The Use of Fused Silica Capillary Gas Chromatography for the Analysis of Acidic-Neutral Drugs in Human Serum

by

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ABSTRACT

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A rapid and practical method for the quantitative analysis of commonly encountered acidic-neutral drugs in human serum is described. The procedure involves the extraction of these compounds from a serum matrix into an organic solvent followed by separation and identification using fused silica capillary gas-liquid chromatography and a nitrogen-phosphorus thermionic detector. Confirmation of peak assignments is accomplished by enzyme immunoassay and thin layer chromatography. The dried concentrated serum extract is introduced onto the column by split injection, and quantitation of drug components is performed by using peak height ratios measured with respect to an internal standard, p-methylphenobarbital. The coefficient of variation for most drugs is under ten percent both within-day and between-days. Fifteen drugs including common benzodiazepines, barbiturates, and anticonvulsants are eluted and well separated within fifteen minutes. The use of a fused silica capillary column as the separation medium affords the toxicologist high resolution, high sensitivity, short analysis time, and the use of small sample volumes while maintaining cost effectiveness.

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I would like to express my sincere thanks to Dr. Francis Smith for his patience and understanding during this project. I am also grateful to Dr. Mahendra Pandya and Dr. James Muirhead-Gould for their kind suggestions and guidance.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF SYMBOLS

SYMBOL	DEFINITION	UNIT
HPLC	High performance liquid chromatography	none
TLC	Thin layer chromatography	none
GLC	Gas liquid chromatography	none
GC	Gas chromatograph	none
N	Total theoretical plates per column	none
t_r	Solute retention time	min.
HETP	Height equivalent to a theoretical plate	mm.
u	Linear gas velocity	cm./sec.
mcL	Microliter	none
mL	Milliliter	none

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

Introduction

The capacity to isolate and identify pharmacologically active agents in human biological fluids has attained great importance during the past decade. This is due in part to both the growing number of compounds routinely used as prescription and over-the-counter medications and to the frequent misuse of these drugs either in accidental overdose or in drug abuse situations. Concurrent with the expanding number of available drugs has been the development of sophisticated analytical techniques within the field of toxicology which has involved primarily the refinement of existing chromatographic methods along with the introduction of enzyme immunoassay. The tremendous growth in technology coupled with the proper training of skilled personnel in pharmacology, pharmacokinetics, and laboratory methods has created a new dimension in patient care which physicians utilize regularly. In the modern clinical setting, a clinician can obtain reliable drug analysis data for a patient usually within a few hours. These data, in conjunction with proper pharmacokinetic data, are used to adjust dosage regiments, assess patient compliance or assess toxicity potential in emergency situations.

The science of toxicology can be roughly divided into three major disciplines: environmental, forensic, and biomedical. Environmental toxicology encompasses the analysis of priority pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and

other organic and inorganic substances which might directly or indirectly endanger life forms in nature. Forensic and biomedical toxicology are similar in that both include the analysis of biological fluids for drugs, but forensic investigations are usually done on cadavers, and they are shrouded with legal considerations as well. Biomedical toxicologists normally perform drug analyses in order to provide useful information on a timely basis to a physician who is treating a patient. The expanding role of biomedical toxicology in the clinical setting is truly formidable. The scope of this science is outlined in Figure (1).

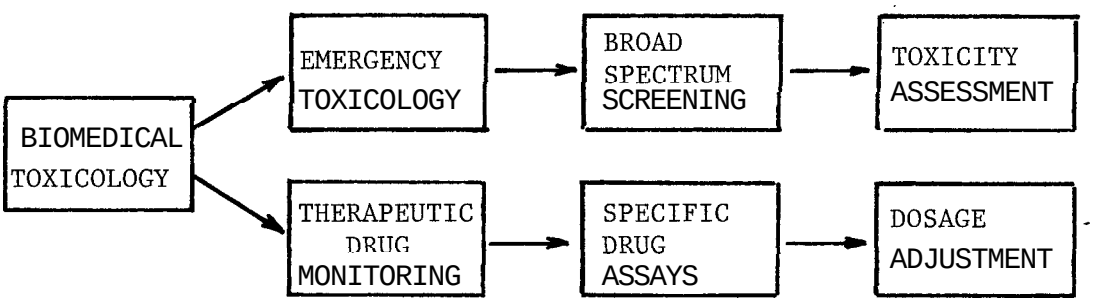

Fig. 1.--The scope of biomedical toxicology

The two subdivisions, therapeutic drug monitoring (TDM) and emergency toxicology, differ primarily in the reasons for which they are utilized. In TDM, the drug (or drugs) to be assayed is known since it is being administered by a physician for a particular disorder. The specimen is always serum or plasma, and the results are always quantitative. Thus, the concentration of the drug in the serum is determined, and the information is used by a physician

to adjust dosage, determine effects of multiple drug therapy, assess patient compliance and in general to help him make intelligent decisions about his choice of treatment. However, the situation in emergency toxicology is quite different. Here, the drug content of the specimen might be a complete mystery, and broad spectrum drug screening must be done in order to identify and perhaps quantitate any compounds present. The specimen can be any biological fluid or material but usually serum, urine and gastric contents are analyzed since they are the easiest to obtain quickly, and the serum is needed for meaningful quantitative work.

The objective in such analyses is to identify the components present as rapidly, unequivocally, and inexpensively as possible while maintaining quantitative capabilities for serum assays with detection limits sensitive enough to provide useful information to the clinician involved. Major innovations in both TDM and emergency toxicology procedures have been implemented during the last decade. These have greatly increased the reliability of the assays performed, while significantly reducing the analysis time and cost. For example, enzyme multiplied immunoassay technique (EMIT®) has proven to be one of the quickest and most reliable methods to assay a wide range of drugs including the major anticonvulsants, theophylline, the aminoglycosides, and a number of others. EMIT® assays are also available for the confirmation of moderate to high levels of certain drug classes in serum. These include the benzodiazepines, barbiturates, and tricyclic antidepressants. Urine assays can be purchased for the confirmation of opiates (conjugated and free), benzoylecgonine, barbiturates, propoxyphene, benzodiazepines, methadone, phencyclidine, and

methaqualone. The EMIT® method, however, requires the purchase of expensive expendable kits for each drug or drug class to be assayed, and some of these assays are not without significant interferences. The confirmatory kits are useful in emergency toxicology when utilized in conjunction with common chromatographic methods.

High performance liquid chromatography (HPLC) has also become quite popular in toxicology for both TDM and drug screening. Multi-component mixtures can be analyzed quickly and inexpensively though the technical skill required of the analyst is much higher than that needed for the EMIT® system. This thesis will not include a detailed description of HPLC theory or techniques, however it is important to note that this analytical tool is now highly refined in that high resolution columns (3 to 5 micron packings), microbore systems, efficient pumping mechanisms, microprocessor-controlled gradient generators, and a wide variety of detectors are now available at moderate cost. A recent review by Giese[1] explains many of the important aspects of HPLC and provides an extensive list of references pertaining to the application of HPLC to drug analyses.

Thin layer chromatography (TLC) has been used in toxicology for many years for qualitative drug screening. Two fundamental TLC procedures are given by sunshine[2] and Blass et al.[3] though many other systems exist. The quantitative analysis of drugs in serum specimens has been done primarily using gas liquid chromatography (GLC) and HPLC, though spectrophotometric methods were popular less than a decade ago.[4] This thesis will present a new approach to one important aspect of biomedical toxicology, quantitative serum drug screening of acidic-neutral type drugs commonly encountered in emergency situations.

The current approach to the quantitative analysis of drugs in serum as a part of a screening procedure has evolved primarily in response to the availability of useful instrumentation and analytical tools such as separation media, detectors and extraction supplies. Gas liquid chromatography, in conjunction with various confirmatory procedures, has gained widespread popularity as the method of choice for serum drug screening. In traditional GLC, coiled glass columns generally three to six feet long filled with a solid support material containing liquid phases like OV-17, OV-101 and SP-2250 are used to accomplish the chromatographic separations, and devices such as the flame ionization detector and others generate signals corresponding to eluted compounds. Table (1) lists some of the packing materials commonly encountered in biomedical toxicology.

TABLE 1

COMMON GLC PACKING MATERIALS

LIQUID PHASE	POLARITY	TYPE
SP-2250	Intermediate	Methyl-phenyl silicone
CARBOWAX 20M	Polar	Polyethylene glycol
SE-54	Non-polar	Methyl-phenyl-vinyl silicone
OV-101	Non-polar	Methyl silicone fluid
OV-1	Non-polar	Methyl silicone gum
OV-17	Intermediate	Methyl-phenyl silicone

The development of these liquid phases and a reliable nitrogen selective detector has greatly enhanced the speed and quality of serum drug screens in today's toxicology laboratories. However, a number of problems exist which must be addressed. The drugs to be included in a screen are selected on the basis of several things including the extraction procedure to be used, the detectability of the drug at both therapeutic and toxic levels, confirmatory methods available, and the abuse potential of the drug. Some of the compounds frequently found in acidic-neutral serum drug screens are listed in Table (2).

TABLE 2

DRUGS COMMONLY INCLUDED IN SERUM SCREENS

GENERIC NAME	COMMON TRADE NAME
Methyprylon	Noludar®
Diazepam	Valium®
Methaqualone	Quaalude®
Phenobarbital	Luminal®
Phenytoin	Dilantin®
Secobarbital	Seconal®
Meprobamate	Equanil®
Aspirin	Anacin®
Amobarbital	Amytal®
Chlordiazepoxide	Librium®
Pentobarbital	Nembutal®
Acetaminophen	Tylenol®
Butabarbital	Rutisol®

Many of these compounds have similar molecular structures[5] and are pharmacologically active in the microgram to nanogram per milliliter range. Thus, two problems arise. First, a formidable number of molecular species, many with similar structures, must be separated sufficiently to allow identification, and secondly, the specimen size must be such that the drugs can be detected at clinically significant levels. Ideally, the compounds in Table (2) should be separated not only from each other, since any two or more of the drugs might be in the same sample, but also from other common potential interferences such as caffeine. Since the specimen is human serum, it is desirable to use a procedure which requires a small sample volume so that analyses on small children and infants can be easily done, and blood loss is minimized for patients who may already be suffering from severe trauma. The total sample volume obtained, however, should produce enough serum to allow repeat analyses. Thus, a total serum volume of two milliliters or less would be appropriate since this can be obtained from the centrifugation of one ten-milliliter vacutainer of whole blood.

Gas liquid chromatography using packed glass columns has long been the technique most commonly employed to perform drug separations and quantitation, but it provides toxicologists with a situation far from ideal. The packing materials popular among toxicologists such as OV-17 and SP-2250, though good for some separations, do not provide enough resolving power for the performance of critical separations in a reasonable amount of time. For example, using either programmed or isothermal conditions, phenytoin cannot be adequately separated from diazepam on either liquid phase without seriously disrupting peak

resolution in other parts of the chromatogram. Since these two drugs are frequently found in combination, an obvious problem exists. Chlordiazepoxide and nordiazepam also superimpose under most conditions, and interferences from unknown compounds are possible since resolution is usually not good enough to show subtle peak distortions. It is possible to use two separate packed columns with dissimilar liquid phases so that interferences on one column might not appear on the other. This requires that two gas chromatographs be used, one containing each column, or that both columns be placed in the same oven. In the latter case, the temperature limits of the two columns must be compatible. It is unlikely that the same temperature program will produce optimum separations on both columns, so analysis time will be increased since two sequential injections will have to be performed. This also increases cost considerably since about twice the usual volumes of gases will be consumed and if a nitrogen-phosphorus thermionic detector (NPD) is used, the collector will require replacement more frequently.

Packed columns also require conditioning prior to their use in analyses. This process involves installing the column in the oven, flowing carrier gas through the column, and then baking the column at its liquid phase temperature limit for some specific period of time designated by the manufacturer. This time period may be only a few hours, or it may involve overnight heating. Conditioning effectively removes excess liquid phase from the support material, and thereby helps reduce baseline noise and the drift commonly associated with temperature programming. Since this process is time consuming and requires the devoted attention of a gas chromatograph, several back-up

columns must be packed and conditioned for immediate installation since most toxicology labs operate twenty-four hours a day all year. This can be a problem when a particular column is needed immediately and someone forgot to condition a spare. Another frequently encountered problem with packed columns is the rapid degeneration of column efficiency due to the decomposition of the liquid phase. This process leaves an increasing number of active sites on the column which cause drug adsorption and subsequent peak tailing. Since liquid phase is lost, the total number of theoretical plates will decrease. Ultimately, peak broadening and extremely poor resolution will occur. Peak tailing and broadening will adversely affect quantitative results, especially when peak height ratios are used, and sensitivity will be reduced since smaller peaks will tend to appear much flatter making them more difficult to differentiate from baseline noise particularly at low concentrations. These problems necessitate frequent column repacking and installation, generally can produce significant quantitative errors, and increase costs both in materials and labor. In toxicology laboratories, either reference or hospital, quantitative drug screening with rapid turn-around time, accurate results, and low cost is crucial if the service is to benefit the patient.

