker, and G.R. Wilkinson. J. Clin. Inv., 55, 347 (1975).

17. S.A. Kaplan, M.L. Jack, K. Alexander, and R.E. Weinfeld. J. Pharm. Sci., 62, 1789 (1973).

18. J.A.F. de Silva, B.A. Koechlin, and G. Bader. J. Pharm. Sci., 55, 692 (1966).

19. E. van der Kleijn, P.J. M. Guelen, C.G.W.M. van Wijk, I. Baars, and T.B. Vree, in G. Levy, ed., Clinical Pharmokinetics: Symposium. Amer. Pharm. Assoc., Acad. Pharmaceutical Sciences (October 1974), p. 79.

20. H. Dasberg. Psychother. Psychosom., 24, 113 (1974).

21. I.A. Zingales. J. Chromatogr., 75, 55 (1973).

22. L. Hillestad, T. Hansen, H. Melsom, and A. Drivenes. Clin. Pharmacol. Ther., 16, 479 (1974).

23. M.A. Schwartz, B.A. Koechlin, E. Postma, S. Palmer, and G. Krol. J. Pharmacol. Exp. Ther., 149, 423 (1965).

24. E. Tsutsumi., T. Inaba, W.A. Mahon, and W. Kalow. Biochem. Pharmacol., 24, 1361 (1975).

25. R. Sellman, J. Kanto, E. Raijola, and A. Pekkarinen. Acta Pharmacol. Toxicol., 36, 33 (1975).

26. M. Linnoila, K. Korttila, and M.J. Mattila. Acta Pharmacol. Toxicol., 36, 181 (1975).

27. M. Mandelli, P.L. Morselli, S. Nordio, G. Pardi, N. Principi, F. Sereni, and G. Tognoni. Clin. Pharmacol. Ther., 17, 564 (1975).

28. G.J. DiGregorio, A.J. Piraino, and E. Ruch. Clin. Pharmacol. Ther., 24, 720 (1978).

29. M. Mandelli, G. Tognoni, and S. Garattini. Clin. Pharmacokinetics, 3, 72 (1978).

30. J.C. Valentour, J.R. Monforte, B. Lorenzo, and I. Sunshine. Clin. Chem., 21, 1976 (1975).

31. P.J. Van der Merwe and J.M. Steyn. J. Chromatogr., 148, 549 (1978).

32. M.A. Brooks, L. D'Arconte, M.R. Hackman, and J.A.F.de Silva. J. Anal. Toxicol., 1, 179 (1977).

33. A. Bugge. J. Chromatogr., 128, 111 (1976).

34. J.J. MacKichan, W.J. Jusko, P.K. Duffner, and M.E. Cohen. Clin. Chem., 25, 856 (1979).

35. M.A. Peat and L. Kopjak. J. Forensic Sci., 24, 46 (1979).

36. J.E. Wallace, H.A. Schwertner, and E.L. Shimek, Jr. Clin. Chem., 25, 1296 (1979).

37. W. Lund, M. Hannisdal, and T. Greibrokk. J. Chromatogr., 173, 249 (1979).

38. B. Peskar and S. Spector. J. Pharmacol. Exp. Ther., 186, 167 (1973).

39. R. Dixon, W. Glover, J. Earley, and M. Delaney. J. Pharm. Sci., 68, 1471 (1979).

40. B.H. Haden, K.G. McNeil, N.A. Huber, W.A. Khan, P. Singh, and R.S. Schneider. Clin. Chem., 22, 1200 (1976).

41. D.M. Hailey. J. Chromatogr., 98, 527 (1974).

42. A.K. Dhar and H. Kutt. Clin. Chem., 25, 137 (1979).

43. J.A.F. de Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks, and R.E. Weinfeld. Anal. Chem., 48, 10 (1976).

44. J.L. Ferguson and D. Couri. J. Anal. Toxicol., 1, 171 (1977).

45. R.E. Weinfeld, H.N. Posmanter, K.C. Khoo, and C.V. Puglisi. J. Chromatogr., 143, 581 (1977).

46. M. Linnoila and F. Dorrity, Jr. Acta Pharmacol. Toxicol., 41, 458 (1977).

47. A. Viala, J.-P. Cano, J.-P. Reynier, and R. Rispe. J. Chromatogr., 147, 349 (1978).

48. D. Haidukewych, E.A. Rodin, and R. Davenport. Clin. Chem., 26, 142 (1980).

49. J.J. De Gier and B.J. 't Hart. J. Chromatogr., 163, 304 (1979).

50. D.J. Greenblatt. Clin. Chem., 24, 1838 (1978).

51. H.H. McCurdy, E.L. Slightom, and J.C. Harrill. J. Anal. Toxicol., 3, 195 (1979).

52. B.M. Yehia, J.S. Oliver and A. Mackay. Vet. Hum. Toxicol., 21 (Suppl.), 167 (1979).

53. M.G. Horning, W.G. Stillwell, J. Nowlin, K. Lertratanangkoon, D. Carroll, I. Dzidic, R.N. Stillwell, and E.C. Horning. J. Chromatogr., 91, 413 (1974).

54. M.S. Greaves. Clin. Chem., 20, 141 (1974).

55. R.C. Baselt, C.B. Stewart, and S.J. Franch. J. Anal. Toxicol., 1, 10 (1977).

56. J.F. Steyn and H.K.L. Hundt. J. Chromatogr., 107, 196 (1975).

57. A.G. Howard, G. Nickless, and D.M. Hailey. J. Chromatogr., 90, 325 (1974).

58. G. Banelli and P. Marini. Boll. Chim. Unione Ital. Lab. Prov., 2, 310 (1976).

59. L.B. Foster and C.S. Frings. Clin. Chem., 16, 177 (1970).

60. H. Sawada, A. Hara, S. Asano, and Y. Matsuaoto. Clin. Chem., 22, 1596 (1976).

61. P.J. Howard. J. Pharm. Pharmacol., 30, 136 (1978).

62. B.J. Gudzinowicz and M.J. Gudzinowicz, Analysis of Drugs and Metabolites by Gas Chromatography-Mass Spectrometry, Vol. 2: Hypnotics, Anticonvulsants, and Sedatives. Marcel Dekker, New York, ch. 2, VII (1977).

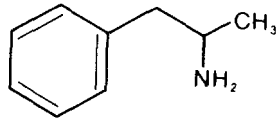
63. A.F. Fentiman, Jr. and R.L. Foltz. J. Labelled Compounds and Radiopharmaceuticals, 13, 579 (1977).

Chapter 10

Amphetamine

For many centuries the khat plant has been cultivated in the Middle East for its central nervous system (CNS) stimulating properties. A synthetic relative of the khat alkaloids, amphetamine (α-methylphenethylamine, I) was first produced in 1887, but its psychopharmacological properties were not described until 1927 (1). For the next 30 years, medical use of amphetamine (as a bronchodilator, as an appetite suppressant, in the treatment of hyperkinesis in children, and for narcolepsy) was extensive (2), but by 1967 the potential for abuse of the drug was causing widespread concern. Regular oral use of amphetamine can cause paranoid psychosis and a disabling dependence, and intravenous use is even more likely to lead to these effects. Primarily because of an initial euphoria, especially following intravenous injection, amphetamine has become a major drug of abuse. Appetite suppression, insomnia, sudden changes in mood, and compulsiveness are the common results of even moderate amphetamine use. Tolerance develops rapidly, so that increasing doses are required to achieve the desired stimulation (3).

Amphetamine is marketed for medical purposes as Dexedrine (d-amphetamine) and as Benzedrine (d,l-amphetamine), or in combination with



I

a barbiturate (e.g. Dexamyl, d-amphetamine + amobarbital) (4). However, because of the relative ease of manufacture, illicit preparations of amphetamine are common in "street" use. As a result, contaminants are often present. Significant quantities of zinc (1-2 percent) have been found in some illicit samples, as well as polycondensation products whose pharmacological and toxicological properties are unknown (5), although there is evidence that the intensity of euphoria following intravenous injection may depend on contaminants (3). Severe reactions can occur in nontolerant persons following a dose of 30 mg of amphetamine, while persons who have developed tolerance can endure doses of 400-500 mg. While true physical dependence has not been proven, very strong psychological habituation to amphetamine is common. In its extreme form, the "speed cycle" of heavy intravenous use for days at a time results

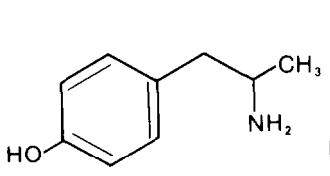
in sleep deprivation (hallucinations, etc.), malnutrition, and often infection (1). For medical purposes, many structural modifications of amphetamine have been made in efforts to eliminate the undesirable effects of the drug while preserving the crucial structural feature (the d-phenethylamine skeleton) for its antifatigue, anorexic, or other pharmacological properties. Structurally related drugs include p-chloroamphetamine, phentermine, fenfluramine, phenmetrazine, and methylphenidate, some of which have also become drugs of abuse (6).

The mechanism of the CNS stimulant action is thought to be a triggering of the release of catecholamines (epinephrine, dopamine, norepinephrine) and blockage of their reuptake (7). Amphetamine also exhibits peripheral α- and ß-adrenergic activity (1). It has been shown to increase cerebral blood flow in rats (8), and in man an increased susceptibility to strokes and cerebral hemorrhages has been reported. Elevated blood pressure and cardiac arrhythmia are the common cardiovascular effects of amphetamine (1).

The optical isomers of amphetamine exhibit different pharmacological effects. With respect to the CNS, the d-isomer is 4-10 times as potent as the 1-isomer, but in cardiovascular effects the 1-isomer is more potent (1). More d-amphetamine is retained in heart and brain tissue, and only the d-isomer leads to the presence of p-hydroxy norephedrine as a metabolite in the brain. It is possible that this metabolite could function as a false neurotransmitter, which might account for the development of psychosis in some individuals after prolonged use of large doses of amphetamine (9).

METABOLISM AND PHARMACOKINETICS

The metabolism of amphetamine in man yields at least two biologically active metabolites, p-hydroxyamphetamine (II) and p-hydroxynorephedrine (III), both of which are found in significant levels in the urine and undoubtedly contribute to the overall pharmacology of the drug (10). ß-Hydxoxylation is carried out by an extra hepatic enzyme located in sympathetic nerve endings (11).



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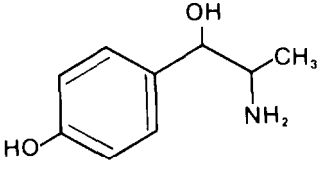

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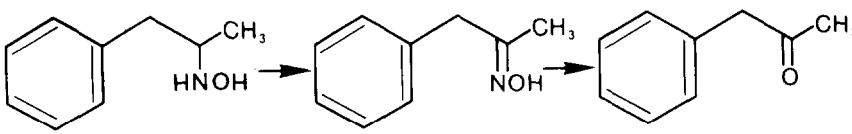
Metabolic N-oxidation of amphetamine has also been reported (12). The hydroxylamine (IV) is unstable and is quickly converted by chemical or metabolic processes to the oxime (V) and to phenylacetone (VI).



IV V VI

Based on the report that 30 percent of an administered dose of d-amphetamine in human subjects was found to be excreted as unchanged drug, 3 percent as II (free and conjugated), 3 percent as phenylacetone, 20 percent as free and conjugated benzoic acid, and 5 percent as III, deamination seems to be the predominant metabolic route in man, although p-hydroxylation is the primary route in rats. Deamination is catalyzed by a liver microsomal enzyme that requires reduced NADP (9). Deamination is stereoselective; the S-(+)-isomer of amphetamine is preferentially metabolized (11).

The pKa of amphetamine is 9.77 (13), so it is ionized at physiological pH; nevertheless, it does readily penetrate the blood-brain barrier. Excretion of the unchanged drug has been shown to fluctuate from 10 percent to 60 percent with changes in urinary pH (14). A computerized method of predicting excretion curves (and by extension, body concentrations) has been developed (15).

Pharmacokinetic studies in humans have confirmed that the drug has a high extravascular concentration in the body. In one of these studies, peak blood concentrations (~35 ng/ml) were reached about 2 hr after ingestion of 10 mg of d-amphetamine sulfate, followed by an exponential decline over the next 48 hr, with a plasma elimination half-life of 11-13 hr. Mean total urinary recovery of unchanged drug was 45 percent of the ingested dose (16). Another report indicated an 8 hr plasma half-life for human subjects on an acid diet, but 22 hr when on an alkaline diet (9). In a study involving patients with amphetamine psychosis, the intensity of psychosis correlated with the concentration of basic polar metabolites of the drug in urine but not with the amphetamine concentration in plasma. Amphetamine metabolism in these patients was followed with the aid of a tritium-labeled analog of the drug. Under metabolic conditions producing acid urine (pH <6.6), renal elimination of unchanged drug (67-73 percent of the dose) was associated with a plasma elimination half-life of 7-14 hr and a rapid reduction in psychotic symptoms. In patients with alkaline urine, the plasma half-life of amphetamine was 18-34 hr, psychosis was of longer duration, and hydroxylated metabolites in urine accounted

for 6-13 percent of the dose as compared with 3-8 percent in acidic urine (17).

In a study using rats as models, inhibition of metabolism increased the brain concentration of amphetamine by a factor of 5, indicating that metabolism is more important than renal excretion as the route of elimination of amphetamine, at least in that species (18). In human subjects receiving 1500 mg of ascorbic acid daily prior to amphetamine administration, the 24-hr excretion of a single 30 mg dose of drug was decreased by an average of 38 percent, regardless of urinary pH, although the acute pharmacological effects of the drug were not altered by the ascorbic acid pretreatment. There was no increase in hydroxylated metabolites to account for the smaller quantity of amphetamine excreted (19).

A definitive compendium of information on the pharmacology and metabolism of amphetamine was published in 1970 (20), and an extensive review of amphetamine metabolism in mammals, including man, appeared in 1976 (11).

