METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

VOLUME 24

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author.

The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the anaylsis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

DAVID GLICK

CONTENTS

Analysis of Morphine and Related Analgesics by Gas Phase Methods

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I. INTRODUCTION

The analgesic virtue of opium was known to early civilizations. Roman literature contains accurate descriptions of its pharmacological activity. In 1804 Sertürner isolated morphine as the main alkaloid from *Papaver sommiferum* (1). Seventy years later, Wright (2) prepared a series of morphine derivatives including the diacetyl, but the name "heroin" for diacetylmorphine was coined by Dreser (3) of the Bayer Company in Germany. Heroin became very popular for the treatment of cough, bronchitis, and asthma, for it was thought to lack the undesirable side effects of morphine such as nausea and vomiting. Although Dreser first claimed that heroin was not habit-forming, Morel-Lavallee (4) warned against its addictive properties as early as 1902. Because of its addictive potential, heroin is no longer prescribed in the United States and its preparation is illegal. The drug is still available legally in many other countries.

In searching for new analgesics, Eislieb and Schauman prepared meperidine in 1939. This analgesic is still commonly used for the relief of labor pains. Methadone, introduced in the late 1940s, is currently used for the treatment of heroin addiction (5).

Following are the main analgesics in current use that are structurally related to morphine: codeine, dihydrocodeine, dihydromorphine, and hydroxydihydrocodeinone (oxycodone). Recently Rice and Jacobson (6) reported the preparation and analgesic activity of O^3, O^6-diacetylnormorphine and O^6-acetylnormorphine.

The literature dealing with morphine analysis is very extensive. Taylor (7) reviewed methods of chemical analysis. Techniques used for morphine analysis include thin-layer chromatography (TLC) (8), spectrofluorometry (9), colorimetry, radioimmunoassay (RIA) (10), free radical assay techniques (FRAT) (11), gas chromatography (GC), and gas chromatography–mass spectrometry (GC-MS). An evaluation of current methods for heroin screen-

ing and morphine analysis has been presented (12,13). Gas chromatography is a well-established method for screening and confirmation of drugs of abuse. When both sensitivity and specificity are required, as for example in forensic sciences, gas chromatography–mass spectrometry is the method of choice. Applications of GC-MS in clinical chemistry have been reviewed recently by Roboz (14).

This chapter describes methods of analysis for morphine and related analgesics by gas phase methods. The literature of the last decade has been reviewed.

II. EXTRACTION PROCEDURES

Many biologic compounds are present in body fluids as ionized species that are not directly amenable to determination by gas chromatography, thus must be extracted as neutral molecules prior to the analysis. The extraction step also serves the purpose of purification and concentration.

Morphine-type analgesics are nitrogen-containing compounds with a secondary or tertiary amino group. Their pK values (15) are little affected by structural changes. Phenolic alkaloids like morphine (pKa 10) present an additional problem with respect to complete extraction. The pH of the aqueous phase must be maintained slightly lower than the pK of the phenol group with an appropriate buffer.

Many methods have been described for the extraction of morphine, most of them derived from the historical procedure of Stas (16). More than a century ago, Stas described a general method for the extraction of alkaloids from biologic tissues. The tissue proteins were precipitated upon heating with an alcoholic solution; then the filtrate was made alkaline with sodium bicarbonate and extracted with diethyl ether. This method is still valid for efficient extraction of numerous drugs. Niyogi (17), in an extensive review, has described various modifications of this original technique.

1. Continuous Solvent Extraction

The continuous extraction of a sample (liquid or solid) with a boiling solvent is a useful method, when large solid samples are analyzed; since more time is necessary for efficient extraction, however, it is not widely used. The large volumes of solvent used in this process must be concentrated. With increasingly sensitive instrumentation, the analysis of large samples is no longer a necessity.

2. Direct Solvent Extraction

In contrast to continuous extraction, the sample can be extracted for a short period of time with a relatively small volume of solvent. At physiological pH's, the drugs are ionized, and since the free base is more soluble than

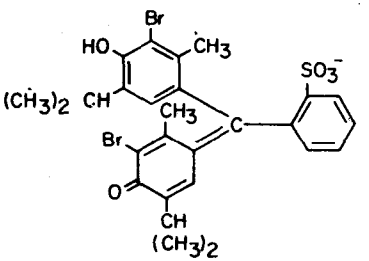
the salt in organic solvents, the extraction is carried out after the pH of the solution has been adjusted to 9–10. Many buffers have been reported in the literature; the most common are bicarbonate, carbonate, or borate buffers. Too high a pH will ionize the phenolic group of morphine and decrease the partition ratio between the aqueous and the organic phases. The addition of salt is often used aid in increase the extraction.