Recently, there has been an increased interest in the application of high resolution gas chromatography to the problem of drug analysis. High resolution or capillary gas chromatography involves the use of long narrow open tubular columns with liquid phase coated on the inner walls. The idea of using open tubular columns for high resolution analyses was first introduced by Golay,[6] but the adaptation

of his ideas to most commercial applications had to wait for the development of technology which would allow scientists to manufacture open tubular columns with good coating efficiencies and to adapt conventional gas chromatographs to the special requirements of capillary operation.

Capillary analyses were previously of little interest to toxicologists since the only columns available were primarily those composed of borosilicate glass. These columns are not particularly suited for routine daily use in drug analyses since they are very fragile, cumbersome to install and remove, loaded with unwanted metal ions, and expensive. Nevertheless, M. Van Boven and I. sunshine[7] describe a method for the quantitation and identification of methyprylon and its metabolites using a wisker-walled polyethyleneglycol-coated glass capillary column and mass spectrometry. This method is time consuming and requires an expensive mass spectrometer. P.O. Edlund[8] describes a capillary method for the determination of opiates in biological fluids, but again the procedure is very cumbersome and impractical for emergency work.

The introduction of fused silica, highly pure silicon dioxide, capillary columns coated with a polyimide shell and having chemically bonded liquid phases, opened the door to a wide range of applications of high resolution chromatography particularly in the area of drug analysis. Fused silica columns are highly flexible, a property which simplifies column installation and handling, and they are virtually devoid of metal ion contamination, which helps insure inertness. Since the liquid phase is both chemically bonded to the fused silica surface (deactivated) and crosslinked within itself,

the columns also exhibit long useful lives. Papers by Dandeneau and Zerenner[9] and Lipsky et al.[10] describe the preparation of fused silica columns and some of the essential considerations. Column preparation, however, is currently the responsibility of manufacturers such as J & W Scientific, Hewlett Packard, Supelco and others since the process is tedious and very expensive. Prefabricated fused silica columns are currently available in a variety of useful lengths and inner diameters. Chromatographers can also choose a bonded liquid phase and liquid phase film thickness to handle their particular separation problem.

Only a few studies have been published which involve the application of fused silica capillary gas chromatography to drug analyses. Kinberger et al.[11] describe a method for the detection of some stimulants and methadone in urine specimens. The authors mention nothing concerning serum assays, and the work was done using SP-2100 as the liquid phase. They also emphasize the need for confirmation by mass spectrometry, a costly venture. A method for the analysis of illicit heroin samples is described by Demedts et al.[12] The work is intended for the analysis of powders and biological specimens are not considered. A screening method for drugs of abuse on SP-2250 was reorted by Pettitt[13] and Plotczyk describes a method for the analysis of some underivatized drugs using a fused silica column.[14] These papers provide an introduction to the potential use of fused silica capillary columns to drug analyses, but they do not present a simple method which can be adapted to daily drug screening in a clinical laboratory setting.

This thesis describes a rapid and simple method for the quantitative analysis of many common acidic-neutral type drugs in human serum. The method involves the use of a fifteen-meter Durabond-5 capillary column (J & W Scientific, Rancho Cordova, Ca.) as the gas chromatographic separation medium. The procedure is designed to be used as a screening analysis in hospital and commercial toxicology laboratories. Peak identities are confirmed using thin layer chromatography and enzyme immunoassay. The entire procedure, including confirmations, can usually be done in less than one hour, and the detection limits for the drugs of interest are more than adequate. Quantitation is accomplished using peak height ratios with respect to an internal standard. The results demonstrate linearity through a wide range of concentrations, and the reproducibility study yields coefficients of variation (CVs) under ten percent for both within-day and between-day analyses. This method of analysis is suggested as a replacement for packed-column GLC in drug screening since resolution and sensitivity are greatly improved, the DB-5 column contains a durable bonded liquid phase which effectively means lower cost in the long run and less liquid phase deterioration occurs which prolongs the detector life.

Conventional gas chromatographs, those designed for packed columns, can be readily converted to capillary operation by the purchase of adaptor kits for the injection system and by some simple replumbing of the detector gases. The movement to high resolution gas chromatography as the method of choice for drug screening is beginning to gain acceptance among toxicologists. At least two excellent texts on high resolution GLC have been published.[15,16]

These books describe both the theoretical and practical approaches to capillary analyses. The method presented in this thesis is part of that growing trend which is aimed at increasing the quality of patient care within a cost containment framework.

CHAPTER II

GAS LIQUID CHROMATOGRAPHY

Theoretical and Practical Considerations

Once a particular gas chromatograph (GC) is chosen by a chromatographer, there are certain items which must be given careful consideration before they are selected. These include the analytical column, the carrier gas, the detector, and the data handling system. The choice of these items, assuming that budgetary problems do not exist, is primarily a function of the substances to be separated, the degree of resolution required, the limits of detection needed for meaningful results, and the time limitations imposed on the analysis. Experimental parameters such as carrier gas flow rate, detector and port temperatures, oven temperature, and in capillary work, the injection technique, will be discussed later.

At the present time, toxicologists choose columns on the basis of both experience and manufacturer's designated applications. The choice of a detector is a function of the sensitivity requirements and the molecular structures of the compounds to be detected. There are three detectors commonly used in drug analyses. These include the flame ionization detector (FID), the nitrogen-phosphorus thermionic detector (NPD), and the electron capture detector (ECD). The NPD and the FID are essentially identical in structure with the exception that the NPD includes a rubidium salt bead which resides in the flame area. This bead enhances the ionization of nitrogen or

phosphorus containing molecules and thus increases the sensitivity of the detector toward these compounds. The NPD is particularly suited for drug analyses since many drugs are amines. There are, however, two important considerations when using the NPD. First, the bead is a consumable item. As it is used, the rubidium salt eventually vaporizes completely, and the bead must be replaced. Second, halogenated compounds such as chloroform, dichloromethane, and chlorobutane will cause a reversible short-lived loss in detector sensitivity. This imposes a small practical limitation in that such solvents cannot be directly injected during an analysis. Since these solvents are commonly used for drug extractions, they must be evaporated to dryness, and the extraction residue reconstituted in a solvent such as methanol or isopropanol prior to injection. The FID, however, is still unsurpassed for the analysis of methanol, ethanol, acetone and isopropanol. These compounds are included in many quantitative toxicology drug screens. The electron capture detector employs a radioactive source (usually Ni^{65}) as an essential part of its mechanics. Its use is therefore subject to certain hazardous materials laws, but it has been successfully used for the analysis of compounds containing electronegative atoms such as halogens. The ECD finds applications in pollutant analyses[17,18] and in the detection of certain derivatized drugs.[19,20]

The selection of a carrier gas is usually dependent upon the detector to be used. For instance, an argon-methane mixture is appropriate for the ECD, but it is far from ideal for an FID. However, when using an FID or NPD, the toxicologist has several carrier gases to choose from including argon, helium, nitrogen and when doing

capillary work, hydrogen. The choice of the proper carrier gas is important since it can significantly influence column efficiency and thus ultimately resolution. This is particulary evident in capillary GC. Some theoretical considerations follow which can be used to select both the proper carrier gas and the carrier flow rate.

Column efficiency, the property which directly affects peak resolution, is usually expressed by the theoretical plate number, (N). A single theoretical plate can be envisioned as a tiny fraction of the column which acts as a partitioning interface. Each interface allows the injected components to partition between the two phases in a manner dependent upon component molecular structure, polarity, and solubility. Each partitioning enhances the separation process by increasing the discriminating effects of the column on the injected solutes. Thus, as the number of theoretical plates per column increases, the resolving power or column efficiency increases. The efficiency is expressed mathematically as the relationship between the length of time a component spends in the column (t_r) and the extent of peak broadening which occurs.

$$N = \frac{t_r}{\sigma} \qquad (1)$$

In equation (1), N is the number of theoretical plates, and σ is the standard deviation of the peak. Figure (2) illustrates the relationship between the standard deviation and the peak width.[21]

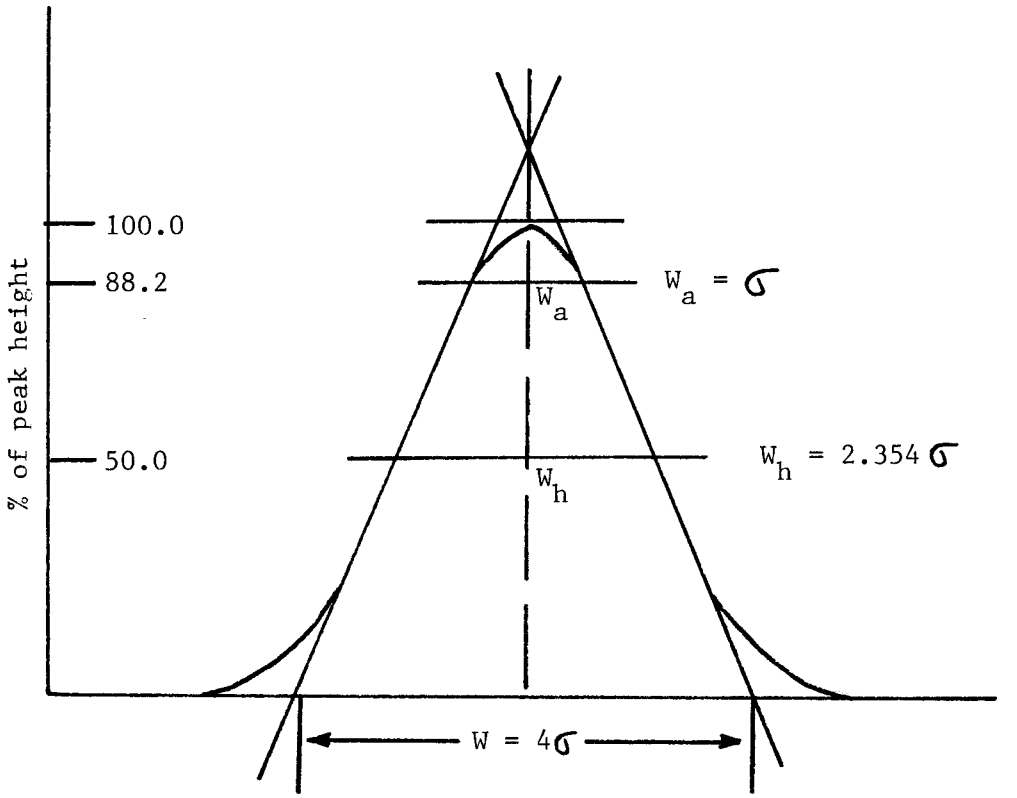

Fig. 2.--Standard deviation and peak width relationship for a Gaussian peak.

Band broadening is usually measured as a function of the peak width at one half the peak height. According to statistical calculations, the peak width at half height for a Gaussian peak is 2.354 σ from the peak center. Thus by substituting into equation (1), an expression is obtained which can be readily used to calculate the theoretical plate number per column. This is shown in equation (2).

$$N = \frac{t_r}{\sigma} = \frac{t_r}{\frac{w_{1/2}}{}} = 5.545 \left(\frac{t_r}{w_{1/2}}\right)^2 \qquad (2)$$

The efficiency of a column can be evaluated by injecting a partially retained compound and measuring its retention time as a function of distance by using a calibrated recording chart. The width at half peak height can be measured using a ruler or a magnifier with calibrations. This calculation can be used to assess column deterioration with usage and to compare two columns which contain the same packing material. It is obviously advantageous to be able to increase the total number of theoretical plates per column since this will increase resolution and sensitivity. This can be done by using long columns, minimizing liquid phase film thickness, or by choosing the proper carrier flow with all other conditions optimized. The packed columns used in GLC usually have between 2,000 to 5,000 plates per meter and column lengths of one or two meters are common.[22]

Another way to express column efficiency has been adopted which is essentially an absolute measure of resolving power since it allows the chromatographer to scrutinize individual theoretical plates. This number, the height equivalent to a theoretical plate (HETP), is obtained by dividing the column length (L) by the number of plates per column (N). This relationship is expressed in equation (3).

$$HETP = \frac{(L)}{(N)} \qquad (3)$$

The absolute efficiency of a column is defined in terms of HETP, but when the column is in actual use, other factors must be considered in the overall performance. Equation (4), the Van Deemter equation, incorporates terms which express the effects of the carrier gas flow,

temperature, and carrier molecular size on the actual plate height.