ANALYTICAL METHODOLOGY

The development of methods sufficiently sensitive, specific, and reproducible for the identification and measurement of amphetamine in biological fluids is important because there is a need to establish therapeutic dosages and plasma concentrations, to understand tolerance and the stereoisomeric effects, to carry out toxicity studies, and simply to identify drug users. The concentrations of amphetamine found in urine, even after small doses, are high enough to be detected by most methods available to clinical laboratories. However, plasma concentrations are usually in the 5-100 ng/ml range. Techniques used have included ultraviolet spectrophotometry (UV), thin-layer chromatography (TLC), fluorometry, immunoassay, gas chromatography (GC), and combined gas chromatography/mass spectrometry (GC/MS).

A quantitative UV method, with the advantages of relative speed and simplicity for screening purposes, was reported in 1968. The amphetamine extract from urine, blood, or tissue homogenate was oxidized with ceric sulfate, using 0.8 N HCl as a catalytic agent, to form a reaction product with a well-defined UV absorption curve (λ_{max} 287 nm, λ_{min} 253 nm in hexane). Sensitivity was 0.5 μg/ml, but the method was unable to distinguish between amphetamine and methamphetamine (21).

Until the early 1970's, fluorometry was the most widely used method for the quantitative analysis of biogenic amines and amphetamines. A fluorometric method, based on conversion of amphetamine to its dihydrolutidine derivative, utilized an automated continuous-flow apparatus for extraction of the derivatized drug from urine (22). The detection limit was 0.15 μg/ml, but 2-phenylethylamine and 2-aminoheptane gave false positive reactions, Fluorometry combined with TLC is widely used, especially for screening urine specimens for amphetamine. For example, the drugs in urine can be separated and concentrated by XAD-2 resin, chromatographed on silica gel in

a suitable solvent system, and sprayed with a color reagent. Sequential spraying with ethanolic ninhydrin-phenylacetaldehyde, aqueous sodium phosphate, and then p-dimethylaminobenzaldehyde has been shown to distinguish amphetamine from other primary amines of forensic and toxicological interest such as mescaline, DOM, phenylpropanolamine, ß-phenethylamine, benzocaine, chlorphentermine , and procaine. A final spray with chromotropic acid was found to be necessary to distinguish amphetamine from 3,4-methylenedioxyamphetamine (MDA). A 2 µg detection limit for amphetamine was reported for this technique (23). A Fluorescamine (4-phenyl- spiro [furan-2(H)-1'-phthalen]- 3,3'dione) spray reagent was found to be more sensitive than ninhydrin, detecting 250 ng of amphetamine in urine extracts following TLC. Methamphetamine does not react with Fluorescamine (24). The Fluorescamine technique was adapted to in situ spectrodensitometry of thin-layer chromatograms for quantitation of amphetamine in urine extracts without elution. A solvent system of ethyl acetate:methanol:water:ammonia (85:13.5:10:0.5) was used to separate amphetamine from extraneous fluorescent material. A concentration of 0.25 µg/ml was detectable (25). 4-Chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD-Cl) has also been used to form a fluorescent derivative of amphetamine in urine and blood extracts before separation by TLC, and the fluorescence intensity used as a quantitative measure of the amphetamine present. Sensitivity is sufficient for measurement of therapeutic concentrations in blood (10-100 ng/ml) (26). Although any primary or secondary amine will yield a fluorescent derivative with NBD-Cl, TLC analysis using a two-solvent system [e.g., pentane: ethyl ether (1:1) or methanol: carbon tetrachloride (80: 30)] can be used to separate amphetamine from compounds likely to yield false results (27).

Some assays developed for amphetamine do not require chromatographic separation. As little as 10 ng/ml of amphetamine in plasma was detected by an enzyme assay based on the N-methylation of the drug to form radioactive methamphetamine by means of a rabbit lung N-methyltransferase and S-adenosyl-L-[methyl-^{3}H] methionine as methyl donor. Sensitivity, specificity, and reproducibility were achieved by a combination of several extraction and drying procedures used prior to liquid scintillation counting (28).

For large numbers of samples or for rapid screening, immunological techniques are often the method of choice. They do not require extensive extraction of biological materials or a high degree of skill in operation. Radioimmunoassay (RIA) depends upon competition between the unlabeled drug in the sample and a known amount of radiolabeled drug for the binding sites on specific drug antibodies in rabbit serum. The antibody in some RIA assays for amphetamine binds equally well with methamphetamine, but the method can detect 30 ng/ml of amphetamine in urine (29). A single RIA takes about 40 min, but several samples can be run concurrently (30). The other common immunoassay, HIT, is based on the linkage of an enzyme (lysozyme) to the amphetamine molecule to form the antigen reagent. Antibody binds the drug and its attached enzyme, inactivating the lysozyme. Any free amphetamine present in the sample competes with the drug-enzyme complex for antibody binding sites; while unbound enzyme is then free to hydrolyze the cell walls of bacteria used as

an indicator in the assay. The clearing of the cell walls suspension (decrease in absorbance) is proportional to the amount of enzyme label displaced from the antibody and hence to the amount of competing free drug present in the specimen. EMIT cannot presently be used for blood, and it is subject to interference by lysozyme naturally present in some urine samples and to false negatives because of inactivation of enzyme in alkaline urines. However, it takes only one minute to perform, it is as sensitive and specific as RIA, and it gives semiquantitative results based on calibration graphs (30).

Gas chromatography (GC) has been used at least since 1969 for measurement of amphetamine in urine and blood. Gas chromatography of extracts, after addition of N,N-dimethylaniline as an internal standard, on a column coated with 5 percent KOH + 10 percent Carbowax 6000 and using a flame ionization detector (FID), was sensitive enough to detect 0.1 μg/ml of amphetamine in urine (31). A rapid GC method developed for routine use in urine screening incorporated diethylamine as the internal standard and confirmation by preparation of a ketone derivative of amphetamine. Detection limits were 0.01 ng/ml (32). For quantitation of amphetamine in blood by GC a number of specific steps were recommended. These included the use of Teflon-coated glassware to minimize absorption, coextraction with a scavenger amine (diethylamine), formation of the nonvolatile HCl salt, preparation of the trifluoroacetamide derivative, and use of an internal standard (1-adamantanamine HCl). This combination of conditions gave a nearly complete (98 percent) recovery of amphetamine from blood, and concentrations of 10-100 ng/ml were measurable (33).

Because of the sensitivity limits of the flame ionization detector, electron capture detection (ECD) after formation of suitable derivatives offers advantages over FID for GC assay of the very low concentrations of amphetamine often present in blood samples. The heptafluorobutyl (34), trichloroacetamide (35), and N-acetyl (36) derivatives, among others, have been reported for this purpose. On-column acylation of amphetamine with N- (trifluoroacetyl) imidazole, or with N-(heptafluorobutyryl) imidazole, has also been reported. The derivatives were formed quantitatively and reproducibly, making the technique suitable for quantitative analysis. The imidazole reagents have the advantage over acyl chlorides or acid anhydrides in that no acids are released into the chromatographic system (37). A comparison study of the ECD response of several fluorinated amphetamine derivatives indicated that a sensitivity of 10 pg for the pentafluorobenzamide derivative was possible using a ^{63}Ni detector, but pure compounds and not biological extracts were used in the study (38). GC-ECD of the trichloroacetate derivative, also using a ^{63}Ni detector, was able to measure concentrations of amphetamine as low as 5 ng/ml in plasma (39). Although EC detectors may improve sensitivity, they can have the disadvantages of excessive background or of severe quenching due to water and solvent. Preliminary studies of the analysis of amines by GC-ECD, using model compounds such as phenethylamine and utilizing glass capillary columns, have suggested that the high resolving power of capillary columns could improve quantitative analysis at the subnanogram level

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of neurotransmitters and related compounds like amphetamine (40).

GC analysis of the metabolite N-hydroxyamphetamine is difficult because of its instability under normal GC conditions, but the decomposition has been shown to be the result of catalytic activity of the solid support rather than thermal conditions or oxidation in the column. Direct GC analysis was found to be possible without derivatization, by the use of a column containing 0.2 percent Carbowax 20M with glass beads as support material, operated at 100°C (41).

For selected ion monitoring analysis of drugs and metabolites in biological fluids preparation of derivatives is often desirable in order to achieve better gas chromatographic characteristics and to generate ions in the mass spectrometer which are more suitable than those generated by the parent drug. Acetylation, trimethylsilylation (TMS), and perfluroacylation give amphetamine derivatives which show good GC and MS characteristics (39).

Some acetylation procedures call for reaction times of up to several hours and are therefore unsuitable for rapid analyses. Recently, a rapid and quantitative acetylation of amphetamine was reported in which trifluoroacetic anhydride was used in the presence of mercuric trifluoroacetate as a catalyst. The reagent acts on amphetamine in the form of either the free base or salt, and the method requires no prior extraction of street drug samples to remove adulterants. Application of this derivatization procedure to GC/MS analysis allowed detection of amphetamine at the picomole level, within about 10 min total analysis time (42).

For purposes of mass spectral identification of primary amines in urine samples, isothiocyanate derivatives have been shown to possess some advantages over TMS derivatives because of a fragmentation pattern which eliminates a CNS-containing radical from the molecular ion and allows for charge retention on the moiety containing the phenyl group (43). The isothiocyanate derivative of amphetamine is claimed to be even more stable than the trifluoroacetamide derivative. By monitoring the molecular ion of the isothiocyanate derivative a signal-to-noise ratio of 3:1 was achieved for 1 ng injected on column (44).

Dansylation of amphetamine with 5-dimethylamino-1-naphthalene sulfonyl chloride yields a UV-absorbing derivative which was employed in an assay for amphetamine in urine and tissue homogenates. Amphetamine-$^{2}H_{5}$ was added to urine (pH 10) before extraction and dansylation. A benzene extract of the dansyl-amphetamine was chromatographed on thin-layer plates developed with benzene:triethylamine (8:1), and the zone identified by UV spectroscopy (365 nm) was eluted with ethyl acetate, dried, and chromatographed on another plate (petroleum ether:toluene:acetic acid:water, 133:67:170:30 v/v). The final extract was analyzed by high resolution mass spectrometry using selected ion monitoring. A linear relationship between amphetamine concentration and ion current response was established over the range 5 pg to 500 ng, and as little as 0.1 ng/ml of amphetamine was detectable in urine. However, because the inter-

nal standard was of poor isotopic purity, reliability of the method suffered at concentrations lower than 2 ng/ml (45).

In a GC/MS procedure involving quantitation of amphetamine as the isothiocyanate derivative, amphetamine-$^2H_5$ and ß-methylphenethylamine were compared as internal standards. The two internal standards were reported to give comparable results (44).

A comparison of an RIA and a GC/MS assay for amphetamine showed that the GC/MS method was about twice as sensitive and gave better precision than the RIA method, However, taking into consideration factors such as cost, time, and degree of training required to perform the assays, RIA performed in duplicate or triplicate was recommended for routine measurements. The measured concentrations in unknown samples by both techniques showed a good correlation, lending confidence in the accuracy of both methods (46).

The optical isomers of amphetamine can be separated by formation of a N-trifluoroacetyl-L-prolyl derivative and separation of the diastereoisomers by TLC. For qualitative purposes, such as forensic differentiation of illicit samples (47), the individual isomers can be measured in urine (48). However, GC/MS, with its high inherent sensitivity, has provided the first means of studying the stereochemical profile of amphetamine in human plasma and saliva at ng/ml concentrations following oral administration of 10 mg of racemic amphetamine. The drug, following extraction, was derivatized by reaction with a chiral agent, N-pentafluorobenzoyl-S-(-)-prolyl-1-imidazole. The resulting diastereoisomers were easily resolved by capillary GC (5 percent OV-275 coated on Chromosorb W-AW) and quantitated by chemical ionization mass spectrometry using methane as the reagent gas. Amphetamine-$^2H_2$ served as the internal standard (49). An analytical procedure developed for simultaneous determination of the concentrations of amphetamine enantiomers in rat urine also utilized GC/MS with selected ion monitoring, but used the N-(S)-α-methoxy-α-(trifluoromethyl)phenylacetyl derivative and amphetamine labeled with deuterium on the methyl group as the internal standard (50).

A general method for extraction of amphetamine from biological materials involves adjustment of the pH to about 10-12 and subsequent extraction with an organic solvent or elution from ion exchange columns. The latter is not as selective and the resulting extracts show many extraneous peaks, so the solvent extraction method is preferred for GC analysis. Solvents used have included chloroform (22) or chloroform: isopropanol (96: 4) (24); pentane (28); diethyl ether, for which recoveries of 70-75 percent from blood and 83 percent from urine have been reported (26,32,51); hexane (89 percent from blood, 97 percent from urine) (21,49), or hexane:isooctane (75:25 v/v) (35); and benzene (33,52). Amphetamine is about 15 percent bound to plasma proteins, but both free and bound forms are extracted equally well, suggesting that losses in recovery are mainly a consequence of the drug's high volatility. Mild conditions are therefore essential for evaporation of the solvent (51).

EXPERIMENTAL PROCEDURE

The experimental procedure for extraction, derivatization, and GC/MS analysis of amphetamine is identical to the procedure described in Chapter 9 for analysis of methamphetamine except for the internal standard (amphetamine-$^2H_3$ is used instead of methamphetamine-$^2H_5$) and the specific ions monitored. Consequently, the concentrations of both drugs can be measured simultaneously by adding deuterated analogs of the two drugs to the biological sample for use as internal standards. Then during data acquisition, the ion currents at four m/z values are monitored. These are m/z 232, 235, 246, and 251, which correspond to the protonated molecule ions for the trifluoroacetamide derivatives of amphetamine, amphetamine-$^2H_3$, methamphetamine, and methamphetamine-$^2H_5$, respectively.

Standards and Reagents

d-Amphetamine hydrochloride can be purchased from Applied Science Laboratories, State College, PA 16801, for preparation of standard solutions. The amphetamine-$^2H_3$ hydrochloride used in the development of this procedure was synthesized by a literature procedure (53). The deuterium-labeled amphetamine had an isotopic purity of 99.9 percent ($^2H_3$).