Many solvents have been used for the extraction of morphine and related compounds. Ether, used by Stas, is no longer widely employed. Chloroform is frequently used. Addition of an alcohol (ethanol, isopropanol, butanol) increases the partition ratio and also prevents adsorption to glassware. Unfortunately the recovery is not always explicitly stated in published methods.

3. Ion-Pair Extraction

In the usual extraction, basic drugs are extracted in un-ionized form. It is, however, possible to transfer ionized compounds into an organic phase if a suitable counterion is added to the aqueous phase. Depending on the nature of the molecule, a cation or an anion is necessary to complete the transfer. It is well known that morphine recovery is improved if ammonium ions are present in the water phase. Horning et al. (18), reported quantitative extraction for a variety of basic drugs including morphine, codeine, and meperidine from blood and urine with ammonium carbonate as the buffer. An ion pair between an ionized phenolic group and the ammonium ion is soluble in organic solvents, and solubility increases if the ammonium ions are tetraalkyl substituted.

The amino group of a basic drug is ionized at physiological pH's; a negatively charged counterion may be used to promote the transfer into an organic phase. Schill and Marsh (19) in an extensive study reported the extraction of some 40 alkaloids with bromothymol blue at pH 7.5. The compounds were subsequently determined photometrically. Boon and Mace



BROMOTHYMOL BLUE

(20) extracted five basic drugs including meperidine at pH 5 with chloroform after addition of a bromothymol blue solution. The ion pair was directly injected into the column and no interference was observed due to the counterion. This interesting technique could be extended to morphine.

4. Clean-up Procedures

Biologic samples such as blood and urine contain hundreds of compounds, and a simple extraction procedure is usually not sufficient to avoid interference from other endogenous compounds. It is often necessary to purify a sample before derivatization, even when high-resolution techniques such as capillary column chromatography and multiple-ion detection are used. The objective of a purification step is to remove most of the "unwanted" substances—endogenous or exogenous—but not at the expense of the recovery of the drug to be measured. This problem becomes very critical when very small amounts are monitored in biologic samples, since endogenous substances may impair the GC separation or may saturate the ion source. With instruments that can detect picogram and even femtogram samples, purification techniques are becoming the limiting factors.

A. DIFFERENTIAL EXTRACTION

Morphine and related compounds, which are bases, are easily separated from acidic and neutral substances. Several approaches are possible. In an acidic medium, neutral and acidic compounds are first removed, then the solution is adjusted to pH 9–10 and the alkaloids are extracted. Ion-pair extraction techniques can be applied for the purification step. After the removal of neutral substances, the counterion is added to the acidic solution and the ion pair is transferred into the organic phase. This method is particularly suitable for alkali-sensitive compounds. A second approach, often called back extraction, consists of extracting the basic compound in unionized form, then washing the organic phase with a solution of a mineral acid. The bases are reextracted from the acid solution after alkalinization as for a direct extraction. This method has been used extensively by many authors with different solvents and acids. The presence of ammonium ions may lead to the formation of ion pairs, which in turn result in poor yields during back extractions. Addition of hexane to the first organic extract results in quantitative transfer to the acid solution.

B. ION EXCHANGE

The basic properties of morphine and its analogs allow these substances to be separated from other endogenous substances by ion-exchange chromatography. Tompsett (21) reported the isolation of morphine alkaloids with a