$$HETP = A + \frac{B}{\bar{\mu}} + C\bar{\mu} \qquad (4)$$

In equation (4), (A) is the Eddy diffusion term, (B) represents longitudinal band broadening effects as diffusion coefficients, (C) is the resistance to mass transfer term and ($\bar{\mu}$) is the average linear velocity of the carrier gas as it flows through the column. The Eddy diffusion term is proportional to the particle size of the packing material. On this basis, smaller particles will decrease (A) which in turn produces a smaller HETP. The band broadening term (B) is a function of solute molecular weight. High molecular weight solutes have smaller values for (B) since they will diffuse more slowly in the direction of the carrier flow. The (C) term is actually the sum of two terms, (C_l) and (C_g). The resistance to mass transfer in the liquid phase (C_l) is primarily a function of the liquid phase film thickness. The resistance to mass transfer in the gas phase (C_g) is a function of solute diffusion coefficients. The (A), (B), and (C) terms of the Van Deemter equation can be estimated by calculations for a given liquid phase and solute mixture, but the most practical way to optimize HETP is to evaluate the relationship between HETP and the carrier gas velocity expressed as ($\bar{\mu}$) for a given column. Figure (3) illustrates a typical Van Deemter plot of HETP versus ($\bar{\mu}$). Note that the curve shows a minimum which indicates the carrier flow rate which produces the smallest HETP for a given set of conditions. Although the curve minimum indicates the flow rate of optimum column performance, the carrier flow may not be practical in terms of analysis time since the optimum

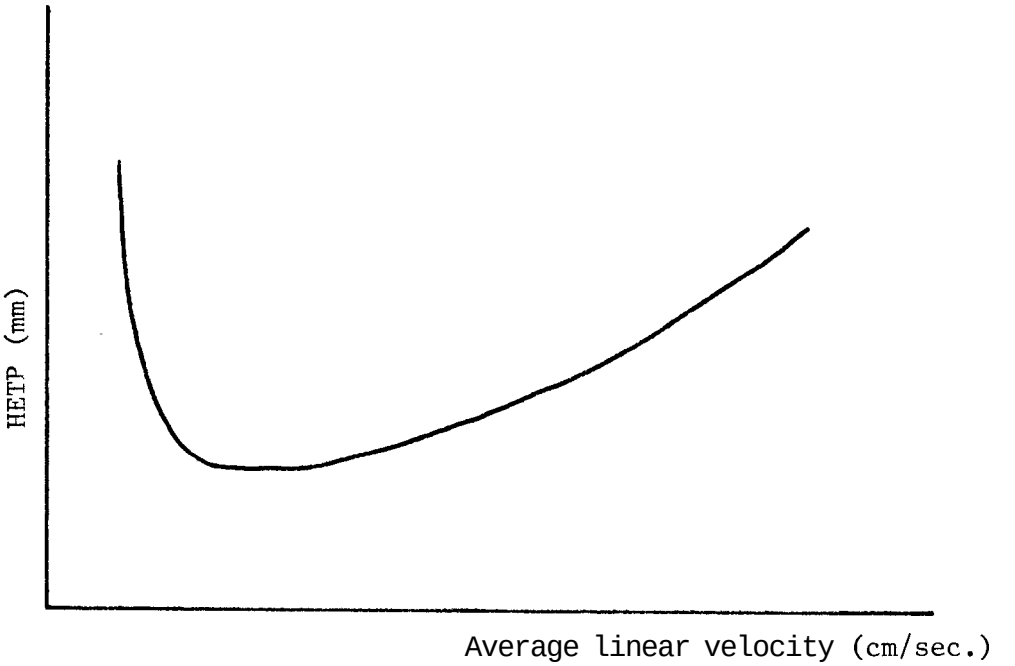

Fig. 3.--A typical Van Deemter plot

linear velocity usually occurs at low carrier flow rates. Therefore, it is also important to consider the relative flatness of the Van Deemter plot. If the slope of the curve following the minimum is small (i.e. the curve is fairly flat), a higher column efficiency is obtained over a larger range of carrier velocities. Van Deemter plots for packed columns predict an increase in HETP for low density carrier gases like helium, hydrogen and nitrogen,[23] but the situation is quite different for capillary columns as will be discussed later. Most chromatographers in toxicology are primarily concerned with the problem of analysis time. Thus, it is common with packed columns to compromise column efficiency for rapid analysis time by running at flow rates much higher than the optimum. Capillary column analysis helps eliminate this problem by delivering high efficiency

and short analysis time since very low carrier flow rates are used, and the total number of theoretical plates per column is significantly larger than that of packed columns.

Other parameters such as injection port temperature, oven temperature (isothermal or programmed), and the detector temperature can be manipulated to enhance the speed and efficiency of the overall analysis. The injection port temperature must be set high enough to insure that the compounds to be chromatographed are flash volatilized upon injection without decomposing their molecular structures. For most drug analyses, temperatures between 200 and 300 Celsius provide enough thermal energy to accomplish complete volatilization. The detector temperature is normally set between 300 and 400 Celsius for drug analyses to insure that deposition of the compounds on detector components is minimized.

The most important temperature setting involves the oven. Since the column resides in the oven, the temperature chosen will directly influence solute retention times and the total analysis time. Under normal circumstances, a chromatographer will experiment with the oven temperature until desired separations are achieved. Analyses can be run either isothermally, at one temperature during the entire analysis, or using a programmed temperature gradient. Most instruments available to toxicologists today are equipped to perform temperature programs with a single ramp. However, most companies now make GCs with multi-ramp programming, a feature which is likely to add a great deal of versatility to many complicated analyses.

CHAPTER III

HIGH RESOLUTION GAS CHROMATOGRAPHY

An Introduction to Open Tubular Columns

Gas chromatography using conventional packed columns is limited by essentially two factors; the rate of solute diffusion along the flow path and the residence time of the solute molecules in the liquid phase.[24] These factors correspond to the Eddy diffusion term (A) and the resistance to mass transfer term (C) respectively as they appear in equation (4). The idea that gas chromatography could be considerably improved by using wall coated open tubular columns (WCOTs) was first proposed by M. Golay in 1957.[25] Golay suggested that long thin tubes of borosilicate glass coated on the inner walls with liquid phase would effectively minimize the limiting factors associated with packed column GLC. Golay's idea could not be properly tested until 1960 when Desty et al.[26] developed a simple but efficient apparatus which produced glass capillary tubing of a consistently good quality. An avalanche of research followed in which many scientists contributed significantly toward advancements in column coating methods, column fabrication, injection techniques, and many of the practical applications of WCOT columns. Noted among these scholars and experimentalists are K. Grob (Sweden), M. Novotny (USA), G. Schomberg (German Federal Republic), and Jennings (USA).[27] As mentioned earlier in this thesis, borosilicate glass capillary columns have been used in many areas of industry, but few methods

related to toxicology have appeared in the literature. The application of capillary GLC to drug analyses essentially began with the avalability of the new fused silica columns. Theoretical and practical aspects of these columns will demonstrate their superiority to packed columns, and provide a rational basis for the proper selection of experimental conditions.

Choosing a Carrier Gas

The Van Deemter equation used for packed columns is modified slightly in capillary work. Since there is no packing material in WCOT columns, the Eddy diffusion term becomes zero. The resistance to mass transfer term becomes primarily a function of the (C_g) component since the liquid phase film thickness of WCOT columns is usually on the order of 0.2 micrometers. The relationship is shown in equation (5).

$$HETP = \frac{\bar{\mu}}{B} + C_g(\bar{\mu}) \qquad (5)$$

Thus, the HETP becomes primarily dependent upon the carrier gas molecular structure and its average linear velocity through the column. Rooney[28] plotted the Van Deemter curves for three common carrier gases, helium, hydrogen, and nitrogen, flowing through a thirty meter WCOT column with a 0.25 millimeter inner diameter and a 0.25 micrometer film thickness. The plots are shown in Figure (4).

As with packed columns, it is important to note the minimum of each curve and the flatness after the minimum. Note that though nitrogen exhibits a lower minimum than either helium or hydrogen, the steep rise occuring after the minimum indicates a significant loss in

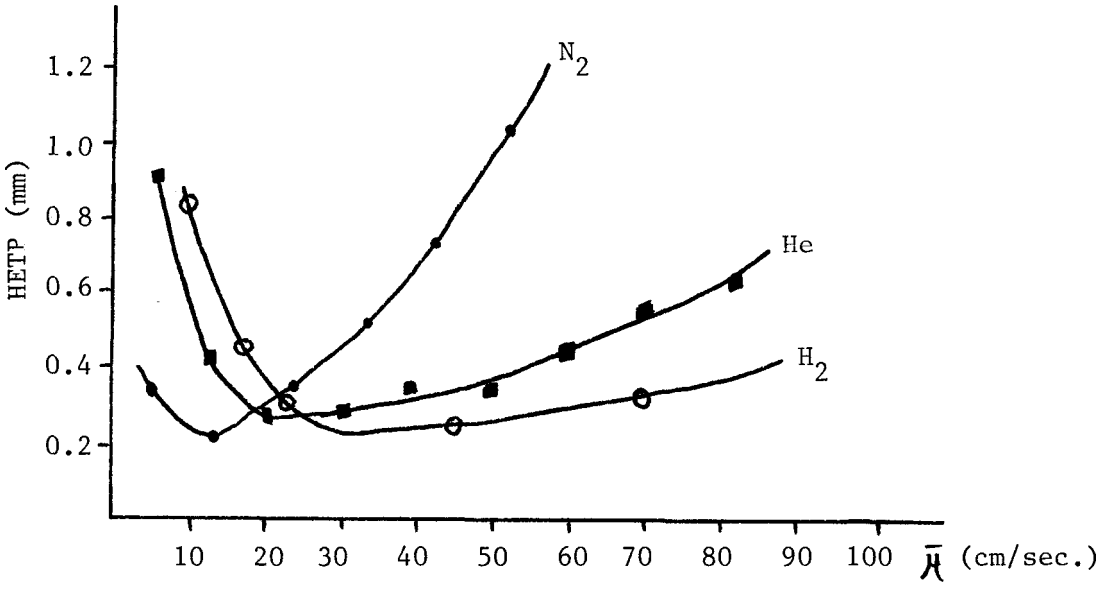

Fig. 4.--Van Deemter plots for helium, hydrogen, and nitrogen flowing through a fused silica column *(30* M x *0.25* mm.)

column efficiency at higher linear velocities. Hydrogen and helium are similar though hydrogen exhibits the smallest decrease in HETP over a wider range of linear velocities. However, hydrogen is highly flammable, and the potential for an explosion exists if a leak develops in the heated oven. On this basis, helium is a good compromise though high-grade helium is expensive.

Flow rates used in most capillary analyses range between 0.5 to *5* mL/min. of carrier gas. These low flow rates can be used while maintaining fast analysis time because the column is essentially an open tube; however, certain modifications in the detector system are required. A FID or NPD usually needs between *20* to *30* mL/min. of carrier flow in order to operate properly. Thus, a "make-up" gas is plumbed to the detector, and its flow is controlled separately. In

many instances, this situation can be used to optimize detector performance without sacrificing column efficiency. Hydrogen or helium can always be used as carrier gases to increase column efficiency, while a specific make up gas can be used depending upon the detector. For instance an argon/methane mixture can be used as a make-up gas for an electron capture detector while the column receives helium.

Helium (ultra high purity) was used as the carrier gas and as the make-up gas in this study. Hydrogen was not chosen because of the explosion hazard. All gas lines, including the air and hydrogen for the NPD, contained moisture traps. An oxygen scrubber (Oxyclear^{R}) was used in the carrier line to protect the liquid phase.

Injection Techniques

When using packed columns, sample volumes from one to ten microliters are usually injected directly onto the column. Packed columns have a large loading capacity when compared to capillary columns. In most instances, even one microliter of a moderately concentrated solution will overload a capillary column causing peak tailing and band broadening. Therefore, though on-column injection can be done with capillary columns under certain conditions, two other injection techniques are more commonly used. The first and most often used is called split injection. This technique requires a special injection port configuration which is represented in Figure (5). Once the specimen is injected, it is flash vaporized and forced through a special split insert liner which causes a high degree of turbulence in the injection port to insure that the injected mixture is homogenous before the split occurs. As the mixture reaches the bottom of the

split insert, part of it is sent through the column, and the remainder is vented to waste. The splitting is usually accomplished by an in-line valve which is controlled by the chromatographer.