For the purpose of preparing calibration graphs and working standards, stock solutions of amphetamine and amphetamine-$^2H_3$ are prepared as follows. Weigh into a 100-ml volumetric flask about 12.6 mg of amphetamine hydrochloride and record its weight to the nearest 0.1 mg The equivalent weight of free base is calculated by multiplying the weight of amphetamine hydrochloride by 0.79. Dissolve the measured amphetamine in about 75 ml of methanol and then add additional methanol to give exactly 100 ml of solution. In the following paragraphs this solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled with its actual concentration based upon the exact measured weight of amphetamine hydrochloride. A series of working standard solutions can be prepared by appropriate dilution of this stock solution as described in Chapter 2.

The stock solution of amphetamine-$^2H_3$ is prepared in the same manner. For the measurement of amphetamine in body fluids within the concentration range 1 to 500 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml amphetamine-$^2H_3$ methanolic solution to each ml of specimen. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of amphetamine-$^2H_3$ to 250 ml with methanol.

Store stock and standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction

Transfer 1 ml of the specimen (whole blood, plasma or urine) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately).

Add 100 µl of the 400-ng/ml amphetamine-$^2H_3$ soiution and vortex for about 10 sec. Allow the sample to equilibrate for about 15 min and then increase the pH to greater than 10 by adding 1 ml of K_2CO_3-saturated distilled water. Extract the basic mixture with 5 ml of 1-chlorobutane by gently mixing the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min and then transfer the organic phase (top) to another 20-ml culture tube by means of a disposable Pasteur pipette. Extract the amphetamine and other organic bases from the organic phase into aqueous acid by adding 3 ml of 0.2 N H_2SO_4 and gently mixing for at least 15 min. Centrifuge and remove the top organic phase by aspiration. Add 0.5 ml of 10 N NaOH to the aqueous phase, mix the contents, and check the pH to make certain the solution is strongly basic (pH > 10). Finally, reextract the organic bases from the aqueous alkaline solution by adding 5 ml of methylene chloride and gently mixing for at least 15 min. Centrifuge for 5 min, aspirate off the upper aqueous layer, and transfer by means of a disposable Pasteur pipette the methylene chloride extract (bottom layer) to a silylated concentrator tube having a volume of at least 5 ml, a conical bottom, and a Teflon-lined screw cap. Add 10 µl of dimethylformamide to act as a "keeper solvent" to minimize evaporative loss of the amphetamine. Remove the methylene chloride by evaporation under a gentle stream of nitrogen or filtered air while heating the tube to no higher than 40°C. When the volume of extract has decreased to about 10 µ1, add 15 µl of N-methyl bis- (trifluoroacetamide) "MBTFA" (Pierce Chemical Co., Rockford, IL 61105). Cap the tube and heat it at 70°C for 15 min. The capped tube can be stored under refrigeration until the GC/MS analysis is ready to be performed. Immediately before the analysis, allow the tube to warm to room temperature.

GC/MS Analysis

The experimental conditions for the GC/MS analysis for amphetamine trifluoroacetamide are as follows:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801).
Carrier and reagent gas:	Methane, 15-20 ml/min
Temperatures:	Injector, 260°C Column, 120°C, isothermal GC/MS transfer line, 260°C Ion source, 160°C

Under these conditions, the amphetamine trifluoroacetamide should elute in 2 to 4 min as a narrow, symmetrical peak.

prior to beginning GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 232 and 235. They correspond to the protonated molecule ions for ampheta-

tamine trifluoroacetamide and amphetamine-$^2H_3$ trifluoroacetamide, respectively. With the divert valve in the divert position, inject 2 to 6 µl of the organic extract into the gas chromatograph. After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the amphetamine trifluoroacetamide peak has eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of amphetamine in the specimen is determined by measuring the heights (or areas) or the amphetamine trifluoroacetamide and amphetamine-$^2H_3$ trifluoroacetamide peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the concentration of amphetamine in the specimen by dividing the measured quantity of amphetamine by the exact volume of specimen extracted.

Discussion of the Experimental Procedure

The electron impact (EI) and chemical ionization (CI) mass spectra of amphetamine trifluoroacetamide are shown in Figure 1. The most abundant ions in the CI mass spectra correspond to the protonated molecule ion (m/z 232) and the ammonia-attachment ion (m/z 249), depending on whether methane alone or methane and ammonia are used as reagent gases. Even though the procedure specifies use of methane alone as the reagent gas, the combination of methane and ammonia is also satisfactory and should provide slightly better sensitivity. Perhaps most important is the fact that the methane and ammonia combination offers an opportunity to monitor a different pair of ion masses if interferences are encountered at m/z 232 or 235 when methane alone is used.

An additional option which can be explored if interferences are encountered in the selected ion monitoring measurements, is to generate a derivative different from the trifluoroacetamide. For example, the acetamide derivative could be prepared by substituting N-acetylimidazole for the N-methyl bis-(trifluoroacetamide). Also, the pentafluoroacetamide or heptafluorobutyramide derivatives could be used. The trifluoroacetamide derivative has proven satisfactory, but the use of other derivatives has not been fully evaluated.

A potential problem associated with concentration of organic extracts containing amphetamine is the loss of the drug through evaporation because of its relatively high volatility. For this reason, some amphetamine assay procedures specify addition of an acid to convert the drug to a nonvolatile salt prior to concentration of the extract. In the procedure described here, losses due to evaporation are minimized by the following means. First, methylene chloride is used in the final extraction because it has a lower boiling point (39°C) than 1-chlorobutane (78°C). Second, a small quantity of dimethylformamide is added just prior to evaporative concentration to act as a "keeper solvent." If these procedures are followed and care is taken not to allow the concentrated residue to remain at an elevated temperature for an extended period, no more than 20 percent of the amphetamine should be lost due to evaporation.

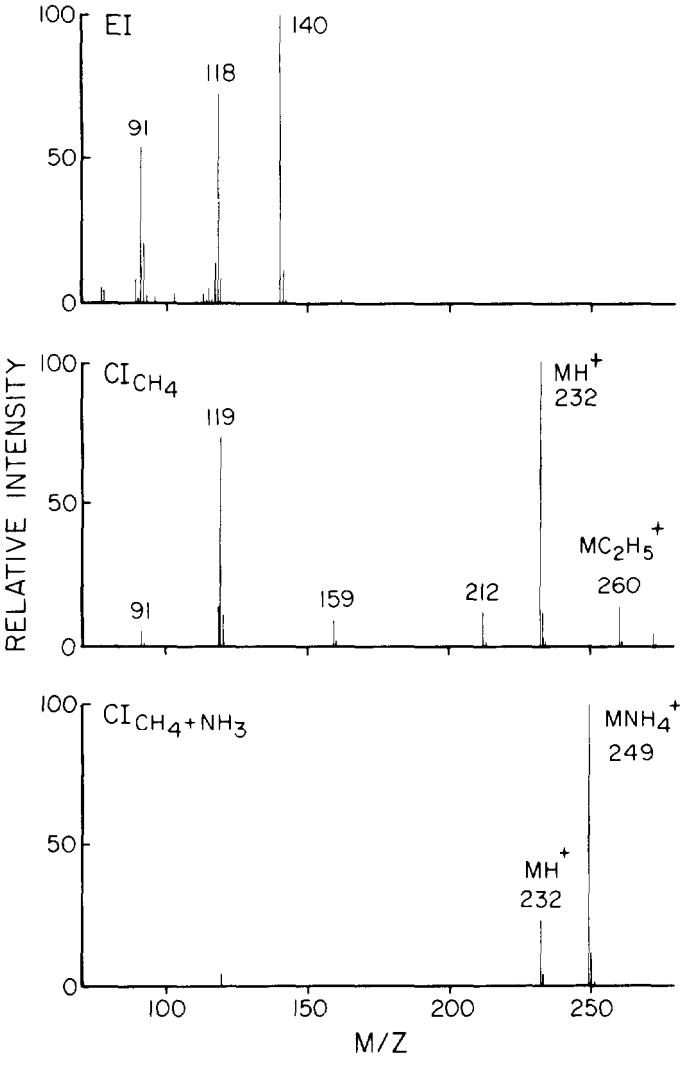


FIGURE 1. MASS SPECTRA OF THE TRIFLUOROACETAMIDE DERIVATIVE OF AMPHETAMINE

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR AMPHETAMINE

Nomenclature

Chemical Name: α-Methylphenethylamine

Empirical Formula: $C_9H_{13}N$

Chemical Abstracts Registry Number: 300-62-9

Trade Names: Actedron, Allodene, Benzedrine, Adipan, Sympatedrine, Psychoedrine, Isomyn, Isoamyne, Mecodrin, Norephedrone, Novydrine, Elastonon, Ortedrine, Phenedrine, Profamina, Propisamine, Sympamine, Simpatedrin

Physical Constants

Appearance: Colorless liquid

Boiling Point: 200-203° (760 mm); 82-85° (13 mm)

Solubility of the free base: Slightly soluble in water; soluble in acid, alcohol, and ether

pKa: 9.77

Specific Rotation: dα-methylphenethylamine sulfate $[\alpha]_D^{20} = +21.8°$ (c=2)

REFFRENCES

1. J.B. Hart and J. Wallace. Clin. Toxicol., 8, 179 (1975).
2. Unsigned. Brit. Med. J., 1, 476 (1975).
3. J.C. Kramer. J. Psychedelic Drugs, 2, 8 (1969).
4. M. Lampe. Drugs: Information for Crisis Treatment. STASH Press, Beloit, WI, p. 9 (1972).
5. J.N. Lomonte, W.T. Lowry, and I.C. Stone. J. Forensic Sci., 2, 575 (1976).
6. J.H. Biel, in E. Costa and S. Garattini, eds., Amphetamines and Related Compounds. Raven Press, New York, p. 3 1970).
7. A. Carlsson, in E. Costa and S. Garattini, eds., Amphetamines and Related Compounds. Raven Press, New York, p. 289 (1970).
8. C. Carlsson, M. Hagerdal, and B.K. Siesjö. Acta Physiol. Scand., 94, 128 (1975).
9. J. Axelrod, in E. Costa and S. Garattini, eds., Amphetamines and Related Compounds. Raven Press, New York, p. 207 (1970).

10. L.G. Dring, R.L. Smith, and R.T. Williams. J. Pharm. Pharmacol., 18, 402 (1966).

11. J. Caldwell. Drug Metab. Revs., 5, 219 (1976).

12. A.H. Beckett, H.T. Coutts, and F.A. Ogunbona. J. Pharm. Pharmacol., 25, 708 (1973).

13. A.H. Beckett and S. Al-Sarraj. J. Pharm. Pharmacol., 25, 328 (1973).

14. J.W. Gorrod. Life Sci., 13, lviii (1973).

15. A.H. Beckett, R.N. Boyes, and G.T. Tucker. J. Pharm. Pharmacol., 20, 277 (1968).

16. M. Rowland. J. Pharm. Sci., 58, 508 (1969).

17. E. Änggård, L.-E. Jonsson, A.-L. Hogmark, and L.-M. Gunne. Clin. Pharmacol. Ther., 14, 870 (1973).

18. A.K. Cho, B.J. Hodshon, B. Lindeke, and G.T. Miwa. J. Pharm. Sci., 62, 1491 (1973).

19. M.P. Kullberg and J.D. Griffith. Fed. Proc., 35, 565 (1976).

20. E. Costa and S. Garattini, eds., Proc. Int. Symp Amphetamines and Related Compounds. Raven Press, New York (1970).

21. J.E. Wallace, J.D. Biggs, and S.L. Ladd. Anal. Chem., 40, 2207 (1968).

22. T.S. Hayes. Clin. Chem., 19, 390 (1973).

23. K.J. Bussey and R.C. Backer. Clin. Chem., 2, 302 (1974).

24. B. Klein, J.E. Sheehan, and E. Grunberg. Clin. Chem., 20, 272 (1974).

25. J. Sherma, M.F. Dobbins, and J.C. Touchstone. J. Chromatogr. Sci., 12, 300 (1974).

26. F. Van Hoof and A. Heyndrickx. Anal. Chem., 46, 286 (1974).

27. T.J. Hopen, R.C. Briner, H.G. Sadler, and R.L. Smith. J. Forensic Sci., 21, 842 (1976).

28. D.S. Kreuz and J. Axelrod. Science, 183, 420 (1974).

29. A. Castro and H. Malkus. Res. Commun. Chem. Pathol. Pharmacol., 16, 291 (1977).

30. R.O. Bost, C.A. Sutheimer, and I. Sunshine. Clin. Chem., 22, 789 (1976).

31. K.D.R. Setchell and J.D.H. Cooper. Clin. Chim. Acta, 35, 67 (1971).

32. J.E. O'Brien, W. Zazulak, V. Abbey, and O. Hinsvark. J. Chromatogr. Sci., 10, 336 (1972).

33. R.B. Bruce and W.R. Maynard, Jr. Anal. Chem., 41, 977 (1969).

34. J.S. Noonan, P.W. Murdick, and R.S. Ray. J. Pharmacol. Exp. Ther., 168, 205 (1969).

35. P.A. Toseland and P.H. Scott. Clin. Chim. Acta, 25, 75 (1969).

36. G. Brugaard and K.E. Rasmussen. J. Chromatogr., 147, 476 (1978).

37. G.R. Wilkinson. Anal. Lett., 3, 289 (1970).

38. E. Änggård, in E. Costa and S. Garattini, eds., Amphetamines and Related Compounds. Raven Press, New York, p. 191 (1970).