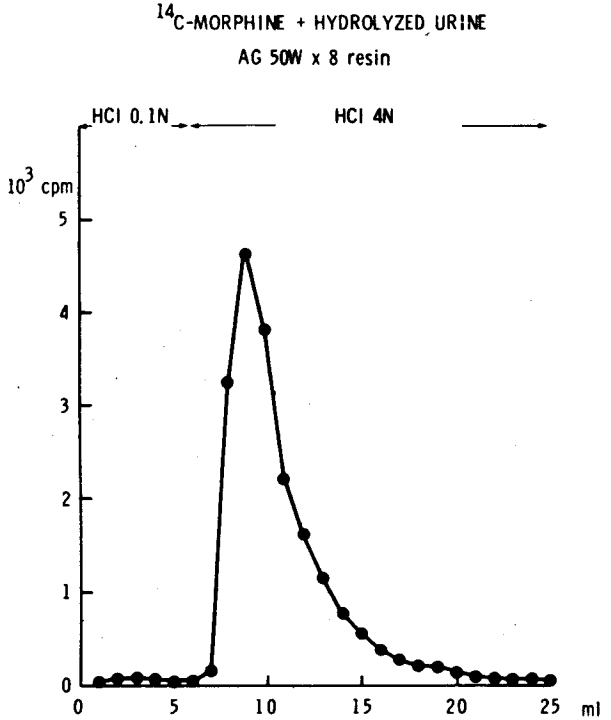


Figure 1. Elution of ^{14}C-morphine in hydrolyzed urine from a small ion-exchange column (0.3 g of AG 50W × 8) with 4*N* hydrochloric acid.

sulfonic acid resin (Dowex 50). These resins have a high capacity of binding, and a very small column is usually sufficient to retain basic compounds contained in biologic fluids. The difficulty lies in elution from the column. Efficient recovery can be obtained when column operating parameters are optimized. Figure 1 illustrates the elution of ^{14}C-morphine in hydrolyzed urine from a small ion-exchange column containing only 0.3 g of the sulfonic acid resin AG 50W × 8, 200-400 mesh (Bio Rad Laboratories, Richmond, Calif.) in a disposable Pasteur pipette. The flow rate was controlled (at the exit) at 1.5 ml/min through the use of a peristaltic pump.

The recent development of high-pressure liquid chromatography (HPLC) makes it possible to obtain good resolution and fast analyses. Opium alkaloids may be separated by HPLC, as described by several authors (22,23).

C. ADSORPTION

Charcoal has been used for many years as an adsorbent for biologic compounds. In a method for the determination of morphine by GC described

by Ikekawa et al. (24), charcoal was used to retain morphine alkaloids from urine.

However synthetic adsorbents, with their more well-defined properties, are replacing natural adsorbents. Florisil, a synthetic magnesium silicate, is often used to purify biologic extracts. With this method, Stolman and Stewart (25) reported quantitative recoveries for morphine, codeine, and heroin. The mechanism of adsorption is not fully understood, and empirical conditions prevail; for example, the optimal pH is different for different specimens: for tissue extracts, between 7 and 8.5; for blood, between 8 and 9; and for urine, between 5 and 6. No rationale is apparent for these variations.

The most widely used adsorbent is currently a styrene-divinylbenzene neutral copolymer (XAD-2). The usefulness of this adsorbent lies in the capacity of the resin to adsorb organic material in aqueous solution. After washing with water, organic solvents release the adsorbed compounds. Extraction and purification are carried out in one step. Several authors (26–28) have applied this technique for the purification of drugs of abuse in human urine. Disposable prefilled columns are commercially available.

5. Conclusion

A review of the principal methods of extraction and purification of morphine alkaloids indicates that no method of extraction has pronounced advantages in all applications. The extraction procedure should be modified to suit the particular analytical problem.

Plasticizers must be kept to a minimum. It is almost impossible, for example, to obtain a blood sample free from plasticizers. Disposable plastic ware should be avoided during the sample preparation. An intense ion at m/e 149 is usually indicative of the presence of a phthalate plasticizer.

III. ANALYSIS BY GAS CHROMATOGRAPHY

The potential value of gas chromatography in the analysis of opium alkaloids was recognized in the early 1960s (29,30) immediately after Vanden Heuvel et al. (31) described the preparation of thin-film GC columns made with a thermostable liquid phase (a dimethylsiloxane polymer) and suitable for the separation of steroids. Morphine and related compounds are directly amenable to gas chromatographic analysis as free bases. Extracts have been injected, without derivatization, in the determination of opium alkaloids (32), thebaine (33) and morphine standards (34,35), legal (36,38) and illegal (39–41) preparations, and biologic samples (8,42–47).

Upon injection of a mixture of morphine and diacetylmorphine, as free bases, a transacetylation may occur in the injector zone with the formation of O^3- and O^6-acetylmorphine. This phenomenon, observed first by Brockmann-Hanssen and Swendsen (30) and Viala et al. (40), is a severe limitation on the quantitative measurement of samples containing both compounds.

In practice, it is usually desirable to prepare a suitable derivative before GC analysis or analysis by GC-MS.