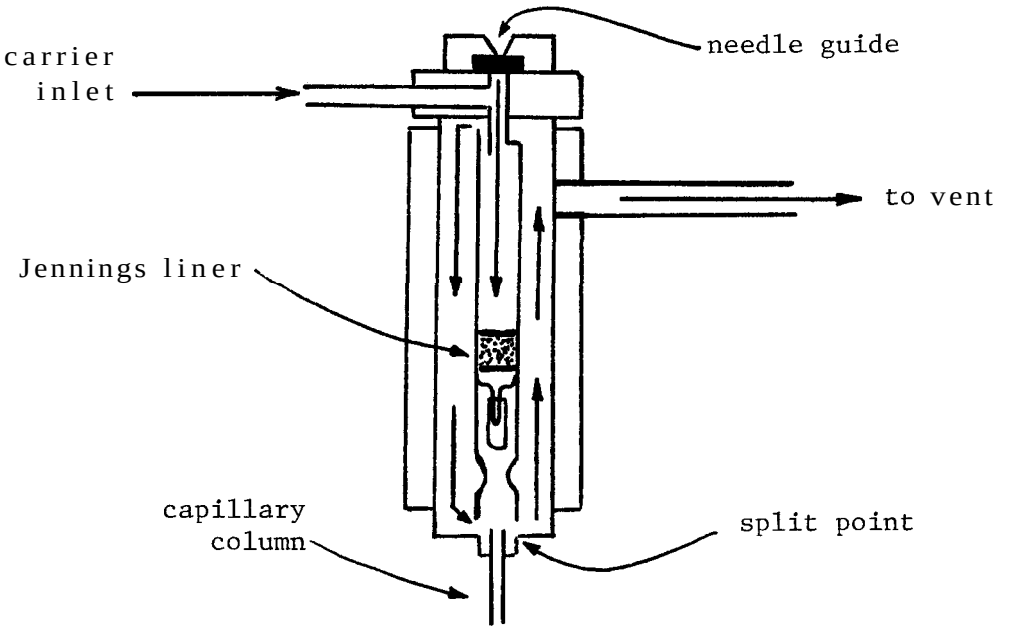

Fig. 5.--A representation of the HP5793A GC split mode injection port.

The glass insert liner is essential since its design can have a dramatic effect upon the reproducibility of the split which ultimately affects quantitative results.[29] The liner used in this study was designed by Jennings[30] and utilizes an inverted glass cup placed in the flow path to create turbulence. Split injection has at least two advantages over other methods. First, the operator can control the split ratio. The split ratio is calculated by dividing the total carrier flow (split vent flow + column flow) by the flow of the carrier through the column. Split ratios usually range from 200:1 to 30:1 depending upon the sample to be analyzed. This effectively allows the chromatographer to determine the optimum split which will give the highest

sensitivity without overloading the column. Secondly, very little specimen actually reaches the column in most cases. This is particularly important when biological specimens are chromatographed since they tend to be "dirty" and thus cause rapid column deterioration.

Splitless injection, the second alternative, involves the use of a hollow glass liner in the injection port. The specimen is injected and at a fixed time later, a purge valve is opened which flushes the injection port with carrier gas. Most of the specimen and the solvent reach the column, and the solutes experience what Grob terms the "solvent effect."[31] The technique is limited since the solvent must have a higher boiling point than the solutes, and thermal focusing of the solutes is recommended for good results.[32]

The third method, on-column injection, involves a more sophisticated technique. On-column injection implies that the solutes are injected directly into the column bypassing the inlet system encountered in split and splitless modes. Fused silica capillary columns used in drug analyses have inner diameters near 0.3 millimeters. In order to inject specimens inside the column, 32 gauge fused silica needles have been devised for use with a special injection apparatus.[33] These needles are much too fragile to pass through a rubber septum so special modifications must also be performed on the injection port.

The split injection mode was used in this study. The helium carrier was split 50:1 using the split insert liner of Jennings. Thermogreen LB-1 septa (Supelco, Inc.) were chosen since they exhibit low bleed and good resistance to thermal aging.

Column Installation

Fused silica capillary columns are highly flexible due to the polyimide coating over the glass. These columns are inherently straight so the column straightening procedures used with coiled borosilicate glass columns are not needed.[34] To install a column, a carbide knife, two graphite ferrules, a magnifier (20X), typewriter correction fluid, a column hanger and two connecting bolts are needed. Initially, the carbide knife should be used to remove a small piece of the column from each end. No particular care need be taken at this point since both ends will be cut again. Careful consideration must be given to the length of column to be inserted into both the injector and the detector. For the split insert liner used in this study, the inlet end of the capillary column should reside about one centimeter below the inverted turbulence cup. Placement of the column in this area will insure a good flow of the carrier-solute mixture into the column. The column inlet must not be allowed to touch the glass liner or uneven flows may cause split discrimination and non-linear results.[35]

The inlet jet of the HP5793A NPD is designed such that the outlet of the fused silica column can be inserted up to the jet tip as shown in Figure (6). This configuration eliminates all contact of solute molecules with the hot metal surface of the jet which prevents solute adsorption and decomposition. This tends to increase analysis sensitivity, and it reduces detector contamination.

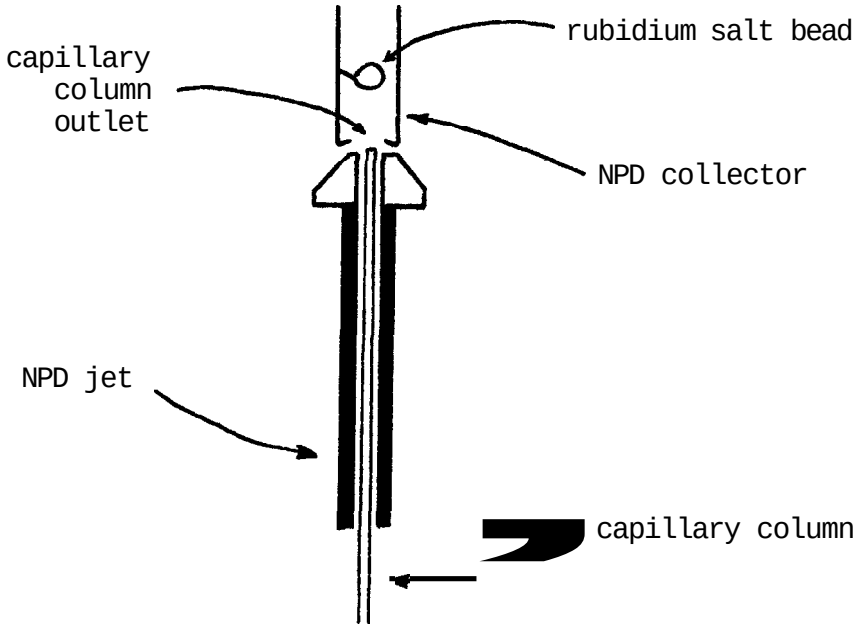

Fig. 6.--Positioning of a fused silica column outlet in the NPD jet of the HP5793A GC.

Certain precautions must be taken when installing the capillary column in the GC. The connecting bolt and graphite ferrule must be placed on the column as a first step. Then prior to connection, a small piece of the column end must be removed since small particles of graphite are usually caught in the end while the ferrule is slipped over the column. At this point the cutting must be done with care to avoid fragmenting the polyimide coating and exposing undeactivated fused silica glass to the flow stream. The magnifier should be used to inspect the column after a cut is made. Once the cut is satisfactory, the column should be inserted to the appropriate height in the injector or detector, and the bolt lightly tightened. The typewriter correction fluid should then be used to make a small mark on the column just under the connecting bolt. The column is then removed and the insertion length checked. The ferrules are then tightened about one

quarter turn past finger tight. Further tightening may crush the ferrules and cause a leak. When both ends of the column have been installed, methanol or an electronic leak detector should be used to check all seals. Soap solutions, such as snoop^{R} and others, should be avoided since trace amounts in the system can have deleterious effects upon the NPD. Before the instrument is started, carrier gas should be allowed to flow through the column for at least fifteen minutes to remove traces of air. The J & W Durabond-5 column used in this study is shipped ready for use.

Confirmatory Methods

Though the use of capillary column GLC considerably reduces the likelihood that two compounds in the same specimen will elute at exactly the same time, the possibility still exists, and it is therefore wise to use appropriate confirmatory methods. Thin layer chromatography and enzyme immunoassay were used in this study to confirm peak assignments. A brief description of each method follows.

Thin Layer Chromatography

As with GLC, TLC is used to separate the compounds in a mixture by means of a dynamic partitioning process. The stationary phase is generally a solid material like silica gel which has been bonded to the surface of a plate. The plate can be plastic, fiber, glass or metal. Glass is commonly used since it is inexpensive, easily coated and cut and somewhat inert. The stationary phase is spread in a thin film (usually 0.2 to 0.5 mm. thick) over the plate surface and allowed to dry uniformly. Precoated plates with various stationary phases are

available from many manufacturers and are generally sold in boxes containing 20% 20 centimeter glass plates. The choice of a stationary phase is based upon the types of compounds to be separated. The mobile phase, usually a mixture of organic solvents, is experimentally optimized once a particular plate is chosen. The mixture to be analyzed is spotted on the plate as a concentrated solution, the plate is developed and the resultant spots visualized by various means.

For drug analyses, many TLC systems have been devised.[36] In the late 1970's, a company in California, Analytical Systems, Inc., marketed a TLC system under the name Toxi-Lab^{R}. This system is very expensive and involves the use of fragile fiber TLC plates. Since it is the only commercially available TLC system which is essentially self-contained, it is quite popular. However, many laboratories still use ninhydrin and iodoplatinate sprays as their only visualization techniques. The system adopted in this work was developed at Accutox Laboratories in Canton, Ohio. The method is similar to Toxi-Lab^{R}, but conventional silica gel-coated glass TLC plates are used, and some of the visualization steps have been modified.[37] This TLC system was used for the confirmation of barbiturates, phenytoin, glutethimide, meprobamate, and certain benzodiazepines.

Enzyme Multiplied Immunoassay Technique (EMIT^{R})

Homogenous enzyme immunoassays were first introduced in the early 1970's[38,39] and their popularity grew immensely as the Syva Company (Palo Alto, Ca.) marketed various drug assay kits under the trade name EMIT^{R}. These kits gained widespread popularity despite their high cost primarily because the assays are extremely simple to

perform, require little manpower and equipment, can be performed quickly, and in most cases, offer a high degree of drug specificity. The chemical principles involved in their use is described by Maggio.[40]

Each of the kits used in this study, the benzodiazepine and the barbiturate, is calibrated using a negative, low and medium control serum supplied by Syva. The kits are calibrated based on their responses to diazepam (benzodiazepine kit) and secobarbital (barbiturate kit). The response of the kits to the other members of each drug class will be discussed later. The kits provide confirmation for medium to high levels of total benzodiazepines or total barbiturates in the serum specimens.

CHAPTER IV

EXPERIMENTAL METHOD

Equipment Required

Hewlett Packard 5793A gas chromatograph equipped with a nitrogen-phosphorus thermionic detector (NPD) and capillary column capabilities (Hewlett Packard Instruments, Palo Alto, Ca.)

2. Syva EMIT^{R} System (semiautomated): This includes a Gilford Stasar III spectrophotometer, CP-5000 microprocessor, a vacuum pump and a semi-automatic pipettor-diluter (Syva Co., Palo Alto, Ca.).

Durabond-5 fused silica capillary column, 15 meters long with a 0.25 mm. inner diameter and a 0.25 micrometer liquid phase film thickness (J & W Scientific, Rancho Cordova, CA.)

4. Omniscribe^{R} strip chart recorder (Houston Instruments, Austin, Tx.)
5. Ames Aliquot Mixer (Ames Company, Elkhart, In.)
6. Transilluminator UV source (Ultraviolet Products, Inc., San Gabriel, Ca.)

Whatman K6-F TLC plates, 250 micrometer film thickness (Whatman Company, Clifton, NJ)

8. Vortex Mixer: Maxi-Mix (Sybron Corporation)
9. Evaporation assembly: hot plate or drilled aluminum block with temperature control and an available nitrogen source.
10. Borosilicate glass culture tubes with Teflon lined caps (Corning Glass Works, Corning, NY)

11. Hamilton 7001N syringe, 1.0 microliter full scale (Hamilton Instruments, Reno, Nevada)
12. Ultra high purity (UHP) gases: helium, air and hydrogen (Liquid Carbonic Corp., Louisville, Ohio)
13. Extra dry nitrogen gas (Liquid Carbonic Corp., Louisville, Ohio)
14. Reacti-vials® 5.0 milliliter (Supelco, Inc., Bellfonte, Pa.)
15. Pipets: MLA 500 microliter volume and SMI precision type ranging from 10 to 250 microliters
16. Brinkman Dispensette® liquid dispenser with a Teflon pump (Brinkman Instruments)
17. Centrifuge
18. Aspirator assembly: this can be made using a vacuum flask, vacuum tubing, and a vacuum pump.

Reagents Required

1. Dichloromethane (Baker Analyzed Reagent® grade)
2. Methanol (Baker Analyzed Reagent® grade)
3. Isopropanol (Baker Analyzed Reagent® grade)
4. Sodium Sulfate, anhydrous powder (Baker Analyzed Reagent® grade)
5. Ammonium Sulfate (Baker Analyzed Reagent® grade)
6. Sodium Azide (Baker Analyzed Reagent® grade)
7. Deionized water
8. EMIT® Benzodiazepine Serum Assay kit (Syva Company, Ca.)
9. EMIT® Barbiturate Serum Assay kit (Syva Company, Ca.)
10. UTAK Serum Hypnotic Controls I, II, and III (UTAK Co., Ca.)
11. 5-ethyl-5-(p-toly1)-barbituric acid (Supelco, Inc.)