39. J.J. Franken and M.M.F. Trijbels. J. Chromatogr., 91, 425 (1974).

40. A.H. Beckett and R. Achari. J. Chromatogr., 135, 200 (1977).

41. A. Wu. Clin. Toxicol., 8, 225 (1975).

42. N. Narasimhachari and P. Vouros. Anal. Biochem., 45, 154 (1972).

43. N. Narasimhachari, R.O. Friedel, F. Schlemmer, and J.M. Davis. J. Chromatogr., 164, 386 (1979).

44. T.J. Danielson and A.A. Boulton. Biomed. Mass Spectr., 1, 159 (1974).

45. K.H. Powers and M.H. Ebert. Biomed. Mass Spectr., 6, 187 (1979).

46. D. Eskes. J. Chromatog., 117, 442 (1976).

47. L.-M. Gunne. Biochem. Pharmacol., 16, 863 (1967).

48. S.B. Matin, S.H. Wan, and J.B. Knight. Biomed. Mass Spectr., 4, 118 (1977).

49. J. Gal. Biomed. Mass Spectr., 5, 32 (1978).

50. D.B. Campbell. J. Pharm. Pharmacol., 21, 129 (1969).

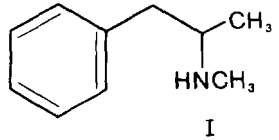
51. A.K. Cho, B. Lindeke, B.J. Hodshon, and D.J. Jenden. Anal. Chem., 45, 570 (1973).

52. B. Lindeke and A.K. Cho. Acta Pharm. Suecica, 9, 363 (1972).

Chapter 11

Methamphetamine

The first synthesis of methamphetamine (d-N,α-dimethylphenethylamine, I), a potent central nervous systerm stimulant, was reported in 1919 (1). Methamphetamine has since been used clinically as an analeptic to counteract alcohol, barbiturate, or narcotic stupor, and also during surgery to maintain the blood pressure of patients under anesthesia (2). Clinical use of the drug has declined in recent years but methamphetamine continues to be abused. It is available on the street as Methedrine tablets or as illicit preparations which are often injected intravenously. Like other stimulants of the amphetamine group, methamphetamine produces an initial state of euphoria. However, this feeling is followed by restlessness, agitation, irritability and sometimes extreme paranoia, as well as depression and exhaustion as the stimulant effects of the drug wear off. In consequence there is a strong tendency to continue use in order to maintain the "high" and avoid the unpleasant after-effects. Tolerance develops rapidly following repeated use and increasing doses are required to maintain the original level of stimulation. Physical dependence has not been proven, but the likelihood of continued use is very high (3). Methamphetamine has been found in human neonatal tissues, and the fetus rarely survives to term when the mother is a methamphetamine abuser (4).



I

For forensic purposes, it may be necessary to use procedures which can identify the stereoisomers, because l-methamphetamine can be found in the urine of persons using at least one of the common, nonprescription inhaler formulations. As in the case of amphetamine, the isomers differ in their pharmacological activity; l-methamphetamine has greater sympathomimetic properties while d-methamphetamine has greater anorexic and stimulant properties (5).

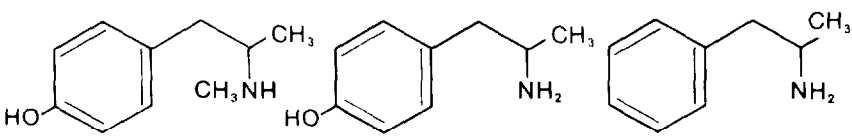
A comparative study in rats showed that the N-methyl group rendered methamphetamine less toxic to liver microsomal enzyme systems than amphetamine, but in these animals the methylated drug was more active, and required a smaller dose to achieve the same pharmaco-

logical effect (6). Also in rats, methamphetamine was found to cause a short-term (less than 6 hr) depletion of norepinephrine and serotonin in brain and a long-lasting (more than 48 hr) depletion of heart norepinephrine. Amphetamine was detected in these tissues after methamphetamine administration, as well as the p-hydroxyl derivative of amphetamine and one or more unidentified metabolite(s) represented by radioactivity. The latter, more polar than the parent drug or amphetamine, persisted at higher concentrations in heart than in brain tissue, paralleling the time course of norepinephrine depletion. Methamphetamine and amphetamine levels were lower in heart than in brain and disappeared from heart tissue within 5 hr (7).

METABOLISM AND PHARMACOKINETICS

A study of ^{14}C-methamphetamine metabolism in man showed that about 90 percent of the ^{14}C was excreted in the urine within four days. An average of 22 percent of the dose was excreted in urine within 24 hr as unchanged drug, and 15 percent was excreted as 4-hydroxymethamphetamine (II).

Minor metabolites include 4-hydroxyamphetamine (III, 1 percent), amphetamine (IV, 2-3 percent), 4-hydroxynorephedrine (V, 1-2 percent), norephedrine (VI, 2 percent), hippuric acid (5 percent), and an acid-labile precursor of benzylmethyl ketone (1 percent). An average of only 62 percent of the radioactivity ingested appeared in urine within 24 hr (8). Radioactivity was not monitored in sweat or saliva, although methamphetamine is known to be excreted by these routes (9). Although its products account for only 4 percent of the dose in man, ß-hydroxylation is significant because norephedrine and 4-hydroxy norephedrine could act as false neurotransmitters (10). The hydroxylated derivatives of methamphetamine are excreted primarily as glucuronic acid conjugates. In those metabolites where both parahydroxylation and N-demethylation have occurred, some evidence indicates that the latter occurs first. N-demethylation is to some degree stereospecific, occurring more readily with the d-isomer of methamphetamine than with the l-isomer (7,11).



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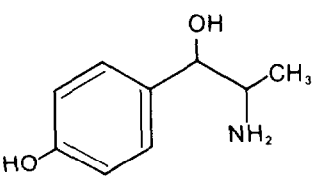

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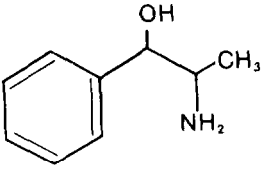


VI

Alteration of urinary pH has been show to affect the amount of unchanged methamphetamine excreted in human urine. When urine was metabolically acidified by ingestion of NH_4Cl or alkalinized by ingestion of $NaHC0_3$, 16-hr excretion of the parent drug was much greater in acid than in alkaline urine. Under alkaline urine conditions, subjective effects of methamphetamine were more prolonged, indicating reabsorption and longer retention in the body (11).

Therapeutic doses of methamphetamine in man are relatively small, 5-10 mg. The drug is a moderately strong organic base (pKa = 9.89) (11), and therefore blood concentrations are usually low (20-60 ng/ml) although as much as 550 ng/ml has been reported in overdose cases .

ANALYTICAL METHODOLOGY

A quantitative method employing thin-layer chromatography (TLC) of urine extracts on alkaline silica gel plates, followed by spectrophotometric determination of a color reaction in the spots developed on TLC, was used to measure methamphetamine in the urine of horses. The intensity of the color was directly proportional to the quantity of drug over the range of 200-2000 μg (12). An improved sensitivity (10 ng/ml) was achieved by ultraviolet spectrophotometry of extracts from urine, serum, or tissue homogenates after oxidation with cerium sulfate, but the procedure was relatively nonspecific for methamphetamine (13).

A fluorometric technique, which could in principle be used for quantitation of methamphetamine if the drug were first purified chromatographically, has been used to measure basic amines in urine. The drugs were converted to highly fluorescent compounds by reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) (14). This technique has in fact been used to screen for methamphetamine in human urine, with specificity determined by the presence of twin fluorescent spots on TLC, corresponding both in color and RF values to the NBD derivatives of methamphetamine and its metabolite, amphetamine (15).

Because of the small amounts of methamphetamine found in blood, improvements in both sensitivity and specificity of assay techniques have been needed. A GC method using electron-capture detection (ECD)

of the heptafluorobutyramide derivative of the drug in blood extracts was sensitive to 20 ng/ml of methamphetamine, the amount found in human blood 6 hr after a 5-mg oral dose (16). In another application of GC-ECD in which therapeutic blood concentrations of methamphetamine were monitored, the trichloroacetamide derivative was prepared before chromatography on 3 percent OV-1. This derivative was superior in chromatographic characteris tics and more sensitive to ECD than either the heptafluorobutyramide or the pentafluorobenzamide. Blood concentrations over a 24-hr period were found to range from 10 to 20 ng/ml following a 12.5-mg dose of methamphetamine, but the method was capable of measuring amounts as low as 25 pg (17).

Human urinary excretion rates of several amphetamine drugs, including methamphetamine, were followed by a GC procedure with the addition of phenethylamine as an internal standard and the use of a flame ionization detector (FID). The drug was chromatographed as its acetamide derivative on a column packing coated with 8 percent ethylene glycol adipate, but the sensitivity was reported only in terms of μg base/mg creatinine (18). In a similar method, blood was first treated with tungstic acid to precipitate proteins, and N-propylamphetamine was added as an internal standard. The supernatant was made strongly basic for ether extraction of methamphetamine and the internal standard before conversion to the acetamide derivatives for GC-FID analysis. This method measured 30 ng/ml methamphetamine in human blood 2 hr after ingestion of a single 10-mg dose (19).

For clinical use, a procedure to measure methamphetamine in urine by GC-FID was &signed to eliminate the false positives caused by interfering substances and to shorten analysis time to 30 min. First, the drug was determined as the free base on a 10 percent Apiezon L-10 percent KOH column. Extracts found positive with reference to GC peaks of standard solutions were then chromatographed on 3 percent OV-17, with on-column derivatization with trifluoroacetic anhydride. ß-Phenethylamine was the internal standard in both GC systems. The amount of methamphetamine was measured with an accuracy of ±7 percent and the sensitivity was 0.1 μg/ml. The procedure was adaptable to rapid analysis of a large number of samples (20,21).

Although chromatographic techniques have proved to be highly sensitive for methamphetamine, the addition of mass spectrometry is often desirable for confirmation of its structure, even in routine analysis such as monitoring in competitive sports. For confirmation purposes of this kind, the trifluoroacetylated derivative of methamphetamine was incorporated into a GC/MS protocol which detected 0.3 μg of methamphetamine/ml of human urine (22). GC/MS had been employed several years earlier as a method of identification, but not quantitation, of methamphetamine in the urine of participants in sporting events, with confirmation based on spectra of the N-acetyl and N-propionyl derivatives (23). Mercuric acetate has been used as a catalyst for rapid (30 sec at 80°C) quantitative formation of trifluoroacetamide derivatives of methamphetamine and other phenylalkylamine drugs prior to GC/MS analysis (24).

Immunoassay techniques have been used in the screening of body fluids for methamphetamine and have the advantages of being highly sensitive and requiring no extraction procedure. However, those in present use cannot distinguish between amphetamine and methamphetamine, and they are subject to interference by other phenylethylamines (21). Radioimmunoassay (RIA) did prove successful in detecting metabolites of methamphetamine in rat saliva following injection of the drug. Neither methamphetamine itself nor its 4-hydroxy derivative was reactive in the RIA procedure, but the rat saliva gave positive RIA reactions which resulted from amphetamine, 4-hydroxynorepnedrine, and 4-hydroxyamphetamine, either alone or in combination (25). A commercial RIA system is available and can be used for quantitative measurement of phenethylamines in both blood and urine (26). However, its dynamic range is very limited and consequently it is primarily used as a screening technique with confirmation and quantitation achieved by other methods. The EMIT technique is widely used for detection of phenethylamines in urine. More than 70 tests per hr can be conducted with a detection level of 2 μg/ml of urine (27).

Extraction solvents used for methamphetamine include diethyl ether (19,22,23), n-hexane (13,17), pentane (16), cyclohexane (28), and chloroform (14,15,18,21), under pH conditions ranging from 8 to 14. Back extraction into acid is sometimes employed (21). Blood proteins can be precipitated and acid substances removed by treatment with tungstic acid prior to extraction of methamphetamine at alkaline pH (17,19). A recovery of 100 percent has been reported for extractions of the drug from blood with pentane (16) and from urine with chloroform (18), both after alkalinization with NaOH.

An extensive review of the GC and GC/MS of amphetamines and their derivatives has been published (29).

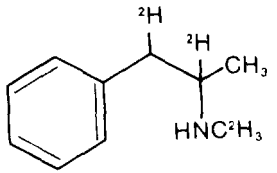
EXPERIMENTAL PROCEDURE

The blood concentrations of methamphetamine following normal dosages are usually in the low ng/ml range. Consequently, the extraction procedure includes a back extraction purification in order to provide a relatively clean extract, which in turn permits GC/MS measurement of the methamphetamine with very high sensitivity and little likelihood of interferences from endogenous components of the blood. After addition of the deuterium-labeled internal standard, the sample is made strongly basic with a saturated potassium carbonate solution and extracted with 1-chlorobutane. The methamphetamine and other organic bases are back extracted into dilute sulfuric acid, which is subsequently made basic with sodium hydroxide and reextracted with 1-chlorobutane. The organic extract is then treated with trifluoroacetic anhydride to convert methamphetamine to its N-trifluoroacetamide derivative prior to concentration by evaporation. Quantitation is achieved by selected ion monitoring using chemical ionization with methane as the reagent gas.

Standards and Reagents

The d,l-methamphetamine hydrochloride used in the development of this method was acquired from the National Institute on Drug Abuse.

It had a melting point range of 170-172°C. No impurities were detected by either gas chromatography or mass spectrometry. d,l-Methamphetamine-$^2H_5$ hydrochloride was synthesized at Battelle Columbus Laboratories using a published procedure (30). The location of the deuterium atoms is indicated in structure VII. On the basis of mass spectral analysis, its isotopic composition was: $^2H_5$, 94 percent; $^2H_4$, 5.7 percent; $^2H_3$, 0.3 percent; $^2H_2$, $^2H_1$ and $^2H_0$ less than 0.1 percent.



VII

For the purpose of preparing calibration graphs and working standards, stock solutions of methamphetamine and methamphetamine-$^2H_5$ are prepared as follows. Weigh into a 100-ml volumetric flask about 12.4 mg of methamphetamine hydrochloride and record its weight to the nearest 0.1 mg. The equivalent weight of free base is calculated by multiplying the weight of methamphetamine hydrochloride by 0.805. Dissolve the measured methamphetamine in about 75 ml of methanol and then add additional methanol to give exactly 100 ml of solution. In the following paragraphs this solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled with its actual concentration based upon the exact measured weight of methamphetamine hydrochloride. A series of working standard solutions can be prepared by appropriate dilution of this stock solution as described in Chapter 2.