1. Derivative Formation

A. ACETATE DERIVATIVES

Anders (48) described a procedure in which acetylation was performed on the column. This technique was also used by Mulé (49). If the proper acid anhydride is added, other esters may be prepared. Acetylation of morphine before injection was reported by Wallace et al. (50). Obviously, acetylation is not the method of choice if the same sample contains any combination of morphine, monoacetylmorphine, and diacetylmorphine.

It is possible to acetylate the phenolic or the allylic group of morphine in specific fashion. In 1954 Welsh (51) described the preparation of O^3-acetylmorphine. O^6-Acetylmorphine is formed by mild hydrolysis of the diacetyl derivative with hydroxylamine hydrochloride in alcohol (52).

Trifluoroacetyl derivatives, prepared by reaction with trifluoroacetic anhydride (53), have a shorter retention time than the acetyl derivatives and may be used for electron capture detection analyses.

Ebbighausen, et al. (54) recently developed an analytical procedure for the study of morphine and codeine based on the use of the trifluoracetyl derivative (morphine) and the heptafluorobutyryl derivative (codeine). Smith and Cole (55) used the O^3-trifluoracetyl derivative of O^6-acetylmorphine in a study of diacetylmorphine metabolism. Ebbighausen et al. (56) also used trifluoracetyl derivatives in a study of codeine metabolism.

B. TRIMETHYLSILYL DERIVATIVES

The most satisfactory derivative of morphine for analytical purposes is the ditrimethylsilyl ether. The phenolic group and the allylic hydroxyl group are readily converted to trimethylsilyl ethers by the usual silylating reagents. Bis-trimethylsilylacetamide, bis-trimethylsilyltrifluoracetamide, or *N*-trimethylsilylimidazole may be used; the reaction is usually catalyzed by the addition of trimethylchlorosilane. The trimethylsilyl (TMS) derivative has good gas chromatographic properties.

This derivative was employed by Wilkinson and Way (57) in an early quantitative study of morphine metabolism, and it has been used many times in later investigations. Although trimethylsilyl ethers undergo hydrolysis relatively easily, they are thermally stable and show little adsorption on GC columns. Column loss may occur if acidic conditions develop on the column packing; the best way of avoiding this circumstance is to employ an initial 1- to 2-cm zone of 10% SE-30 packing, according to the practice described by Thénot and Horning (58). The TMS derivative was employed in a recent method described by Clarke and Foltz (59). Other studies (60–66) have also been based on the use of this derivative.

Codeine forms a O^6-trimethylsilyl ether; this derivative is suitable for analytical studies. Diacetylmorphine does not require derivative formation. O^6-Acetylmorphine forms a O^3-trimethylsilyl ether. Normorphine forms a ditrimethylsilyl ether, in the same fashion as morphine. The secondary amino group also reacts with most silylating reagents, but not with *N*-trimethylsilylimidazole (67), to yield an *N*-trimethylsilyl derivative. Compounds of this type are active silylating agents, and when they are employed as derivatives it is not unusual to find both the free amine and the *N*-trimethylsilyl derivative present during the GC separation.

The phenolic group may be selectively silylated in the presence of other alcohol groups with isopropenyltrimethylsilane (67).

C. ALKYL DERIVATIVES

Peralkylation of morphine may be carried out according to a procedure first described by Corey (68,69) and later applied by Hakomori (70) for the permethylation of sugars and by Haegele et al. (71) for the peralkylation of peptides and amino acids.

Procedure. In the experimental preparation of diethylmorphine, morphine hydrochloride (10.7 mg, 0.1 mmole) was dissolved in 600 μl of dimethylsulfoxide (distilled over calcium hydride). To this solution, 150 μl of a 1*M* solution of methylsulfinylmethide carbanion was added. The reaction mixture was sonicated for 10 min to break gel particles that were formed. This was followed by the addition of 10.5 μl of ethyl iodide (eqmolar excess), and the reaction mixture was sonicated for 50 min. Ice and water were added (approximately 1 ml) and the diethylmorphine was extracted with 2 ml of chloroform. The chloroform solution was washed three times with 1-ml portions of water, and the solvent was removed with a stream of nitrogen. The reaction is conveniently carried out in a 3.5-ml screw-cap vial, which is flushed with nitrogen when reagents are added, since the carbanion solution is extremely sensitive to moisture and to oxygen. The yield was 10.9 mg (96%).