Preparation of Injection Standards

Pure drug standards were obtained from both the Applied Science Company and the United States Pharmacopeial Convention (USP). All drug standards to be injected were prepared as 100 mg/dL solutions in methanol. The drugs were weighed to 0.1 mg and placed into separate 100-mL class (A) volumetric flasks which had been repeatedly rinsed in reagent grade methanol. The flasks were filled to their calibration marks with reagent grade methanol. These solutions were stored in amber bottles and refrigerated until used.

Preparation of the Extraction Buffer

1.5 molar ammonium sulfate was prepared by dissolving 19.8 grams of the salt in deionized water such that the total volume was 100-mL. The pH was adjusted to 5.0 using 2 molar sulfuric acid. The solution was stored in an amber bottle at room temperature.

Preparation of the Internal Standard Solution

5-ethyl-5-(p-toly1)-barbituric acid was prepared as a 3 mg/dL solution in deionized water. Three milliliters of a 1 mg/mL methanolic solution was pipetted into a 100-mL class (A) volumatric flask. Deionized water was added to the calibration mark and the solution stored in an amber bottle under refrigeration.

Preparation of Serum Controls

Two serum controls (high and low) were prepared using the methanolic injection standards, SMI precision pipets, 50-mL class (A) volumetric flasks, and serum obtained from the blood bank of a local hospital. The serum was centrifuged to remove any red cells, and then it was extracted to check for the presence of drugs or interfering peaks. If the chromatogram was satisfactory, the serum was mixed nine parts to one with a 1% aqueous sodium azide solution which served as a preservative. Various volumes of the injection standards were pipetted into two clean 50mL class (A) volumetric flasks according to Table (3). The methanol was then slowly evaporated to a tiny volume using a stream of dry nitrogen. Drug free serum was added to each flask up to the calibration mark, and the solutions were thoroughly mixed. The control sera were then divided into 10-mL aliquots, placed into small polyethylene bottles and frozen until used. Once reconstituted, the controls were usually consumed within one week.

The UTAK Hypnotic controls were purchased lypholized. Each vial was reconstituted with 5.0 mL of deionized water, mixed well, and allowed to stand at room temperature for at least fifteen minutes prior to use. These were refrigerated until used.

Selection of the Extraction pH

The extraction process involves the interfacing of two immiscible solvents such that drug molecules present in either solvent redistribute themselves into the two solvents to varying degrees. the re: distribution process is dictated by a drug's differing solubility in the

TABLE 3

METHANOLIC STANDARD VOLUMES[a]
FOR SERUM CONTROL PREPARATION (50 mL)

DRUG	LOW		HIGH	
	Std. Vol. (mcL)	Serum Conc. (mcg/mL)	Std. Vol. (mcL)	Serum Conc. (mcg/mL)
Methyprylon	100	2	500	10
Aprobarbital	100	2	500	10
Butabarbital	100	2	500	10
Amobarbital	100	2	500	10
Pentobarbital	100	2	500	10
Secobarbital	100	2	500	10
Mephobarbital	250	5	500	10
Phenobarbital	500	10	2500	50
Methaqualone	100	2	500	10
Primidone	250	5	500	10
Phenytoin	500	10	250	15
Diazepam	25	0.5	100	2
N-desalkylflurazepam	25	0.5	100	2
Nordiazepam	25	0.5	100	2
Chlordiazepoxide	25	0.5	250	5

[a]All standards are 100 mg/dL in methanol.

two solvents. A drug's molecular structure, polarity and extent of acid-base ionization are primary factors which determine its solubility in a particular solvent. Many drugs are weak organic acids or bases and thus exhibit a pH-dependent equilibrium between a polar ionized form and a non-ionized form. Therefore, one can influence the solubility of many drug molecules in aqueous or organic (non-aqueous) solvents by selecting an appropriate buffer. An extraction performed at an optimized pH will both enhance the extraction efficiency of the desired compounds and hopefully exclude potentially interfering substances. Most of the compounds included in this study are considered weakly acidic or neutral. It follows that they will extract into a non-polar organic solvent from an aqueous solvent buffered at a slightly acidic pH. The use of phosphate buffers (pH 5-6) is common, but Thoma et. al.[41] suggest the use of 1.5 molar ammonium sulfate at an acidic pH to suppress the extraction of fatty acids and cholesterol. Thus, 1.5 molar ammonium sulfate was prepared at pH 4 to 7 and recovery studies and blank serum analyses were done to optimize conditions. Values of pH higher than 7 were not considered since the coextraction of many basic drugs is likely to be enhanced.

Selection of the Extraction Solvent

Ideally, the solvent chosen should efficiently extract the drugs from the serum matrix, evaporate readily, be non-toxic and inexpensive. Most organic solvents fail the non-toxicity criterion, but proper venting and careful handling can minimize risk. Some of the solvents most commonly used in drug extractions include ethers, hexane, chloroform and dichloromethane. The use of ethers in a hospital laboratory is not recommended since various people are likely to be handling

the solvents during shifts, and many instruments are running and producing heat in the lab. The potential for an explosion or fire is obvious since ethers are very volatile and highly flammable. Hexane was tried, but it gives extremely poor extraction efficiency at pH 5. In fact, only the benzodiazepines extracted at all. Chloroform and dichloromethane exhibited similar extraction efficiencies, but since dichloromethane has a lower boiling point, it will evaporate faster. Also, when using chlorinated methanes, the aqueous layer resides on top of the solvent interface. This allows easy access to the aqueous layer for removal by aspiration. On the basis of its volatility, efficiency, and density with respect to the aqueous phase, dichloromethane was chosen as the extraction solvent in this study. A solvent to serum ratio of 10:1 was used to insure adequate recovery of the drugs. Ratios below 5:1 resulted in frequent emulsion formation.

Chromatographic Conditions

Adjustable parameters critical to capillary chromatography include the injection port temperature and split ratio (with split injection), the oven temperature, the carrier gas flow rate, the column, the carrier gas, the detector temperature, and the make-up gas flow rate. The rationale for the conditions used in this study will be described.

Injection Port Temperature

The HP5793A GC is equipped with a microprocessor which controls all temperature parameters. Injection port temperatures between 200 and 300 Celsius are commonly used in drug analyses since most drugs will flash volatilize in this range. Considerations also must be made con-

cerning septum bleed and softening. Thermogreen **LB-1** septa were used in this study at a port temperature of **250** Celsius. Thermogreen septa are recommended for use between **200** and **300** Celsius. The septum was changed twice weekly to minimize column damage due to air leakage.

The Column

The J & W Durabond-5 **(DB-5)** fused silica capillary column was chosen for this work since it has a liquid phase similar to SE-54, a liquid phase frequently used in packed column drug analyses, and the manufacturer claimed that the column would exhibit minimal bleed and a long useful life. The shortest column available, fifteen meters, was selected to reduce cost and analysis time. A film thickness of **0.25** micrometers and an inner diameter of **0.25** millimeters represent the dimensions of a general purpose column, and this constituted a good starting point.

Oven Temperature

On the basis of my prior experience with packed columns and from the ranges most commonly found in the literature, programming the oven temperature over the range between **100** to **300** Celsius was considered likely to give the best results for the compounds of interest in this study. A mixture of the drugs listed in Table (4) was prepared in methanol for use as an injection standard. This mixture was injected repeatedly while oven temperature and carrier flow adjustments were made until satisfactory separations were accomplished. The critical separations involved the diazepam-N-desalkylflurazepam, nordiazepam-chlordiazepoxide decomposition product, phenytoin-oxazepam, butabarbital-acetaminophen and the phenobarbital-theophylline pairs. A temperature program

TABLE 4

DRUGS INCLUDED IN THE ANALYSIS AND CRITICAL SERUM CONCENTRATIONS[42]

DRUG	TOXICITY LIKELY ABOVE (mcg/mL)
Methyprylon	30
Aprobarbital	30
Butabarbital	30
Amobarbital	15
Pentobarbital	10
Secobarbital	3
Mephobarbital	20
Phenobarbital	40
Methaqualone	10
Primidone	12
Phenytoin	20
Diazepam	2
N-desalkylflurazepam	1
Nordiazepam	2
Chlordiazepoxide	3

starting at 130°C climbing to 290°C at 13°C/min. with a final hold of 2 minutes accomplished most of the separations. Phenytoin and oxazepam were not completely resolved, however, this is not a-major problem since detectable quantities of oxazepam rarely appear in serum, and the two drugs are not frequently given together in a medication regimen. Theophylline and phenobarbital also coelute, but this problem will be discussed later. The column head pressure was set at 19 psi which corresponded to 1.3 mL/min. of carrier gas going through the column. These conditions were satisfactory for good resolution in most cases and a quick analysis.

Detector Temperature and Make-Up Gas Flow

The NPD should always be set at a temperature high enough to prevent condensation of sample molecules on the jet and the collector. Hewlett Packard recommends 300 Celsius for their NPD in order to optimize both response and sample combustion. Since it was recommended by the manufacturer and since the oven temperature reaches 290 celsius, 300 Celsius was chosen as the detector temperature. The collector bead voltage and the make-up gas flow were set according to the manufacturer's recommendations. The make-up gas flow rate was varied between 20 and 60 mL/min., and it was found that the sensitivity decreased at higher flows. Therefore, the lower limit, 20 mL/min. was used. The collector bead voltage was initially set at sixteen and adjusted upward as needed. As the rubidium salt vaporized, the voltage had to be slowly increased to maintain detector sensitivity.

Analytical Procedure

1. Obtain two clean 16 x 100 mm borosilicate glass culture tubes with teflon lined screw caps.
2. Rinse each tube twice with dichloromethane.
3. Add 500 mcL of serum to each tube.
4. Label one tube "TLC" and the other "GLC".
5. Add 100 mcL of ammonium sulfate buffer to each tube.
6. Add 500 mcL of the internal standard solution to the tube labelled "GLC".
7. Vortex the tubes briefly to mix their contents.
8. Add 5 mL of dichloromethane to each tube and cap them tightly.

9. Place the tubes on the Ames rocker for five minutes and allow the extraction to occur.
10. Centrifuge the tubes for two minutes at 2500 rpm.
11. Remove the aqueous layer in both tubes by aspiration.
12. Using a small spatula, add anhydrous sodium sulfate to each tube until the powder no longer cakes.
13. Centrifuge the tubes for one minute at 2500 rpm.
14. Obtain two 5-mL Reactivials® and rinse them twice with dichloromethane. Label one vial "TLC" and the other "GLC".
15. Remove the culture tubes from the centrifuge and decant the solvent from each tube into the appropriately labelled Reactivial®.
16. Place the tubes in the wells of the hot plate or heating block and add water until the wells are about half-filled. Maintain the well temperature between 40 and 50 Celsius.
17. Evaporate the dichloromethane to dryness under a stream of dry nitrogen.
18. Reconstitute the residue in the "TLC" vial with 25 mcL of methanol and that in the "GLC" vial with 25 mcL of the retention marker solution (100 mg/dL barbital and 30 mg/dL clonazepam in 2-propanol).
19. Spot the contents of the "TLC" vial on the TLC plate according to the Accutox procedure. Develop the plate and visualize the spots while the GLC analysis is performed.
20. Inject 1.0 mcL of the contents of the "GLC" vial into the GC and initiate the temperature program and the strip chart recorder.
21. While the GLC analysis is progressing, perform the EMIT® benzodiazepine assay on the serum. The barbiturate assay is done only if the other data indicates the presence of a barbiturate.

22. Analyze the data from all three methods. Confirm peak identities and perform the quantitative calculations. The total analysis time should be less than one hour if no repeat assay is needed.

CHAPTER V

EXPERIMENTAL RESULTS

Chromatographic Data

The chromatogram in Figure (7) is obtained from the analysis of the high control serum. Peak identities in a sample are established by superimposing this control chromatogram on that of the sample, and by analyzing the EMIT® and TLC data. Since an integrator was not available for routine use, quantitation was accomplished using peak height ratios with respect to the internal standard, p-methyl-phenobarbital. The peak heights were measured using a clear plastic ruler calibrated in millimeters. Standard curves prepared from the control sera were used to obtain response factors for quantitation. The linearity data are shown in Table (5), and a sample calculation is given in Figure (8).

Peak height ratios are considered acceptable for quantitation provided that the peaks are sharp and symmetrical. Kipiniak[43] published a study of peak height versus peak area measurements and concluded that in many cases peak height measurements produced better results though neither method is infallible. All peak height measurements in this study were estimated to the nearest 0.5 millimeters, and all calculations were done using a Hewlett Packard 33C calculator.