The stock solution of methamphetamine-$^2H_5$ is prepared in the sane manner. For the measurement of methamphetamine in body fluids within the concentration range 1 to 500 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml methamphetamine-$^2H_5$ methanolic solution to each ml of specimen. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of methamphetamine-$^2H_5$ to 250 ml with methanol.

Store stock and standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction

Transfer 1 ml of body fluid (whole blood, plasma or urine) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately). Add 100 µl of the 400-ng/ml methamphetamine-$^2H_5$ solution and vortex for

about 10 sec. Allow the sample to equilibrate for about 15 min and then increase the pH to greater than 10 by adding 1 ml of K_2CO_3-saturated distilled water. Extract the basic mixture with 5 ml of 1-chlorobutane by gently mixing the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min and then transfer the organic phase (top) to another 20-ml culture tube by means of a disposable Pasteur pipette. Extract the methamphetamine and other organic bases from the organic phase into aqueous acid by adding 3 ml of 0.2 N H_2SO_4 and gently mixing for at least 15 min. Centrifuge and remove the top organic phase by aspiration. Add 0.5 ml of 10 N NaOH to the aqueous phase, mix the contents, and check the pH to make certain the solution is strongly basic (pH> 10). Finally, reextract the organic bases from the aqueous alkaline solution by adding 5 ml of methylene chloride and gently mixing for at least 15 min. Centrifuge for five minutes, aspirate off the upper aqueous layer, and transfer by means of a disposable Pasteur pipette the methylene chloride extract (bottom layer) to a silylated concentrator tube having a volume of at least 5 ml, a conical bottom, and a Teflon-lined screw cap. Add 10 µl of dimethylformamide to act as a "keeper solvent" to minimize evaporative loss of the methamphetamine. Remove the methylene chloride by evaporation under a gentle stream of nitrogen or filtered air while heating the tube to no higher than 40°C. When the volume of extract has decreased to about 10 µl, add 15 µl of N-methyl bis-(trifluoroacetamide) 'MBTFA" (Pierce Chemical Co., Rockford, IL 61105). Cap the tube and heat it at 70°C for 15 min. The capped tube can be stored under refrigeration until the GC/MS analysis is ready to be performed. Immediately before the analysis, allow the tube to warm to room temperature .

GC/MS Analysis

The experimental conditions for the GC/MS analysis for methamphetamine trifluoroacetamide are as follows:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier and reagent gas:	Methane, 15-20 ml/min
Temperatures:	Injector, 260°C Column, 120°C, isothermal GC/MS transfer line, 260°C Ion source, 160°C

Under these conditions, the methamphetamine trifluoroacetamide should elute in two to four minutes as a narrow, symmetrical peak.

Prior to beginning GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 246 and 251. They correspond to the protonated molecule ions for methamphetamine trifluoroacetamide and methamphetamine-$^2H_5$ trifluoroacetamide,

respectively. With the divert valve in the divert position, inject 2 to 6 μl of the organic extract into the gas chromatograph. After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the methamphetamine trifluoroacetamide peak has eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of methamphetamine in the specimen is determined by measuring the heights (or areas) or the methamphetamine trifluoroacetamide and methamphetamine-$^2H_5$ trifluoroacetamide peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the concentration of methamphetamine in the specimen by dividing the measured quantity of methamphetamine by the exact volume of specimen extracted.

Discussion of the Experimental Procedure

The procedure for analysis of amphetamine (Chapter 10) is virtually identical to this procedure, except for the ions monitored. Consequently, the concentrations of both drugs can be measured simultaneously by adding deuterated analogs of the two drugs to the biological sample for use as internal standards. Then during data acquisition, the ion currents at four m/z values are monitored. These are m/z 232, 235, 246, and 251, which correspond to the protonated molecule ions for the trifluoroacetamide derivatives of amphetamine, amphetamine-$^2H_3$, methamphetamine, and methamphetamine-$^2H_5$, respectively.

The electron impact (EI) and chemical ionization (CI) mass spectra of metamphetamine trifluoroacetamide are shown in Figure 1. The relative sensitivities achieved by monitoring the ion current at the m/z value corresponding to the most abundant ion generated by each of the ionization processes are shown in Table 1. In this case, the highest response was obtained by monitoring the fragment ion at m/z 154 generated by EI ionization. Nevertheless, CI should give effective sensitivities at least as high as EI ionization because of the lower background usually experienced in CI. The most abundant ions in the CI mass spectra correspond to the protonated molecule ion (m/z 246) and the ammonia-attachment ion (m/z 263), depending on whether methane alone or methane and ammonia are used as reagent gases. Even though the procedure specifies use of methane alone as the reagent gas, the combination of methane and ammonia is also satisfactory and should provide slightly better sensitivity. Perhaps most important is the fact that the methane and ammonia combination offers an opportunity to monitor a different ion mass if interferences are encountered at m/z 246 or 251 when methane alone is used.

An additional option which can be explored if interferences are encountered in the selected ion monitoring measurements, is to generate a derivative different from the trifluoroacetamide. For example, the acetamide derivative could be prepared by substituting N-acetylimidazole for the N-methyl bis-(trifluoroacetamide). Also the pentafluoroacetamide or heptafluorobutyramide derivatives could

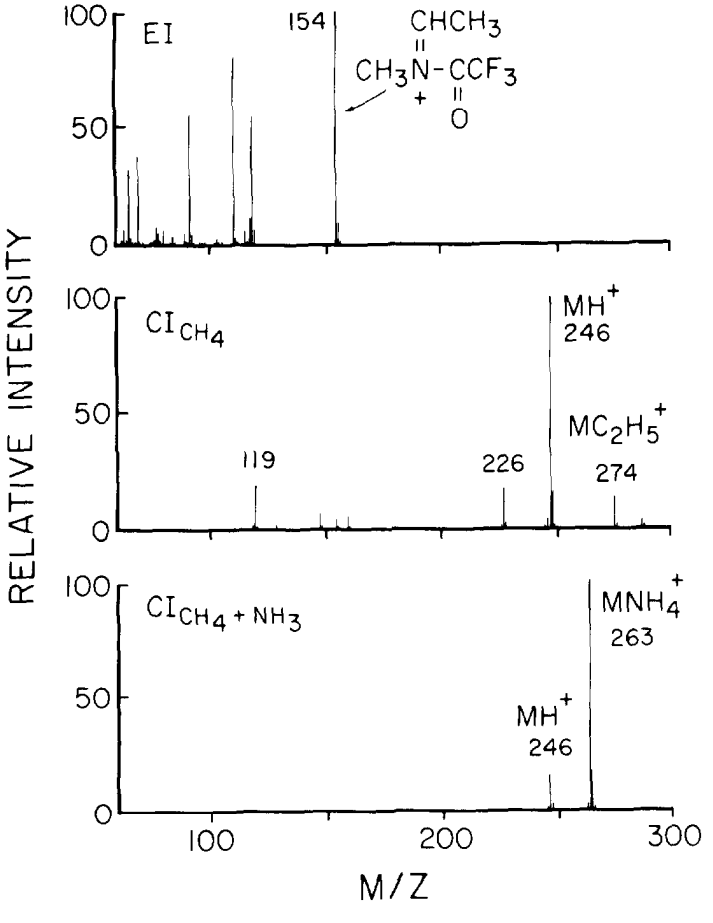


FIGURE 1. MASS SPECTRA OF METHAMPHETAMINE TRIFLUOROACETAMIDE

be used. The trifluoroacetamide derivative has proven satisfactory, but the use of other derivatives has not been fully evaluated.

TABLE 1. RELATIVE RESPONSES FOR PROMINENT IONS IN THE EI AND CI MASS SPECTRA OF THE TRIFLUOROACETAMIDE DERIVATIVE OF METHAMPHETAMINE

Method	m/z Monitored	% of Sample Ion Current	Relative Response Per Unit Weight of Drug
EI	154	18	100
CI (CH_4)	264	50	32
CI (CH_4 + NH_3)	263	72	59

A potential problem associated with concentration of organic extracts containing methamphetamine is the loss of the drug through evaporation because of its relatively high volatility. For this reason, some methamphetamine assay procedures specify addition of an acid to convert the drug to a nonvolatile salt prior to concentration of the extract. In the procedure described here, losses due to evaporation are minimized by the following means. First, methylene chloride is used in the final extraction because it has a lower boiling point (39°C) than 1-chlorobutane (78°C). Second, the methamphetamine is converted to the less volatile trifluoroacetamide derivative prior to removing the solvent. Finally, a small quantity of dimethylformamide is added just prior to evaporative concentration to act as a "keeper solvent."

A comparison study showed that all of the organic solvents investigated except diethyl ether are effective in extracting methamphetamine from 2.5 N sodium hydroxide (Table 2). 1-Chlorobutane was selected as the solvent to extract methamphetamine from biological fluids. It represents a compromise of several factors including extraction efficiency, solvent volatility, cleanliness of the extract, and ease of separation of the organic phase from the biological fluid. Diethyl ether, ethyl acetate, and ethylene chloride were eliminated from consideration because of their relatively low partition coefficients (4.3, 25.8, and 41.9, respectively). Chloroform:isopxopanol 9:1 (v/v), chloroform, and methylene chloride all had high partition coefficients (82.8, > 90, and 78.5, respectively), but they form the lower layer of an aqueous/organic two-phase system and it is therefore difficult to separate them from body fluids using a pipette without introducing contamination from the aqueous phase. 1-Chlorobutane, hexane, and cyclohexane all have relatively high partition coefficients (70.3, 81.3, and 74.3, respectively) and all form the upper layer of the aqueous/organic two-phase system. Of these, hexane was found to cause emulsion problems and

was eliminated from further consideration. Although 1-chlorobutane and cyclohexane have similar partition coefficients, 1-chlorobutane was selected for methamphetamine extraction based on previous successful experience using this solvent for drug extractions.

At pH 10, the percent of methamphetamine extracted from aqueous solution reaches a maximum (about 97 percent) and this does not significantly change at the higher pH region.

TABLE 2. PARTITION COEFFICIENTS FOR METHAMPHETAMINE BETWEEN 2.5 N SODIUM HYDROXIDE AND VARIOUS ORGANIC SOLVENTS (a)

Organic Solvent	Partition Coefficient	Percent Methamphetamine in Organic Phase
Diethyl Ether	4.3	81.1
Ethyl Acetate	25.8	96.3
Chloroform:Isopropanol 9:1 (v/v)	82.8	98.8
Methylene Chloride	78.5	98.7
Chloroform	90	100
Ethylene Chloride	41.9	97.7
1-Chlorobutane	70.3	98.6
Hexane	81.3	98.8
Cyclohexane	74.3	98.7

(a) Equal volumes of 2.5 N sodium hydroxide and organic phase were used to determine the partition coefficient.

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR METHAMPHETAMINE

Nomenclature

Chemical Name: d-N, α-Dimethylphenethylamine

Empirical Formula: $C_{10}H_{15}N$

Chemical Abstracts Registry Numbers: d-Methamphetamine: [537-46-2]
d-Methamphetamine Hydrochloride: [51-57-0]

Trade Names: Adipex, Amphedroxyn, Desfedrin, Desoxyfed, Desoxyn, Destim, Dexoxal, Doxephrin, Drinalfa, Efroxine, Gerobit, Hiropon, Isophen, Madrine, Methedrine, Methylisomyn, Pervitin, Semoxydrine, Soxysympamine, Syndrox, Tonedron

Physical Constants

Melting Points: d-Methamphetamine Hydrochloride: 173-175°C

Specific Rotation: d-Methamphetamine $[\alpha]_D^{25}$ = + 14° to + 20°(C = 1)

Solubility of d-Methaqhetamine Hydrochloride: 1 g/2 ml Water, 1 g/3 ml Ethanol, 1 g/5 ml Chloroform

Acid Dissociation Constant: d-Methamphetamine Hydrochloride pKa = 9.89

Refractive Index: d-Methamphetamine Hydrochloride (n_D^{25}) = 1.5101

Boiling Point: d-Methamphetamine 212°C

REFERENCES

1. A. Ogata. J. Pharm. Soc. Japan, 451, 751 (1919).
2. T.J. Haley. J. Am. Pharm. Assn., 36, 161 (1947).
3. M. Lampe. Drugs: Information for Crisis Treatment. STASH Press, Beloit, WI (1972).
4. J.C. Garriott and F.G. Spruill. J. Forensic Sci., 18, 434 (1973).
5. M.D. Solomon and J.A. Wright. Clin. Chem., 23, 1504 (1977).
6. G. Feuer, D.R. Miller, S.D. Cooper, F.A. de la Iglesia, and G. Lumb. Int. J. Clin. Pharmacol., Ther., and Toxicol., 7, 13 (1973).