Alkyl derivatives are more stable than TMS derivatives and do not increase the molecular weight significantly. However, they have not been used for analytical purposes.

D. CARBAMATE DERIVATIVES

Chloroformates react with tertiary amines to yield carbamates.

$$R_1R_2NCH_3 + ClCOOR_3 \rightarrow R_1R_2NCOOR_3 \qquad [1]$$

The carbamates of morphine have been used in synthetic work for the preparation of the secondary amine (72–74) or for isotope labeling (75). Hartvig and Vessman (76,77) described methods for the determination of

tertiary amines after demethylation to secondary amines and subsequent perfluoroacylation before electron capture gas chromatography. The reaction may be carried out in one step with the appropriate chloroformate (e.g., pentafluorobenzylchloroformate) (78).

2. Column Selection

Since morphine and morphinelike analgesics are basic compounds, it is necessary to avoid any accumulation of acidic material on the column. Repeated injections may result in erosion of the stationary phase at the head of the column; the best way of avoiding this circumstance is to employ an initial 1- to 2-cm zone of 10% SE-30 packing, according to the practice of Thénot and Horning (58).

A base-treated support may be used to prepare the packing. However in our experience tailing is not observed with a 3 to 5% coating.

A survey of the literature shows that silicone phases (OV-17, SE-30) are the most widely used of all liquid phases. Moffat (79) reported the retention index of 180 drugs on SE-30, which he called the "preferred stationary phase" for the identification of basic drugs. With a nonpolar phase like SE-30, compounds are eluted according to their size and some predictions of retention behavior can be made. Normorphine diTMS, morphine diTMS, and normorphine triTMS are eluted from a SE-30 column in that order, as expected from the size of the molecule: $H < CH_3 < Si(CH_3)_3$.

3. Detectors

A. FLAME IONIZATION DETECTOR

The flame ionization detector (FID) is the most widely used of all GC detectors because of its stability, its large dynamic response range, and its universal applicability. No special derivative is required for FID. The sensitivity is quite adequate for most applications; Ikekawa et al. (24) reported 10 ng as a detectable amount of morphine TMS.

B. ELECTRON CAPTURE DETECTOR

Several orders of magnitude in sensitivity of detection may be gained over the FID with the electron capture detector. However it is necessary to prepare special derivatives to obtain such an improvement. Perfluoroacyl derivatives are suitable for electron capture detection. With the pentafluoropropionyl derivative, Dahlström and Paalzow (80) obtained a sensitivity limit of about 5 pg for morphine: 100 pg could be determined in a 30-mg brain tissue sample.

C. NITROGEN DETECTOR

A specific response to compounds containing nitrogen (and phosphorus) may be obtained when the collector plate of a conventional flame ionization detector is replaced by an alkali-coated ceramic. The exact mechanism of the response is not fully understood.

The selective detection of nitrogen-containing compounds is an attractive technique. Previous difficulties (mainly lack of stability) have prevented the full investigation of the use of the nitrogen detector. Recent improvements in technology should result in a wider use of this detector. Applications to morphine-type compounds were reported by Riedmann (81) and Smith and Cole (82).

4. Quantification

Quantification by gas chromatography is carried out by adding a suitable internal standard. This standard should be chemically related; it should also have a similar retention behavior, and it should be added at the first steps of the sample preparation.

Tetraphenylethylene was used in the early work of Wilkinson and Way (57), but ethylmorphine (55) and nalorphine (53) are more appropriate internal standards.

IV. ANALYSIS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—COMPUTER SYSTEMS

1. Introduction

Analytical systems based on a combination of a gas chromatograph, a mass spectrometer, and a computer, and operated as a single instrumental system, provide the most powerful and most reliable method of analysis now known for the study of complex mixtures of biologic origin. They are particularly valuable in studies of drugs and drug metabolism. The function of the gas chromatograph is to separate components of the mixtures under investigation. For example, most drugs yield multiple metabolites; some metabolites may have a physiological action related to that of the original drug, some may have toxic properties due to their specific structure, and some may be inactive. The structural differences introduced through metabolic transformations are usually such that separation of the parent drug and individual metabolites is possible with ordinary GC columns. It is usually necessary to prepare derivatives before the instrumental analysis step, since many metabolites contain polar groups that would lead to undue adsorption if derivatives were not prepared. The mass spectrometer provides an intermittent or continuous record of mass spectral data. Quantitative analyses are usually carried out by using the mass spectrometer as a specific ion detector.