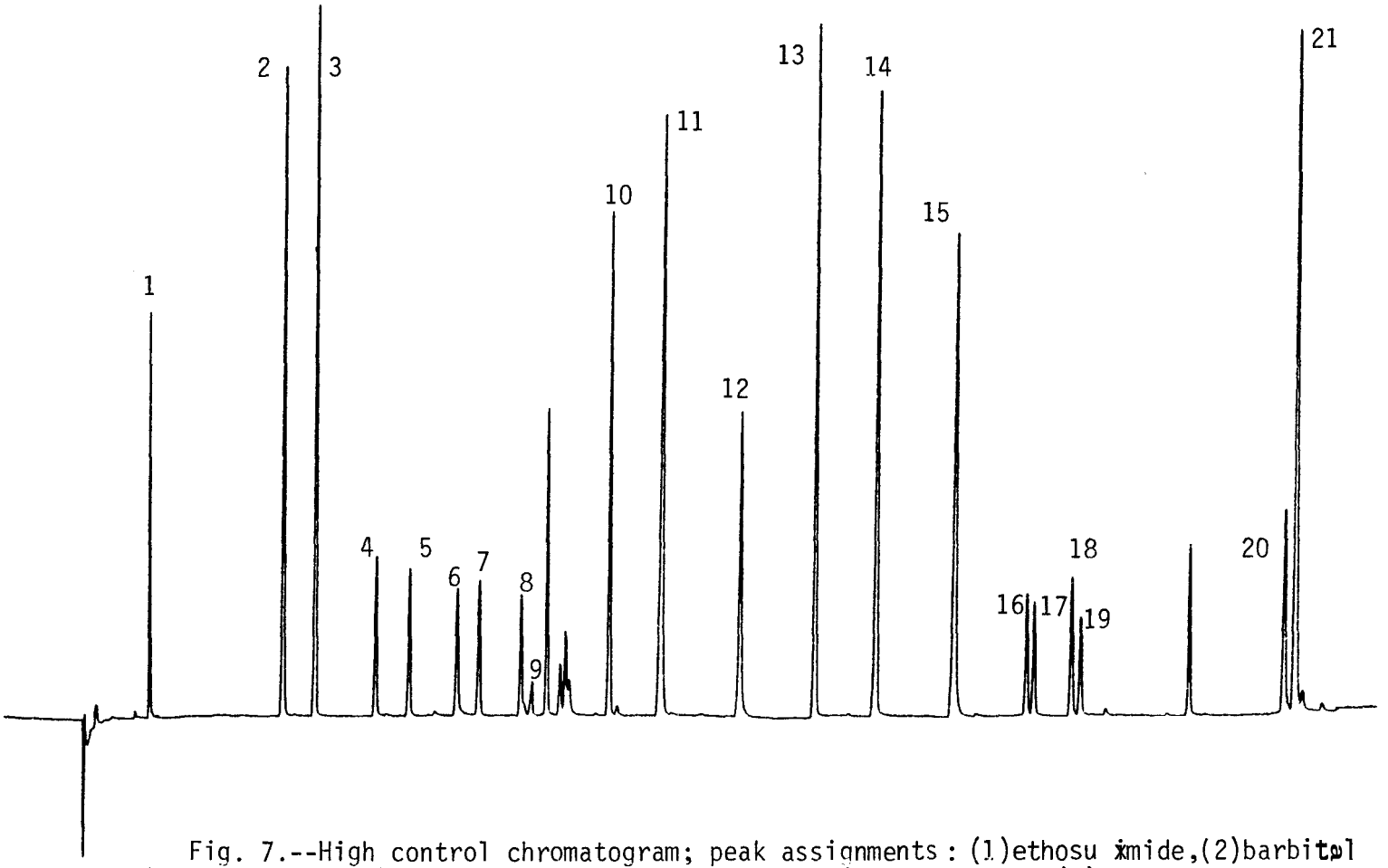

Fig. 7.--High control chromatogram; peak assignments: (1)ethosuximide, (2)barbital (3)methyprylon, (4)aprobarbital, (5)butabarbital, (6)amobarbital (7) pentobarbital (8)secobarbital, (9)meprobamate, (10)mephobarbital, (11)phenobarbital (12)internal std (13)methaqualone, (14)primidone, (15) phenytoin, (16)diazepam, (17)N desalkylflurazepam (18)nordiazepam, (19)chlordiazepoxide decomposition product, (20)chlordiazepoxide (21)clonazepam

TABLE 5

CONTROL LINEARITY DATA[a]

DRUGS	$\bar{x}$ (½ low)	$\bar{x}$ (low)	$\bar{x}$ (½ high)	$\bar{x}$ (high)	slope	R^2
Methyprylon	*0.29*	*0.64*	*1.67*	*3.14*	*0.316*	*0.999*
Aprobarbital	*0.07*	*0.18*	*0.31*	*0.70*	*0.068*	*0.994*
Butabarbital	*0.08*	*0.16*	*0.42*	*0.79*	*0.079*	*0.999*
Amobarbital	*0.09*	*0.16*	*0.40*	*0.70*	*0.068*	*0.997*
Pentobarbital	*0.06*	*0.14*	*0.31*	*0.67*	*0.067*	*0.999*
Secobarbital	*0.06*	*0.14*	*0.33*	*0.67*	*0.067*	*0.999*
Mephobarbital	*0.63*	*1.14*	*1.77*	*2.30*	*0.226*	*0.999*
Phenobarbital	*0.30*	*0.54*	*1.16*	*2.38*	*0.046*	*0.999*
Methaqualone	*0.21*	*0.43*	*1.15*	*2.09*	*0.209*	*0.998*
Primidone	*0.51*	*1.21*	*1.74*	*2.44*	*0.253*	*0.998*
Phenytoin	*0.48*	*0.81*	*1.05*	*1.44*	*0.094*	*0.994*
Diazepam	*0.06*	*0.11*	*0.23*	*0.41*	*0.0002*	*0.998*
N-desalkylflurazepam	*0.05*	*0.10*	*0.24*	*0.46*	*0.0002*	*0.999*
Nordiazepam	*0.07*	*0.10*	*0.22*	*0.45*	*0.0002*	*0.998*
Chlordiazepoxide	*0.10*	*0.18*	*0.83*	*1.79*	*0.355*	*0.999*

[a]The data are peak height ratios, and there are five data points per mean ($\bar{x}$).

Drug peak height = *76.5* mm (measured)

I.S. peak height = *80.0* mm (measured)

Standard curve slope = *0.096*

$$\text{Concentration of drug (mcg/mL)} = \frac{76.5 \text{ mm}}{80.0 \text{ mm}} \times \frac{1}{0.096} = 9.9 \text{ mcg/mL}$$

Fig. 8.--Sample quantitative calculation

Method Accuracy

Unlike standard quantitative chemical analysis where standard deviations greater than 1% are usually considered unsatisfactory, the accuracy requirements in drug assays are far less demanding especially in emergency toxicology where broad spectrum drug screening is done. This is largely due to the fact that most drugs exhibit a range of concentrations which is considered therapeutic. Unfortunately, therapeutic ranges are really only statistical averages, and individual patients may respond to any given drug in an abnormal manner. However, a physician can use serum drug levels from toxicological analyses to assess the potential role the drug may play in a patient's condition.

Though high quantitative accuracy is analytically desirable, it is usually not clinically significant, especially at very low drug levels, and the excessive costs involved in a rigid high accuracy quality control program make it impractical in most circumstances. If a serum concentration is assayed within 10% of its actual value, the accuracy is sufficient to make the results clinically useful in emergency toxicology and in most TDM assays. For example, diazepam has a therapeutic range roughly between 500 and 2000 ng/mL. If a patient actually having 1200 ng/mL of diazepam in his serum is assayed as having 1090 ng/mL, the result is clinically accurate since the physician becomes aware that the level is mid-range therapeutic and not high-range therapeutic or toxic. Secondly, the results from these screens must be obtained quickly to be of any value. This usually means that only one assay is done with no duplicates to obtain an average. Therefore, on

this basis, the data obtained from the reproducibility studies, both within-day and between-day, are are acceptable for screening purposes. Tables (6) and (7) show the within-day variation of drug peak height ratios for the low and high controls respectively, and Tables (8) and (9) provide the between-day data.

TABLE 6

LOW CONTROL WITHIN-DAY
PEAK HEIGHT RATIO VARIATION (N=10)

DRUG	MEAN ($\bar{x}$)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (CV)
Methyprylon	0.64	0.030	4.7%
Aprobarbital	0.18	0.008	4.4%
Butabarbital	0.16	0.009	6.0%
Amobarbital	0.16	0.008	5.3%
Pentobarbital	0.14	0.007	5.0%
Secobarbital	0.14	0.008	5.9%
Mephobarbital	1.14	0.055	4.8%
Phenobarbital	0.54	0.024	4.5%
Methaqualone	0.43	0.033	7.6%
Primidone	1.21	0.055	4.5%
Phenytoin	1.05	0.060	5.7%
Diazepam	0.10	0.009	9.7%
N-desalkylflurazepam	0.10	0.007	7.0%
Nordiazepam	0.10	0.007	7.0%
Chlordiazepoxide[a]	0.18	0.015	8.0%

[a]Expressed as the sum of the parent peak and the decomposition product

TABLE 7

HIGH CONTROL WITHIN-DAY PEAK HEIGHT RATIO VARIATION (N=10)

DRUG	MEAN ($\bar{x}$)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (CV)
Methyprylon	3.14	0.180	5.7%
Aprobarbital	0.70	0.027	3.9%
Butabarbital	0.79	0.040	5.0%
Amobarbital	0.70	0.035	5.0%
Pentobarbital	0.67	0.030	4.4%
Secobarbital	0.67	0.025	3.8%
Mephobarbital	2.30	0.116	5.5%
Phenobarbital	2.38	0.130	5.5%
Methaqualone	2.09	0.140	6.7%
Primidone	2.44	0.170	6.9%
Phenytoin	1.44	0.037	2.6%
Diazepam	0.41	0.018	4.5%
N-desalkylflurazepam	0.46	0.025	5.5%
Nordiazepam	0.45	0.021	4.6%
Chlordiazepoxide[a]	1.79	0.045	2.5%

[a]Expressed as the sum of the parent peak and the decomposition product.

TABLE 8

LOW CONTROL BETWEEN-DAY PEAK HEIGHT RATIO VARIATION (N=10)

DRUG	MEAN ($\bar{x}$)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (CV)
Methyprylon	0.67	0.041	6.7%
Aprobarbital	0.15	0.008	5.6%
Butabarbital	0.16	0.010	6.4%
Amobarbital	0.16	0.011	6.6%
Pentobarbital	0.13	0.009	6.3%
Secobarbital	0.14	0.009	6.2%
Mephobarbital	1.13	0.060	5.3%
Phenobarbital	0.56	0.029	5.1%
Methaqualone	0.45	0.033	7.3%
Primidone	1.19	0.052	4.4%
Phenytoin	1.02	0.068	6.7%
Diazepam	0.11	0.010	9.4%
N-desalkylflurazepam	0.10	0.006	6.1%
Nordiazepam	0.11	0.007	6.3%
Chlordiazepoxide[a]	0.18	0.016	8.8%

[a]Expressed as the sum of the parent peak and the decomposition product

TABLE 9

HIGH CONTROL BETWEEN-DAY
PEAK HEIGHT RATIO VARIATION (N=10)

DRUG	MEAN ($\bar{x}$)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (CV)
Methyprylon	3.21	0.140	4.5%
Aprobarbital	0.65	0.019	2.9%
Butabarbital	0.78	0.035	4.4%
Amobarbital	0.69	0.036	5.2%
Pentobarbital	0.63	0.032	5.1%
Secobarbital	0.64	0.033	5.2%
Mephobarbital	2.27	0.092	4.0%
Phenobarbital	2.32	0.120	5.1%
Methaqualone	2.20	0.130	5.9%
Primidone	2.37	0.140	6.1%
Phenytoin	1.45	0.049	3.4%
Diazepam	0.42	0.026	6.3%
N-desalkylflurazepam	0.44	0.022	5.0%
Nordiazepam	0.43	0.020	4.7%
Chlordiazepoxide[a]	1.77	0.048	2.7%

[a]Expressed as the sum of the parent peak and the decomposition product

The accuracy of some of the standard curve data in Table (5) was evaluated using controls purchased from UTAK Laboratories. These controls were assayed over a ten day period, and the results are shown in Table (10). Chromatograms of these controls are shown in Figures (9-1 1).

TABLE 10

UTAK HYPNOTIC CONTROL ASSAYS
BETWEEN-DAY (N=10)

DRUG	ASSIGNED VALUE (mcg/mL)	PHR[a] $\bar{X} \pm s$	CALCULATED CONC. (mcg/mL) $\pm$ s
HYPNOTIC I			
Methyprylon	40	11.9 ± 0.9	37.7 ± 2.9
Secobarbital	10	0.63 ± 0.05	9.4 ± 0.7
Methaqualone	10	2.15 ± 0.18	10.3 ± 0.8
HYPNOTIC II			
Phenobarbital	40	1.72 ± 0.17	37.4 ± 3.6
Chlordiazepoxide[b]	10	3.63 ± 0.36	10.2 ± 1.0
HYPNOTIC III			
Pentobarbital	20	1.41 ± 0.08	21.0 ± 1.2
Diazepam	10	2.11 ± 0.12	10.5 ± 0.6

[a] Peak Height Ratio: mean ($\bar{X}$) plus or minus the standard deviation

[b] Expressed as the sum of the parent peak and the decomposition product

The assayed values for the UTAK controls agree within 10% of the spiked values indicated by UTAK. This method was also tested by

performing assays on the March 1983 College of American Pathologists (CAP) Advanced Toxicology Proficiency Survey specimens. These specimens are spiked with known amounts of drugs and sent to participating laboratories as unknowns. The specimens are assayed and returned for evaluation. The results from this method and those reported by the CAP are shown in Table (11).