7. C.D. Morgan, F. Cattabeni, and E. Costa. J. Pharmacol. Exp. Ther., 180, 127 (1972).

8. J. Caldwell, L.G. Dring, and R.T. Williams. Biochem. J., 129, 11 (1972).

9. T.B. Vree, T.J.M. Muskens, and J.M. Van Rossum. Arch. Int. Pharmacodynamie, 199, 311 (1972).

10. H. Thoenen, A. Hürlimann, K.F. Gey, and W. Haefely. Life Sci., 5, 1715 (1966).

11. A.H. Beckett and M. Kowland. J. Pharm. Pharmacol., 17, suppl.. 109S (1965).

12. M.S. Karawya, M.A. El-Keiy, S.K. Wahba, and A.R. Kozman. J. Pharm. Pharmacol., 20, 650 (1968).

13. J.E. Wallace, J.D. Biggs, and S.L. Ladd. Anal. Chem., 40, 2207 (1968).

14. J. Monforte, R.J. Bath, and I. Sunshine. Clin. Chem., 18, 1329 (1972).

15. R.N. Gupta, B.G. Chittim, and P.M. Keane. J. Chromatogr. Sci., 12, 67 (1974).

16. R.B. Bruce and W.R. Maynard, Jr. Anal. Chem., 41, 977 (1969).

17. R.C. Driscoll, F.S. Barr, B.J. Gragg, and G.W. Moore. J. Pharm. Sci., 60, 1492 (1971).

18. J.W. Schweitzer and A.J. Friedhoff. Clin. Chem., 16, 786 (1970).

19. P. Lebish, B.S. Finkle, and J.W. Brackett, Jr. Clin. Chem., 16, 195 (1970).

20. N.C. Jain, T.C. Sneath, and R.D. Budd. Clin. Chem., 20, 1460 (1974).

21. N.C. Jain, R.D. Budd, and T.C. Sneath. Clin. Toxicol., 8, 211 (1975).

22. K. Kamei, M. Murata, K. Ishii, M. Namekata, and A. Momose. Chem. Pharm. Bull., 21, 1996 (1973).

23. A.H. Beckett, G.T. Tucker, and A.C. Moffat. J. Pharm. Pharmacol., 19, 273 (1967).

24. A. Wu. Clin. Toxicol., 8, 225 (1975).

25. G.J. Pigregorio and E.K. Kniaz. Drug and Alcohol Dependence, 1, 377 (1976).

26. Abuscreen. Roche Diagnostics, Nutley, NJ 07110.

27. EMIT. Syva, Palo Alto, CA 94304.

28. J.W. Blake, R.S. Ray, J.S. Noonan, and P.W. Murdick. Anal. Chem., 46, 288 (1974).

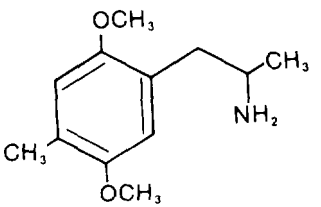
29. B.J. Gudzinowicz and M.J. Gudzinowicz. Analysis of Drugs and Metabolites by Gas Chromatography - Mass Spectrometry, Vol. 4. Marcel Dekker, Inc., New York (1978).

30. A.F. Fentiman, Jr. and R.L. Foltz. J. Labelled Compounds and Radiopharmaceuticals, 12, 69 (1976).

Chapter 12

2, 5-Dimethoxy-4-methylamphetamine (DOM)

In the late 1960's a street drug known to users as "STP" was extensively circulated in the United States as a hallucinogen (1). The psychotomimetic component of the illicit preparations was identified as 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (I), a structure more conveniently referred to as 2,5-dimethoxy-4-methylamphetamine or DOM, which had been developed as an experimental compound by Dow Chemical Company. Early tests of its psychotropic activity in mice showed behavioral responses very similar to those evoked by mescaline, but which required only about one-fiftieth of the dose for an equivalent response (2). Controlled dosage studies in human volunteers indicated a hallucinogenic potency 100 times greater than mescaline but only about one-thirtieth that of LSD. Effects of the drug included elevation of pulse rate, systolic blood pressure and temperature, as well as nausea, paresthesia, and tremors. Psychoactivity began with moderate euphoria within 1-2 hr after administration of as little as 2 mg; and doses in excess of 5 mg always produced marked hallucinogenic effects of dose-related intensity which subsided within 8 hr (1).

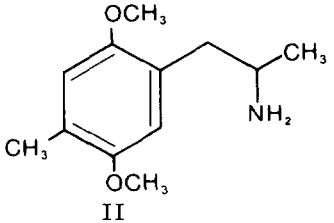


I

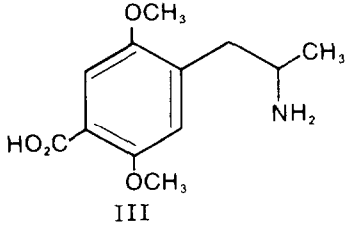
The sensory and hallucinogenic effects of DOM are limited to the R-(-) enantiomer (3). In vivo metabolism of DOM in rabbits is stereoselective (4), and in rabbit liver preparations S-(+)-DOM-$^{2}H_{6}$ is metabolized more rapidly than R-(-)-DOM by microsomal enzymes (5). Several methoxyl and methyl analogs were prepared for a study of comparative pharmacological activity in mice. These studies showed that DOM was almost as effective as amphetamine in decreasing pentobarbital-induced sleep time, and that the 4-methyl group was apparently essential for the psychotomimetic activity of DOM while the 2-methoxyl group was not (6). DOM has no known therapeutic value but has been used for research investigations concerning model psychoses.

METABOLISM AND PHARMACOKINETICS

In vivo studies in rats, using radiolabeled tracers, showed that the major metabolic pathway of DOM in that species is hydroxylation of the 4-methyl group to 1-(2,5-dimethoxy-4-hydrovthylphenyl)-2-aminopropane (II), followed by oxidation of II to 1-(2, 5-dimethoxy-4-carboxyphenyl)-2-aminopropane (III). Nearly 50 percent of the urinary radioactivity was present as II plus its glucuronide and sulfate conjugates. Unchanged DOM accounted for 8 percent of urinary radioactivity and III accounted for 28 percent. 1-(2,5-Dimethoxy-4-methylphenyl)-2-propanone (IV) was detected in trace amounts. In feces, 83 percent of the radioactivity was due to III; 6 percent was due to II; only a trace amount was due to unchanged DOM (7). Identification of the metabolites was based on comparison of TLC Rf values with those of synthesized standards (8). The same metabolic pathways for DOM, including the minor one involving oxidative deamination, have been demonstrated in rabbits in vivo (4). N-Hydroxylation (V) and O-demethylation reactions have been reported in rabbit liver microsomal preparations incubated with DOM (9). The O-demethylated derivative of DOM might be of interest in light of speculation that a bis-O-demethylated metabolite could be oxidized to the corresponding p-hydroquinone which in turn could cyclize to form 5-hydroxy-2,6-dimethylindole in a manner analogous to that observed for 6-hydroxydopamine. The 5-hydroxy-2,6-dimethylindole has been suggested as a possible cause of hallucinogenic activity (10).



II



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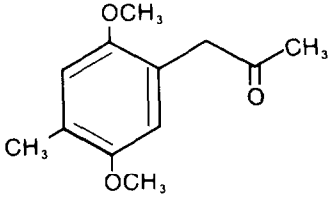
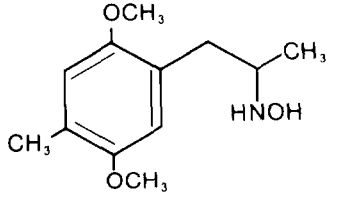


IV



V

In the human study previously mentioned, about 20 percent of the injected dose appeared in the urine within 24 hr as the unchanged drug. The peak of urinary excretion, between 3 and 6 hr after administration, corresponded to the peak clinical effects (1). In rats, excretion of radiolabeled DOM and its metabolites was nearly complete within 24 hr (7). Plasma concentration of DOM monitored over a 24-hr period in two 9-kg dogs given 2 mg and 4 mg of DOM respectively, suggested a bi-phasic elimination process, with an initial rapid decrease and a slower phase corresponding to a half-life of 1.5 hr (11).

ANALYTICAL METHODOLOGY

Even though DOM has been a recognized drug of abuse for at least ten years, little effort appears to have been devoted to development of methods for identification and quantitation of the drug in biological media for forensic or research purposes. In 1968, several authors reported data on the physical and chemical characteristics for DOM including its TLC Rf values and its ultraviolet, infrared, nuclear magnetic resonance, and mass spectra. These analytical techniques were used for identification of the drug in confiscated illicit preparations (2,12). An ultraviolet spectrophotometric method for determination of DOM following oxidation by cerium sulfate was reported (13). However, UV and IR spectra were unsatisfactory for samples containing impurities because of inadequate specificity. Mass spectrometry was suggested as a reliable means of identification of DOM in confiscated drugs (14).

A fluorometric method has been used for the analysis of unchanged DOM in the urine of human volunteers during a study of hallucinogenic reactions (1). More recently, a highly sensitive radioimmunoassay (RIA) has been reported in which a radioiodinated derivative of DOM was used as the test antigen. The specificity of the antibody, raised in rabbits by DOM conjugated to human serum albumin, was such that structurally related molecules (i.e., amphetamine, mescaline, catecholamine metabolites, or N,N-diethyltryptamine) were several orders of magnitude less effective than DOM as inhibitors (15).

In vivo metabolic studies in rats measured excretion of tritium-labeled DOM and its metabolites in urine and in fecal homogenates by liquid scintillation counting. Metabolites were extracted with diethyl ether and identified by comparison of TLC Rf values with those of synthesized standards. The major metabolites were further characterized by gas chromatography with a flame-ionization detector (GC-FID) on columns of 3 percent OV-1 or 3 percent SE-30 maintained at 160°C. Quantitation of individual metabolites was done by scintillation counting of the appropriate spots cut from paper chromatograms (7). Similarly, concentrations of urinary DOM and metabolites from rabbits receiving carbon-14 labeled DOM were measured by isotope dilution analysis during a study of stereoselective metabolism. DOM was extracted from the urine with diethyl ether and recrystallized for measurement of radioactivity (4).

In vitro studies of stereoselective metabolism have utilized direct-probe chemical ionization mass spectrometry with deuterium-labeled DOM as an internal standard for quantitation of DOM and its metabolites in rabbit liver preparations (5). In other metabolic studies with rabbit liver microsomes, the technique of selected ion monitoring was used to measure DOM metabolite formation, with DOM-$^2H_6$ as substrate, and a synthetic N-hydroxylated metabolite of DOM as the internal standard. The deuteratcd metabolite produced by the enzyme system was identified as its bis-(trifluoroacetyl) derivative by GC/MS. Electron impact mass spectra were obtained following GC separation on a 2 percent Dexil 300 column maintained at 160°C. GC separations prior to isobutane chemical ionization mass spectrometry were done on 3 percent OV-25 or 3 percent OV-1 columns (9).

In published studies which involved extraction of DOM from biological specimens, the extraction solvent has usually been diethyl ether, with no extraction efficiencies reported (4,7).

EXPERIMENTAL PROCEDURE

The procedure for extraction, derivatization, and GC/MS quantitation of DOM is the same as that described for the analysis of methamphetamine (Chapter 9) and the other phenylalkylamine drugs (i.e., amphetamine and mescaline). After addition of the deuterium-labeled internal standard, the sample is made strongly basic with a saturated potassium carbonate solution and extracted with 1-chlorobutane. The DOM and other organic bases arc back extracted into dilute sulfuric acid, which is subsequently made basic with sodium hydroxide and reextracted with 1-chlorobutane. The organic extract is then treated with trifluoroacetic anhydride to convert DOM to its N-trifluoroacetamide derivative prior to concentration by evaporation. Quantitation is achieved by selected ion monitoring using chemical ionization with methane as the reagent gas.

Standards and Reagents

The DOM•HCl used in the validation of this procedure was obtained from the National Institute on Drug Abuse. It had a melting point of 187-189°C. The DOM•HCl can be purchased from Applied Science Laboratories, State College, PA 16801. DOM-$^2H_6$•HCl was synthesized at Battelle Columbus Laboratories using a published procedure (15). On the basis of mass spectral analysis the DOM-$^2H_6$ had the following isotopic composition: $^2H_6$ 96.1 percent; $^2H_5$, 3.1 percent; $^2H_4$, 0.6 percent; $^2H_3$, 0.2 percent; $^2H_2$, $^2H_1$, and $^2H_0$, <0.1 percent.

For the purpose of preparing calibration graphs and working standards, stock solutions of DOM and DOM-$^2H_6$ are prepared as follows. Weigh into a 100-ml volumetric flask about 11.7 mg of DOM•HCl and record its weight to the nearest 0.1 mg. The equivalent weight of free base is calculated by multiplying the weight of DOM•HCl by 0.85. Dissolve the measured DOM in about 75 ml of methanol and then add additional methanol to give exactly 100 ml of solution. In the following paragraphs this solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled with its actual

concentration based upon the exact measured weight of DOM hydrochloride. A series of working standard solutions can be prepared by appropriate dilution of this stock solution as described in Chapter 2.

The stock solution of DOM-$^2H_6$ is prepared in the same manner. For the measurement of DOM in body fluids within the concentration range 1 to 500 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml DOM-$^2H_6$ methanolic solution to each ml of specimen. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-mg/ml stock solution of DOM-$^2H_6$ to 250 ml with methanol.

Store stock and standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction

Transfer 1 ml of body fluid (whole blood, plasma or urine) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately). Add 100 µl of the 400-ng/ml DOM$^2H_6$ solution and vortex for about 10 sec. Allow the sample to equilibrate for about 15 min and then increase the pH to greater than 10 by adding 1 ml of K_2CO_3- Saturated distilled water. Extract the basic mixture with 5 ml of l-chlorobutane by gently mixing the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min and then transfer the organic phase (top) to another 20-ml culture tube by means of a disposable Pasteur pipette. Extract the DOM and other organic bases from the organic phase into aqueous acid by adding 3 ml of 0.2 N H_2SO_4 and gently mixing for at least 15 min. Centrifuge and remove the top organic phase by aspiration. Add 0.5 ml of 10 N NaOH to the aqueous phase, mix the contents, and check the pH to make certain the solution is strongly basic (pH > 10). Finally, rcextract the organic bases from the aqueous alkaline solution by adding 5 ml of methylene chloride and gently mixing for at least 15 min. Centrifuge for 5 min, aspirate off the upper aqueous layer, and transfer by means of a disposable Pasteur pipette the methylene chloride extract (bottom layer) to a silylated concentrator tube having a volume of at least 5 ml, a conical bottom, and a Teflon-lined screw cap. Add 10 µl of dimethylformamide to act as a "keeper solvent" to minimize evaporative loss of the DOM. Remove the methylene chloride by evaporation under a gentle stream of nitrogen or filtered air while heating the tube to no higher than 40°C. When the volume of extract has decreased to about 10 µl, add 15 µl of N-methyl bis-(trifluoroacetamide) "MBTFA" (Pierce Chemical Co., Rockford, IL 61105). Cap the tube and heat it to 70°C for 15 min. The capped tube can be stored under refrigeration until the GC/MS analysis is ready to be performed. Immediately before the analysis, allow the tube to warm to room temperature.

GC/MS Analysis

The experimental conditions for the GC/MS analysis for DOM trifluoroacetamide are as follows:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science laboratory, State College, PA 16801)
Carrier and reagent gas:	Methane, 15-20 ml/min
Temperatures:	Injector, 260°C Column, 165°C, isothermal GC/MS transfer line, 260°C Ion source, 160°C

Under these conditions, the DOM trifluoroacetamide should elute at about 4 min as a narrow, symmetrical peak.

Prior to beginning GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 306 and 312. They correspond to the protonated molecule ions for DOM trifluoroacetamide and DOM-$^2H_6$ trifluoroacetamide, respectively. With the divert valve in the divert position, inject 2 to 6 µl of the organic extract into the gas chromatograph. After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the DOM trifluoroacetamide peak has eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of DOM in the specimen is determined by measuring the heights (or areas) or the DOM. trifluoroacetamide and DOM-$^2H_6$ trifluoroacetamide peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the concentration of DOM in the specimen by dividing the measured quantity of DOM by the exact volume of specimen extracted.

Discussion of the Experimental Procedure

The methane chemical ionization mass spectrum of the trifluoroacetamide derivative of DOM is shown in Figure 1. In addition to the protonated molecule ion at m/z 306, there is also an abundant fragment ion (m/z 193) which is formed by loss of H_2NCOCF_3 from the protonated molecule ion. These ions are shifted to m/z 312 and 199 in the methane CI mass spectrum of DOM-$^2H_6$. Normally only the ion currents at m/z 306 and 312 are monitored during GC/MS analysis, but the ion currents at m/z 193 and 199 can also be monitored if additional specificity is desired. The slope of the calibration graph obtained by measuring the m/z 193/199 ratio for samples containing known concentrations of DOM and DOM-$^2H_6$ may be slightly different

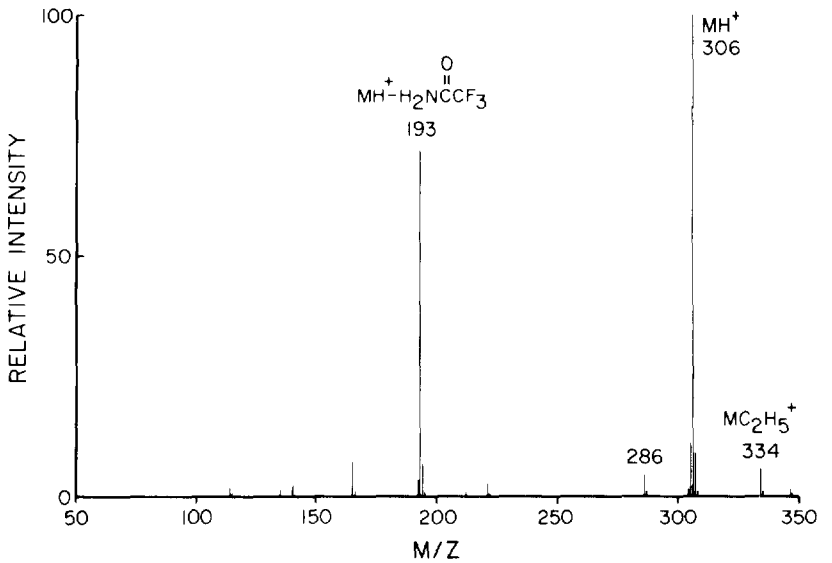


FIGURE 1. METHANE CI MASS SPECTRUM OF THE TRIFLUOROACETAMIDE DERIVATIVE OF DOM

from the slope obtained for the m/z 306/312 ratio. However, if the concentration of DOM calculated from the m/z 193/199, peak height ratio differs significantly from the concentration based on the measured m/z 306/312 peak height ratio, it is likely that an interfering compound is contributing to one or more of the DOM-TFA peaks in the ion current profiles. When this occurs, usually a visual examination of the ion current profiles will disclose which of the peak heights is inaccurate due to the interference; i.e. the peak will have a shoulder, or will be perceptibly broadened, or will have an abnormal retention time.

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR DOM

Nomenclature

Chemical Name: 2,5-Dimethoxy-4-methylamphetamine

Empirical Formula: $C_{12}H_{19}NO_2$

Chemical Abstracts Registry Numbcr: 15588-95-1

Physical Constants

Appearance: White crystals

Melting Point: 61°C
Hydrochloride, 190°C

Specific Rotation: Synthetic material is usually racemic

Solubility: Insoluble in water, soluble in chloroform

W Absorption: λ_{max} = 214, 225 and 288 nm (in dilute HCl)

REFERENCES

1. S.H. Snyder, L. Faillace, L. Hollister. Science, 158, 669 (1967).
2. G.F. Phillips and R.J. Mesley. J. Pharm. Pharmacol., 21, 9 (1969).
3. A.T. Shulgin. J. Pharm. Pharmacol., 25, 271 (1973).
4. S.B. Matin, P.S. Callery, J.S. Zwieg, A. O'Brien, R. Rapoport, and N. Castagnoli, Jr. J. Med. Chem., 17, 877 (1974).
5. K.J. Weinkam, P. Callery, J. Gal, and N. Castagnoli, Jr. 22nd Ann. Conf. MS and Allied Topics, Philadelphia. Abst., p. 174 May 19-24, 1974.
6. B.T. Ho, W.M. McIsaac, R. An, L.W. Tansey, K.E. Walker, L.F. Englert, Jr., and M.B. Noel. J. Med. Chem., 13, 26 (1970).
7. B.T. Ho, V. Estevez, L.W. Tansey, L.F. Englert, P.J. Creaven, and W.M. McIsaac. J. Med. Chem., 14, 158 (1971).
8. B.T. Ho and L.W. Tansey. J. Med. Chem., 14, 156 (1971).
9. J. Gal, L.D. Gruenke, and N. Castagnoli, Jr. J. Med. Chem., 18, 683 (1975).
10. J.S. Zweig and N. Castagnoli, Jr. J. Med. Chem., 17, 747 (1974).

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11. R.J. Martin and T.G. Alexander. J. Assoc. Off. Anal. Chem., 51, 159 (1968).

12. J.F. Wallace, J.D. Biggs, and S.L. Ladd. Anal. Chem., 40, 2207 (1968).

13. S.W. Bellman. J. Assoc. Off. Anal. Chem., 51, 164 (1968).

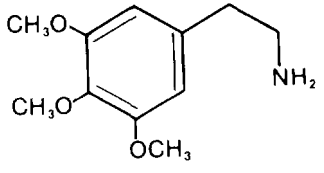
14. L.J. Riceberg, H. van Vunakis, and L. Levine. Anal. Biochem., 60, 551 (1974).

15. A.F. Fentiman, Jr., and R.L. Foltz. J. labelled Compounds and Radiopharmaceuticals, 12, 69 (1976).

Chapter 13

Mescaline

Mescaline (3,4,5-trimethoxyphenylethylamine, I) is the hallucinogenic constituent of the peyote cactus Lophophora williamsii, a plant which has been employed by Indians in northern Mexico from the earliest recorded time as an adjunct to religious rites, to relieve hunger and fatigue, and for treatment of disease (1). In 1889, a tincture of the peyote buttons appeared in the Parke-Davis catalog as a cardiac and respiratory stimulant for treatment of angina pectoris and pneumothorax. Mescaline was first isolated from peyote in 1896 but a complete synthesis was not reported until 1918. During the 1940's mescaline was one of several hallucinogens used in research concerning "model" psychoses (1). At present the use of mescaline, except in a religious context by certain North American Indians, is primarily as an illicit hallucinogen. However, street drugs sold as mescaline often contain little or none of the drug. Instead they may contain amphetamines, DOM, belladonna alkaloids, LSD, or phencyclidine (PCP) as the active ingredient.



I

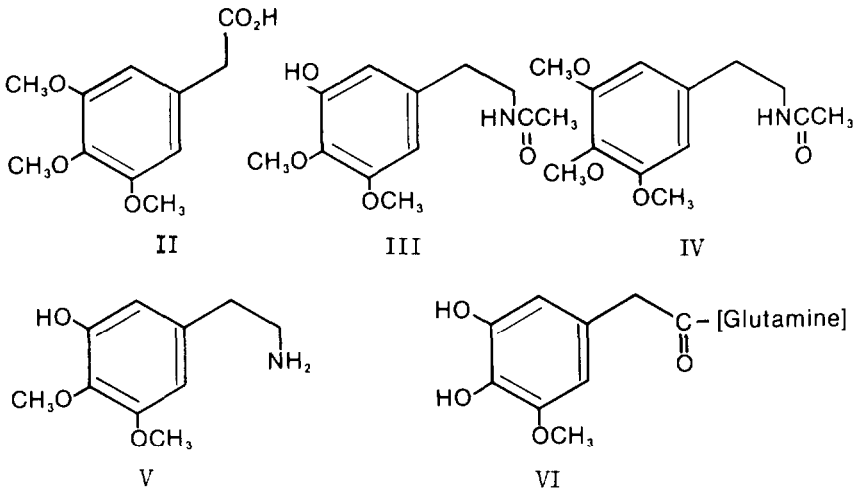
Mescaline, unlike most other hallucinogenic drugs which are indole compounds, is chemically reiated to the hormone neurotransmitters, epinephrine and norepinephrine. Pharmacological effects vary among animal species. Physical dependence has not been demonstrated in man, although repeated doses of mescaline cause tolerance to the physiological, subjective, and psychological effects of the drug, within 3 to 6 days. Cross tolerance to these effects has been demonstrated between mescaline and LSD or psilocybin. Certain drugs, such as insulin and barbiturates, potentiate the toxicity of mescaline. Mescaline poisoning may cause abdominal cramps, nausea, vomiting, and other symptoms which can be mistaken for acute gastroenteritis, appendicitis, or pancreatitis (2). An hallucinogenic dose of mescaline is about 500-1500 mg (1). Distribution studies of mescaline-^{14}C in cat brains showed the highest concentration of radioactivity in cortical and subcortical grey matter (3). In vitro experiments with sheep umbilical vasculature (4) and in vivo studies of rat responses (5) have shown that antagonists of serotonin also antagonize the action of mescaline, supporting the hypothesis that mesca-

line initiates its pharmacological activity via serotonin receptors. Efforts to synthesize specific antagonists to mescaline have led to production of the N-allyl and N-n-propyl derivatives of mescaline which alone have no effect on the swim-maze behavior of mice but do antagonize the effect of mescaline on mouse behavior. No correlation has been demonstrated, however, between the swim-maze effects of mescaline in mice and psychotomimetic potency in humans (6).

METABOLISM AND PHARMACOKINETICS

Mescaline metabolism has been studied in several species, stimulated by evidence that the hallucinogenic properties of mescaline are actually caused by one or more of its metabolites. This is suggested because the psychotomimetic effects typically take several hours to reach their maximum and the hallucinogenic dose is so large. Cases of "flashbacks" over a period of several months following a single dose of mescaline have also been reported (7). The major urinary metabolic product in mammals is 3,4,5-trimethoxyphenylacetic acid (II) produced by oxidative deamination by way of the corresponding aldehyde. The aldehyde can also be reduced to 3,4,5-trimethoxyethanol, which has been detected in rat urine and brain, although the aldehyde intermediate itself has not been isolated (8). The enzyme involved in deamination differs from monoamine oxidase in that it is cyanide-sensitive and resistant to octanol, and it has been characterized as a type-II enzyme (diamine oxidase) (9,10). However, mescaline is not extensively metabolized in man or in laboratory animals. Metabolic studies with mescaline-^{14}C in human subjects showed that 55-60 percent of the drug was excreted unchanged in the urine. Trimethoxyphenylacetic acid accounted for 27-30 percent of urinary radioactivity, while N-acetyl-ß-(3,4-dimethoxy-5-hydroxyphenyl) ethylamine (III), in the form of a conjugate, represented 5 percent and N-acetylmescaline (IV) less than 1 percent. The proportions varied with time following administration. Five other minor metabolites were only partially characterized (11). Other studies have shown the presence of 3,4-dimethoxy-5-hydroxyphenethylamine (V) and the glutamine conjugate of 3,4-dihydroxy-5-methoxyphenylacetic acid (VI) in human urine following administration of mescaline (7,12).

Compounds I, II, III, and IV were also found in human cerebrospinal fluid (CSF) after oral administration of mescaline-^{14}C. Mescaline represented at least 50 percent of the CSF radioactivity, and the concentration of radioactivity in the CSF appeared to coincide with the peak of behavioral manifestations. The average biological half-life of radioactivity ingested as mescaline in this study was 6 hr, with 87 percent of the dose excreted within 24 hr and 92 percent within 48 hr (11). Neither trimethoxyphenylacetic acid nor N-acetylmescaline, in doses as high as 12 mg/kg, had any physiological or psychological effects in human subjects, indicating that N-acetylation and deamination are both detoxification pathways for mescaline. The discrepancy in the relative amount of trimethoxyphenylacetic acid and N-acetylmescaline in CSF as compared to urine suggested that in the central nervous system N-acetylation is a more important pathway (11). N-Acetylmescaline has been identified in



the CNS of mice (13) and of rats, in which acetylation appeared to occur predominantly in the soluble cell fractions (14). 0-Demethylation of mescaline has been reported; however, 0-demethylation can occur spontaneously under acidic conditions, which casts doubt on the authenticity of these urinary metabolites (12).

In the continuing effort to identify the mechanism of hallucinogenic activity of mescaline, rats trained for discriminative responses were given inhibitors of aldehyde dehydrogenase or amine oxidase before treatment with mescaline or three of its major rat metabolites. A role in mescaline-induced interoceptive stimuli could not be established for any of the metabolites (8); however, in mouse brain, side-chain degradation of mescaline to 3,4,5-trimethoxybenzoic acid has been demonstrated in vivo and in vitro, catalyzed by enzymes in the nuclear and microsomal fractions of brain tissue (15). The authors of this report suggest that complete elucidation of the mechanism of the oxidative degradation of mescaline to 3,4,5-trimethoxybenzoic acid could provide a basis for disclosure of the assumed relationship between the hallucinogenic properties of mescaline and its metabolism.