TABLE 11

MARCH 1983
CAP SURVEY RESULTS

SPECIMEN	DRUGS PRESENT	PARTICIPANT MEAN ASSAY VALUE	ANALYSIS RESULTS
	Diazepam	1.3 mcg/mL	1.3 mcg/mL
T2	Methaqualone	2.3 mcg/mL	2.8 mcg/mL
	Nordiazepam	2.0 mcg/mL	2.0 mcg/mL
T3	Secobarbital	3.1 mcg/mL	3.8 mcg/mL

On the basis of these studies, the method can be considered accurate enough for quantitative emergency toxicology.

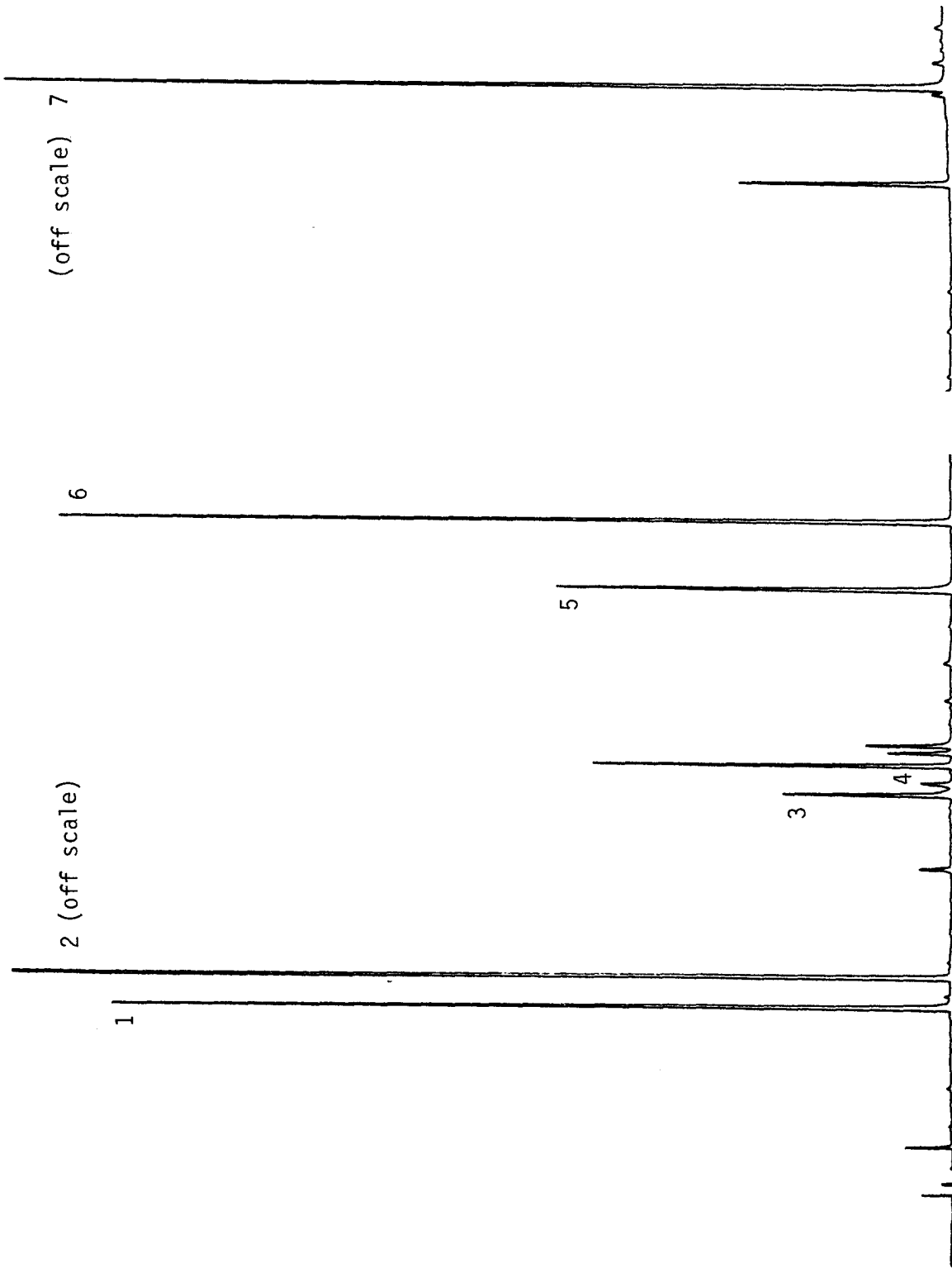

Fig. 9.--UTAK Hypnotic I; peak assignments: (1)barbital, (2)methyprylon, (3)secobarbital, (4)meprobamate, (5)internal std., (6)methaqualone, (7)clonazepam

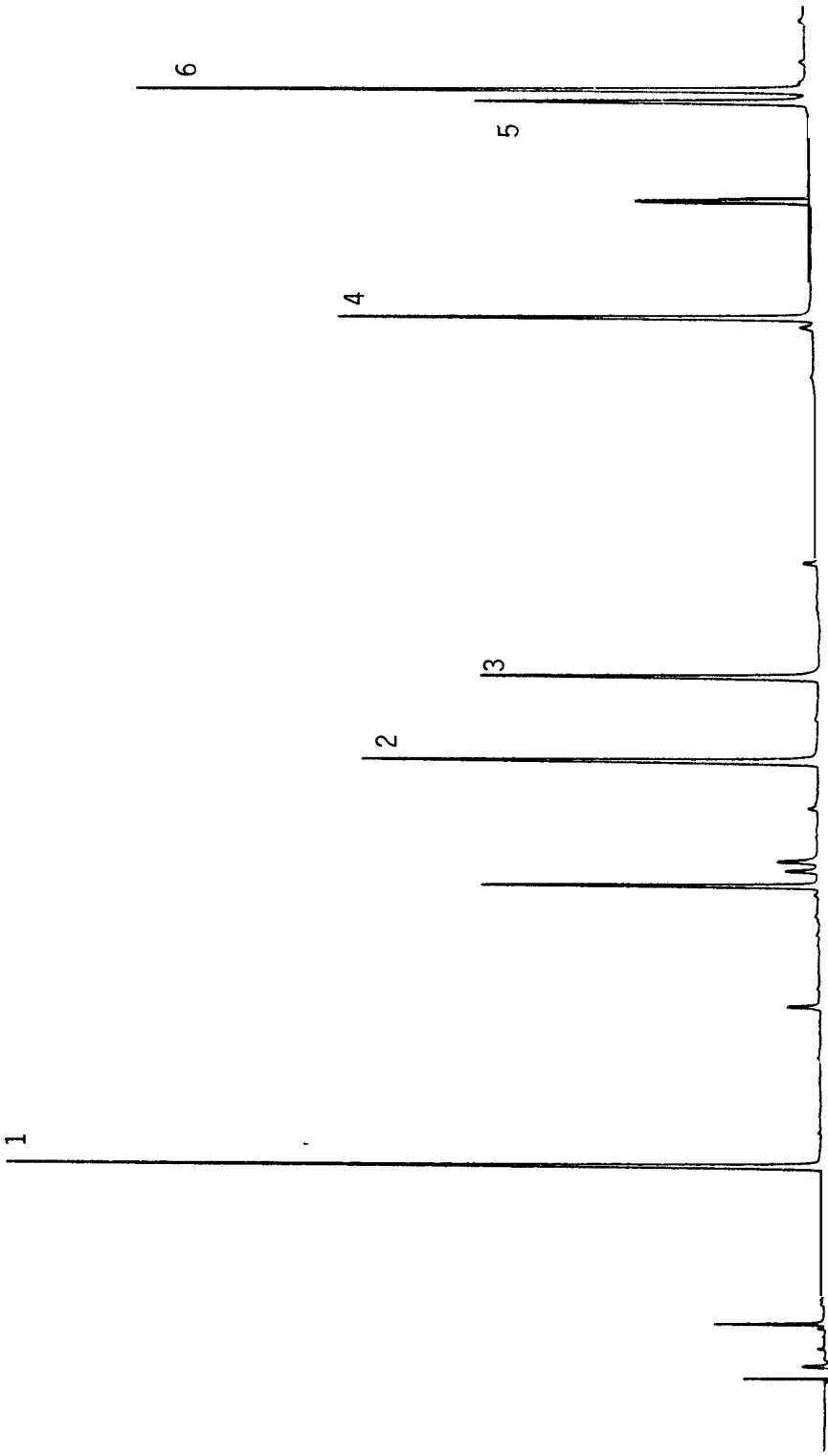

Fig. 10.--UTAK Hypnotic II chromatogram; peak assignments: (1)barbital, (2)phenobarbital, (3)internal std., (4)chlordiazepoxide decomp. product, (5)chlordiazepoxide, (6)clonazepam

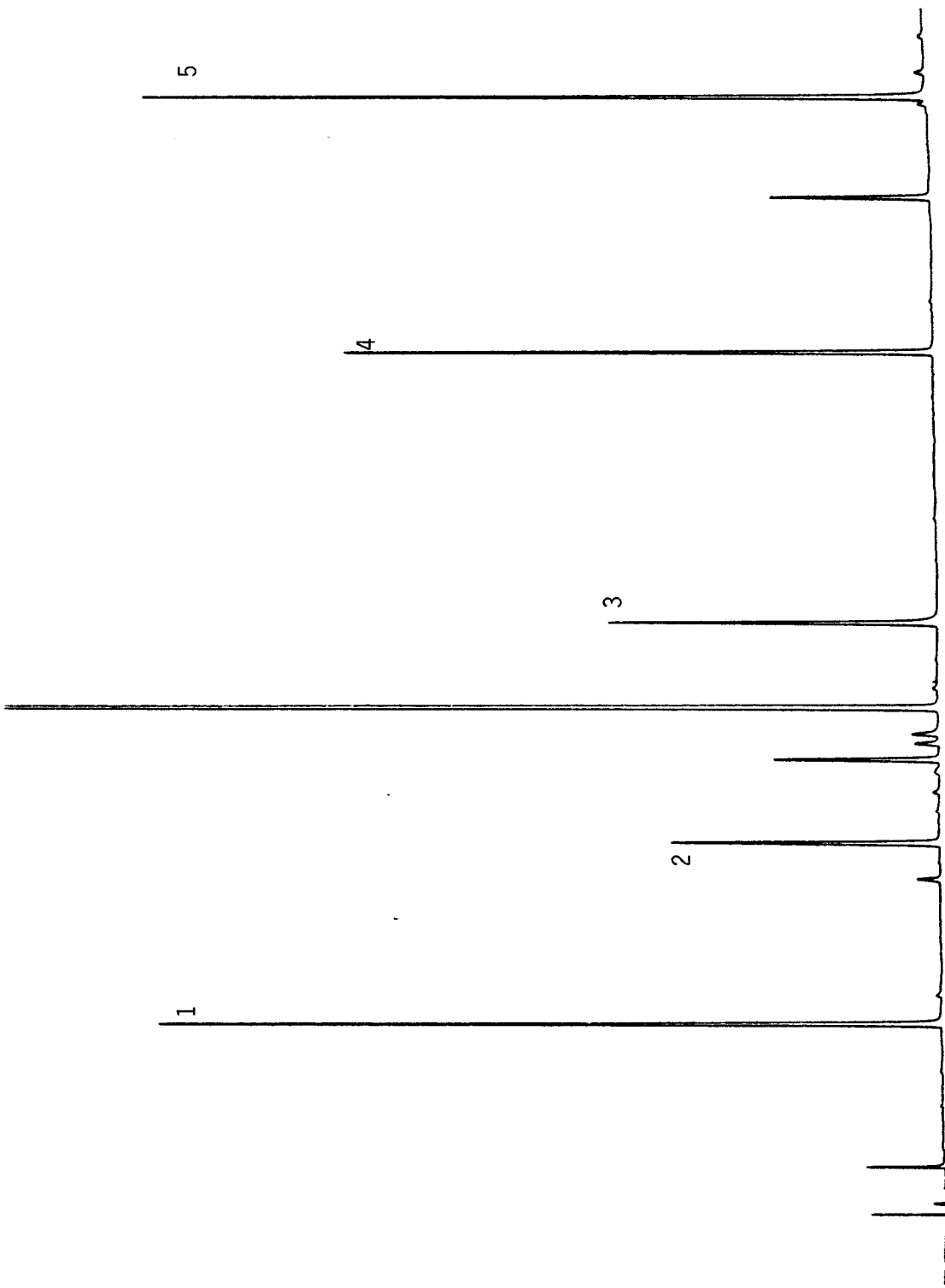

Fig. 11.--UTAK Hypnotic III chromatogram; peak assignments: (1)barbital (2)pentobarbital, (3)internal std., (4)diazepam, (5)clonazepam (there is a slight baseline drift in this chromatogram)