ANALYTICAL METHODOLOGY

Thin-layer chromatography (TLC) and gas chromatography (GC) have been the methods used in most studies for isolation and identification of mescaline in biological fluids or plant materials (16,17). For TLC, mescaline extracted from urine can be developed in ethyl

acetate:methanol:NH_4OH (85:5:5) and sprayed with ninhydrin before irradiation under 350 nm light, or in chloroform:methanol (9:1) treated with Marquis reagent. The detection limit reported for TLC is 40 ng/ml. GC conditions recommended for confirmation of mescaline in urine extracts are 3 percent SE-30 on Chromosorb W-AW 100/200 mesh in a Teflon-lined aluminum column, and an oven temperature of 150°C (2). A recent study of mescaline metabolism in mouse brain tissue used mass spectrometry to confirm the identification of 3,4,5-trimethoxybenzoic acid as its brominated derivative after TLC separation (15). A quantitative radioimmunoassay, which can distinguish between mescaline and its most common urinary metabolite, trimethoxyphenylacetic acid, has been reported. The assay detected as little as 100 pg of mescaline (18).

A very sensitive GC/MS method for quantitation of mescaline in blood or urine has been reported (19). Blood plasma was made basic (pH 10) and extracted with benzene. The extracted mescaline was converted to its trifluoroacetyl derivative which was measured by GC/MS using a 2.5 percent OV-1 on Varaport column packing and a column temperature of 195°C. Deuterated mescaline was used as an internal standard. The limit of detection was reported to be 0.5 ng injected on column.

Mescaline can be separated from its acid metabolites in urine by extraction of the latter from acid solution with ethyl acetate. Mescaline remains in the aqueous phase and can be quantitatively extracted with benzene after the solution is made strongly basic (11). Mescaline extraction from urine at pH 8.5 in n-butanol has also been reported, with a recovery of 65 percent after back-extraction into 0.1N HCl (20). There are also reports of mescaline extraction from urine buffered at pH 9.2 using chloroform or chloroform:isopropanol (3:1) (2,3) and with ethylene dichloride:isoamyl alcohol at pH 10 (12)

EXPERIMENTAL PROCEDURE

The procedure for extraction, derivatization, and GC/MS quantitation of mescaline is the same as that described for the analysis of methamphetamine (Chapter 11) and the other phenylalkylamine drugs (i.e., amphetamine and DOM). After addition of the deuterium-labeled internal standard, the sample is made strongly basic with a saturated potassium carbonate solution and extracted with 1-chlorobutane. The mescaline and other organic bases are back-extracted into dilute sulfuric acid, which is subsequently made basic with sodium hydroxide and reextracted with 1-chlorobutane. The organic extract is then treated with trifluoroacetic anhydride to convert mescaline to its N-trifluoroacetamide derivative prior to concentration by evaporation. Quantitation is achieved by selected ion monitoring using chemical ionization with methane as the reagent gas.

Standards and Reagents

The mescaline hydrochloride (m.p. 183-184) and the mescaline-$^2H_3$ hydrochloride (m.p. 179-182) used in the validation of this procedure were obtained from the National Institute on Drug Abuse. No impuri-

ties were detected in either compound by gas chromatography. The mescaline-$^2H_3$ HCl was further examined by thin-layer chromatography (silica gel plates, 1.5 percent ammonia in methanol, Rf = 0.19) and found to give a single spot. The three deuterium atoms in mescaline-$^2H_3$ were located on the 4-methoxy group. Its isotopic composition was: $^2H_3$, 93 percent; $^2H_2$, 3 percent; $^2H_1$, 3 percent; $^2H_0$, < 1 percent.

For the purpose of preparing calibration graphs and working standards stock solutions of mescaline and mescaline-$^2H_3$ are prepared as follows. Weigh into a 100-ml volumetric flask about 11.7 mg of mescaline hydrochloride and record its weight to the nearest 0.1 mg. The equivalent weight of free base is calculated by multiplying the weight of mescaline hydrochloride by 0.85. Dissolve the measured mescaline in about 75 ml of methanol and then add additional methanol to give exactly 100 ml of solution. In the following paragraphs this solution will be referred to as the "0.10-mg/ml stock solution" although it should be labeled with its actual concentration based upon the exact measured weight of mescaline hydrochloride. A series of working standard solutions can be prepared by appropriate dilution of this stock solution as described in Chapter 2.

The stock solution of mescaline-$^2H_3$ is prepared in the same manner. For the measurement of mescaline in body fluids within the concentration range 1 to 500 ng/ml, addition of approximately 40 ng of the internal standard to each ml of specimen is satisfactory. This can be conveniently done by adding 100 µl of a 400-ng/ml mescaline methanolic solution to each ml of specimen. Prepare the 400-ng/ml internal standard solution by diluting 1 ml of the 0.10-ng/ml stock solution of mescaline-$^2H_3$ to 250 ml with methanol.

Store stock and standard solutions in well-stoppered or capped glass vessels in the dark at less than 0°C.

Extraction

Transfer 1 ml of body fluid (whole blood, plasma or urine) to a 20-ml culture tube equipped with a Teflon-lined screw cap (0.5 to 2 ml of specimen can be used, but it must be measured accurately). Add 100 µl of the 400-ng/ml mescaline-$^2H_3$ solution and vortex for about 10 sec. Allow the sample to equilibrate for about 15 min and then increase the pH to greater than 10 by adding 1 ml of K_2CO_3-saturated distilled water. Extract the basic mixture with 5 ml of 1-chlorobutane by gently mixing the contents for 30 min using a motorized rocker or rotator. Centrifuge for 5 min and then transfer the organic phase (top) to another 20-ml culture tube by means of a disposable Pasteur pipette. Extract the mescaline and other organic bases from the organic phase into aqueous acid by adding 3 ml of 0.2 N H_2SO_4 and gently mixing for at least 15 min. Centrifuge and remove the top organic phase by aspiration. Add 0.5 ml of 10 N Na0H to the aqueous phase, mix the contents, and check the pH to make certain the solution is strongly basic (pH > 10). Finally, reextract the organic bases from the aqueous alkaline solution by adding 5 ml of methylene chloride and gently mixing for at least 15 min.

Centrifuge for 5 min, aspirate off the upper aqueous layer, and transfer by means of a disposable Pasteur pipette the methylene chloride extract (bottom layer) to a silylated concentrator tube having a volume of at least 5 ml, a conical bottom, and a Teflon-lined screw cap. Add 10 µl of dimethylformamide to act as a "keeper solvent" to minimize evaporative loss of the mescaline. Remove the methylene chloride by evaporation under a gentle stream of nitrogen or filtered air while heating the tube to no higher than 40°C. When the volume of extract has decreased to about 10 µl, add 15 µl of N-methyl bis-(trifluoroacetamide) "MBTFA" (Pierce Chemical Co., Rockford, IL 61105). Cap the tube and heat it at 70°C for 15 min. The capped tube can be stored under refrigeration until the GC/MS analysis is ready to be performed. Immediately before the analysis, allow the tube to warm to room temperature.

GC/MS Analysis

The experimental conditions for the GC/MS analysis for mescaline trifluoroacetamide are as follows:

GC column:	1.8 m x 2 mm (I.D.) glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, State College, PA 16801)
Carrier and reagent gas:	Methane, 15-20 ml/min
Temperatures:	Injector, 260°C Column, 160°C, isothermal GC/MS transfer line, 260°C Ion source, 160°C

Under these conditions, the mescaline trifluoroacetamide should elute at about 4 min as a narrow, symmetrical peak.

Prior to beginning GC/MS analysis of samples, the performance of the total GC/MS system should be evaluated and optimized as described in Chapter 2. The ions to be monitored occur at m/z 308 and 311. They correspond to the protonated molecule ions for mescaline trifluoroacetamide and mescaline-$^{2}H_{3}$ trifluoroacetamide, respectively. With the divert valve in the divert position, inject 2 to 6 µl of the organic extract into the gas chromatograph. After approximately 1 min, switch the divert valve so that the entire carrier gas flow enters the ion source of the mass spectrometer, and begin data acquisition. When the mescaline trifluoroacetamide peak has eluted, cease data acquisition and return the divert valve to the divert position.

The quantity of mescaline in the specimen is determined by measuring the heights (or areas) or the mescaline trifluoroacetamide and mescaline-$^{2}H_{3}$ trifluoroacetamide peaks in the selected ion current profiles ("ion chromatograms") and relating the ratio of peak heights to a calibration graph. Calculate the concentration of mescaline in the specimen by dividing the measured quantity of mescaline by the exact volume of specimen extracted.

Discussion of the Experimental Procedure

The methane chemical ionization mass spectrum of the trifluoroacetamide derivative of mescaline is shown in Figure 1. The only abundant ion in the mass spectrum is the protonated molecule ion at m/z 308. The fragment ion formed by loss of H_2NCOCF_3 from the protonated molecule ion is considerably less abundant than it is in the methane CI mass spectra of the other phenylalkylamine drugs included in this monograph. This is probably because the amide nitrogen is attached to a primary carbon, rather than a secondary carbon as is the case in amphetamine, methamphetamine, and DOM. Elimination of H_2NCOCF_3 from a secondary carbon would lead to a more stable carbonium ion.

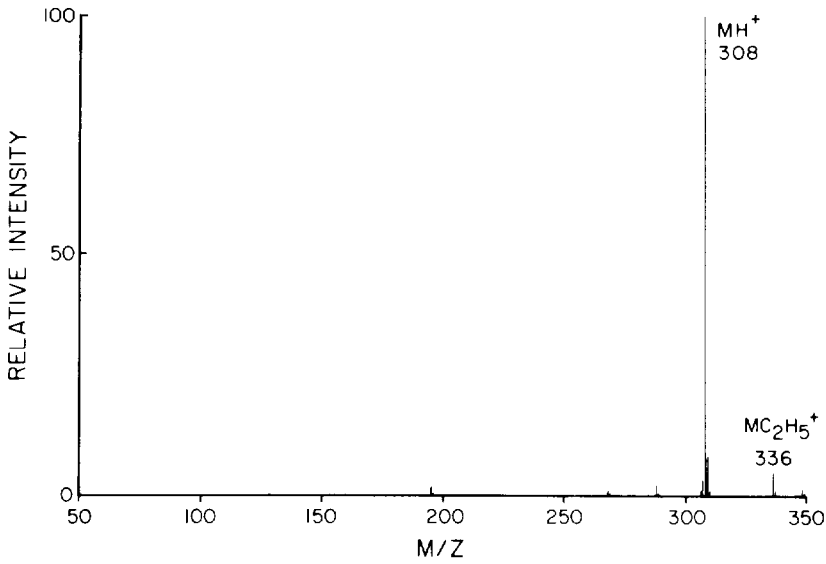


FIGURE 1. METHANE CI MASS SPECTRUM OF: THE TRIFLUORO-ACETAMIDE DERIVATIVE OF MESCALINE

PHYSICAL, CHEMICAL AND SPECTROMETRIC DATA FOR MESCALINE

Nomenclature

Chemical Name: 3,4,5-Trimethoxybenzenethanamine or
3,4,5-Trimethoxyphenethylamine

Empirical Formula: $C_{11}H_{17}NO_3$

Chemical Abstracts Registry Number: 54-04-6

Physical Constants

Appearance: White crystals

Melting Point: 35-36°C
Hydrochloride, 181°C

Boiling Point: 180° (12mm)

Specific Rotation: Optically inactive

Solubility: Moderately soluble in water; soluble in alcohol, chloroform, benzene; almost insoluble in ether and petroleum ether

UV Absorption: λ_{max} = 268($A^{1\%}_{1\ cm}$ = 30)

REFERENCES

1. Staff of Student Association for the Study of Hallucinogens, Mescaline and Peyote: A Literature Review. STASH Press, Madison, WI (1974).

2. D.T. Teitelbaum and D.C. Wingeleth. J. Anal. Toxicol., 1, 36 (1977).

3. N. Neff, G.V. Rossi, G.D. Chase, and J.L. Rabinowitz. J. Pharmacol. Exp. Ther., 144, 1 (1964).

4. D.C. Dyer, R. Benington, and R.D. Morin. Arch. Int. Pharmacodyn. Ther., 217, 197 (1975).

5. J.C. Winter. Arch. Int. Pharmacodyn. Ther., 214, 250 (1975).

6. F. DeSantis, Jr. and K.A. Nieforth. J. Pharm. Sci., 65, 1479 (1976).

7. J. Harley-Mason, A.H. Laird, and J.R. Smythies. Confinia Neurologica, 18, 152 (1958).

8. R.G. Browne and B.T. Ho. Pharmacol. Biochem. and Behavior, 3, 109 (1975).

9. E.A. Zeller, J. Barsky, E.R. Berman, M.S. Cherkas, and J.R. Fouts. J. Pharmacol. Exp. Ther., 124, 282 (1958).

10. L.J. Riceberg, M. Simon, H. Van Vunakis, and R.H. Abeles. Biochem. Pharmacol., 24, 119 (1975).

11. K.D. Charalampous, K.E. Walker, and J. Kinross-Wright. Psychopharmacologia, 9, 48 (1966).

12. J. Ratcliff'e and P. Smith. Chem. Ind., p. 925 (July 18, 1959).

13. N.S. Shah and H.E. Himwich. Neuropharmacol., 10, 547 (1971).

14. B.T. Ho, S.F. Pong, R.G. Browne, and K.E. Walker. Experientia, 29, 275 (1973).

15. L. Demisch and N. Seiler. Biochem. Pharmacol., 24, 575 (1975).

16. G.F. Phillips and J. Gardiner. J. Pharm. Pharmacol., 21, 793 (1969).

17. J. Lundstrom and S. Agurell. J. Chromatogr., 36, 105 (1968).

18. L.J. Riceberg, H.Van Vunakis, and L. Levine. Anal. Biochem. 60, 551 (1974).

19. C. Van Peteghem, M. Van den Heede, and A. Heyndrickx in A. Frigerio, ed., Recent Dev. Mass Spectr. Biochem. Med., 4th Volume. Plenum Press, New York, p. 153 (1978).

20. J.M. Musacchio and M. Goldstein. Biochem. Pharmacol., 16, 963 (1967).

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