Interference Study

Methanolic solutions of the drugs available were prepared and injected individually under the chromatographic conditions of this procedure. An integrator was used to obtain accurate retention times. The retention data for the drugs tested appear in Table (12). On the basis of this data, the only compounds not included in the screen which might interfere with the compounds of interest include theophylline, oxazepam, butalbital and acetaminophen. Of these, theophylline is the only real problem. Butalbital and acetaminophen elute very shortly after butabarbital and very nearly together. However, acetaminophen tends to tail, and one is immediately alerted to the possibility of its presence. When this occurs, an acetaminophen assay, which could be done using this method, must be done separately. Acetaminophen was not included as a quantitative part of the GLC screen because it has a very strong detector response even at low levels, and it tends to mask butabarbital. Acetaminophen can be readily quantitated by HPLC or EMIT^{R} but a butabarbital determination is more difficult. Butalbital can be differentiated from butabarbital since it elutes just slightly later. The subtle retention shift can be seen when a control chromatogram is superimposed. Butalbital is simply reported as "present" since no data are available concerning its therapeutic range,[44] and hence, quantitative results would have little meaning. Oxazepam was discussed earlier with respect to phenytoin. Theophylline presents a problem since it coelutes with phenobarbital. Theophylline is commonly prescribed as a bronchiodilator and phenobarbital is, along with phenytoin, a drug of - choice for many epilepsies. The possibility that these two compounds

TABLE 12

DRUG RETENTION TIMES (MIN.)[a]

DRUG	RETENTION TIME	DRUG	RETENTION TIME
Acetaminophen	4.55	Methadone	9.00
Amitriptyline	9.62	Methapyriline	8.38
Amobarbital	4.95	Methaqualone	8.83
Aprobarbital	4.10	Methsuximide	3.86
Barbital	3.08	Methylnitrazepam	12.61
Benzphetamine	6.24	Methylphenidate	5.05
Butabarbital	4.48	Methyprylon	3.48
Butalbital	4.52	Nitrazepam	13.23
Caffeine	6.17	N-acetylprocainamide	11.72
Carbamazepine	10.08	N-desalkylflurazepam	11.02
Chlordiazepoxide	13.72	Nordiazepam	11.38
Chlordiazepoxide DP[b]	11.48	Norpropoxyphene	11.67
Clonazepam	13.85	Nortriptyline	9.80
Codeine	10.56	Oxazepam	10.17
Desipramine	9.44	p-methylphenobarbital	7.99
Diazepam	10.92	Pentobarbital	5.18
Ethinamate	2.26	Phenacetin	4.73
Ethosuximide	1.48	Phencyclidine	6.43
Flurazepam	13.25	Phenobarbital	7.20
Glutethimide	6.05	Primidone	9.40
Imipramine	9.03	Procainamide	9.40
Lidocaine	6.45	Propoxyphene	9.55
Lorazepam	12.38	Protriptyline	9.42
Meperidine	4.86	Secobarbital	5.62
Mephobarbital	6.67	Theophylline	7.18
Meprobamate	5.75	Trimipramine	9.73

[a] J & W DB-5 column, 15 meters, 0.25 mm i.d., 0.25 micron film temperature program: 130 to 290 celsius at 13°C/min.

[b] Chlordiazepoxide GC decomposition product

might be in the same sample is reasonable, and therefore care must be used when a peak assignment is made. Fortunately, a little good judgement can all but prevent the problem. Theophylline has a very high NPD response (10 mcg/mL gives a peak height ratio of 3.5). Since the therapeutic range of theophylline is from 10 to 20 mcg/mL, it is likely

that a very large peak will result if theophylline is present. Phenobarbital can be detected to a level of 2 mcg/mL using the TLC analysis. Thus, if a light to medium spot appears on the TLC plate, and a large peak appears on the chromatogram, the presence of theophylline is probable and separate methods (HPLC and EMIT) can be used to confirm the suspicion. The EMIT® barbiturate assay can be used to confirm only very high levels of phenobarbital. In any event, one may choose to use judgement and report results, or a "phenobarbital" peak may be confirmed by a second quantitative method. Experience is the best guide, though information concerning the patient's current medications can be helpful.

Interferences as coeluting peaks are only a problem if they can't be identified readily. Any good toxicologist is always suspicious and confirms high levels of drugs and investigates any odd behavior shown on the chromatogram. The high resolution afforded by the fused silica capillary column helps to reduce coelution problems by both enhancing separations and by making peak distortions more obvious.

Confirmatory Procedures

Confirmation of GLC peak identities is a necessary and sometimes time consuming process. Ideally, these procedures should be run concurrent with the GLC analysis, and they should consume small quantities of serum. Many possible methods are available including HPLC, TLC, spectrophotometric, chemical color tests, immunoassay, and others. In this study, the barbiturates and benzodiazepines are confirmed using TLC and EMIT® methods. Phenytoin, meprobamate, and

glutethimide confirmations involve TLC alone. Methaqualone can be confirmed using a spectrophotometric method,[45] but no GLC interferences have been encountered to merit the use of such a time consuming and interference-ridden procedure. Methyprylon is not confirmed since no simple way is available other than to use a second GC equipped with a different column. Primidone can be confirmed using HPLC or EMIT^{R}.

In many cases, a urine specimen is also analyzed with the serum, and this aids immensely in compound identification since drugs tend to accumulate in the urine. However, assuming that no urine is available, the confirmatory methods used in this study are adequate. The sensitivity of the two methods toward the drugs of interest is shown in Table(13).

TABLE 13

DRUG DETECTION LIMITS
FOR THE TLC AND EMIT ANALYSES

DRUGS	TLC METHOD (mcg/mL)	EMIT^{R} METHOD (mcg/mL)
Amobarital	1	15
Aprobarbital	1	-
Butabarbital	1	7
Butalbital	1	8
Chlordiazepoxide	0.5	5
Diazepam	0.5	0.3
Glutethimide	2	
Meprobamate	3	-
N-desalkylflurazepam	0.5	2
Nordiazepam	0.5	2
Pentobarbital	1	1
Phenobarbital	2	20
Secobarbital	1	3

The TLC data were obtained by analyzing spiked sera, and the EMIT^{R} data were extrapolated from studies done by Syva.[46,47] These confirmatory methods cover most of the drugs found in serum screens. The other compounds not included in the confirmatory methods are free of interferences on the basis of this study, but confirmations may be necessary in special cases where peak distortion, patient history, or urine analysis results cast doubt upon the GLC data.

GLC Detection Limits

The detection limits of the analysis were assigned on the basis of both clinical significance and peak detectability. Detection limits were set at one fifth the lower limit of the drug's therapeutic range unless this was below the lowest measurable peak height. It is felt that if detection limits are set too low, insignificant results will be frequently generated, and the potential for a false positive increases since small noise peaks might be misconstrued as drugs. Also, the GLC analysis is usually more sensitive than the TLC or EMIT^{R} methods. Thus, if the detection limits on the GLC are set too low, confirmation becomes difficult. The drug detection limits for the GLC analysis are listed in Table (14).

Extraction Recovery Study

Recovery was evaluated by comparing the responses of the extracted drugs to those of the same drugs directly injected with no extraction. The recovery standard was a methanolic solution containing the drugs of interest in concentrations identical to those in the high control serum. A 60 mg/dL solution of the internal standard compound was

TABLE 14

DRUG GLC DETECTION LIMITS

DRUG	DETECTION LIMIT (mcg/mL)
Methyprylon	2
Aprobarbital	1
Butabarbital	1
Amobarbital	1
Pentobarbital	1
Secobarbital	1
Mephobarbital	2
Phenobarbital	2
Methaqualone	1
Primidone	1
Phenytoin	2
Diazepam	0.2
N-desalkylflurazepam	0.1
Nordiazepam	0.2
Chlordiazepoxide	0.2

prepared in isopropanol. 500 mcL of the methanolic recovery standard was pipetted into a Reactivial^{R}, and the methanol was evaporated to dryness under dry nitrogen. 25 mcL of the alcoholic internal standard was then added to the vial. This solution was injected and served as the 100% recovery reference. 500 mcL of the high control was mixed with 500 mcL of deionized water and 100 mcL of buffer. The specimen was extracted, aspirated, and dried. The dichloromethane was evaporated to dryness, and 25 mcL of the alcoholic internal standard was added. The mixture was injected at the same attenuation as the 100% recovery standard. The peak height ratios of the control drugs were

compared to those of the standard. The results are shown in Table (15).

TABLE 15

PERCENT RECOVERY RESULTS:
DICHLOROMETHANE EXTRACTION

DRUG	pH 4	pH 5	pH 6	pH 7
Methyprylon	93	96	90	91
Aprobarbital	94	94	90	85
Butabarbital	99	98	96	92
Amobarbital	96	94	92	93
Pentobarbital	93	95	94	92
Secobarbital	97	93	94	92
Mephobarbital	90	92	93	90
Phenobarbital	91	94	90	89
Methaqualone	93	98	96	98
Primidone	92	90	88	82
Phenytoin	89	92	90	90
Diazepam	71	95	96	94
N-desalkylflurazepam	70	90	93	94
Nordiazepam	66	89	91	93
Chlordiazepoxide	89	92	90	90

On the basis of these data, the extraction could be done at pH 5,6 or 7 with no significant loss of efficiency. However,-it is felt that the coextraction of basic compounds would be maximally suppressed if the lower pH is used. Hence, the extraction was done at pH 5.

Column Durability

The DB-5 column was received in September, 1982, and the manufacturer claimed that the column possessed 95,850 theoretical plates using hydrogen as the carrier gas and methyl undecanoate as the solute. The column was evaluated in October, 1982, December, 1982 and March, 1983 by using p-methylphenobarbital and equation (2). Table (16) shows the results of the evaluations.

TABLE 16

EFFICIENCY DATA FOR THE DB-5 COLUMN

DATE	t_r (min.)	$W_{\frac{1}{2}h}$ (min.)	$N = t_r/W_{\frac{1}{2}h} \times 5.545$
10-08-82	8.02	0.00049	91,000
12-14-83	7.99	0.00049	90,000
03-11-83	8.00	0.00049	90,000

A calibrated magnifier was used to measure the peak width at a chart speed of 20 cm/min. As the data indicates, the column has lost very little efficiency even though it has been in continuous daily use since September, 1982. This is evidence in support of the cost effectiveness of the column. The column can be purchased new for $215, but it is likely to last two years or longer on the basis of the data obtained. The long life of the DB-5 column simplifies quality control since fewer recalibrations must be performed when new columns are installed, reducing instrument down-time.

CHAPTER VI

SUMMARY

Conclusions

The method presented in this study is simple to perform, but requires some technical skill and interpretative ability. The capillary column is durable, easy to install and maintain, and it offers high resolving power with fast analysis time. Quantitative results and drug detection limits are within clinically acceptable ranges and interferences are minimal. The TLC method used in this study provides good confirmatory data though other TLC procedures, such as Toxi-Lab could be substituted.

Since conventional GCs can be readily converted to capillary operation, this method can be adapted to current hospital and clinical laboratory settings were toxicology is practiced.

Recommendations

Five more drug quantitations need to be added to the screen, namely meprobamate, glutethimide, acetaminophen, salicylates, and ethanol. Of these, acetaminophen is the only one which can be done using the proposed system. A very simple screen for serum salicylates can be used as an adjunct to the chromatography. By mixing 500 mcL of serum with 2 mL of Trinder's reagent,[48] salicylate levels of 10 mg/dL or higher can be detected by the appearance of the characteristic violet-purple color. Since levels above 40 mg/dL are

considered toxic in adults, this method eliminates the need to run a complete colorimetric quantitative analysis on every specimen. Only those which are positive need be done.

Meprobamate presents a special problem because of its very low NPD response. This drug could be readily detected and quantitated using this method if the NPD were replaced with an FID. This would, however, have a deleterious effect upon the detection limits of the other drugs and hydrocarbon (lipid) interferences would be more pronounced. Ethanol can be quantitated spectrophotometrically[49] or by using GLC. In either case, it requires a separate analysis and a specimen of whole blood for meaningful results. Glutethimide can be quantitated using HPLC or a different GC column. There are, however, other interesting concepts arising.

The idea of using two fused silica columns placed into the same injection port but going to separate detectors has been explored by Pandya.[50] He used two columns of different polarities (DB-5 and DB-1701 both from J & W Scientific) so that certain retention shifts were seen for compounds like methaqualone and methyprylon, which are not readily confirmed by TLC or EMIT®. On the DB-1701 column, glutethimide elutes in a position which is free of the interferences found on the DB-5 column. The two columns have compatible temperature limits though the DB-1701 tends to bleed somewhat above 240 celsius.

The critical part of the dual column approach is apparently related to the placement of the two columns in the injector. Commercially available two-holed ferrules were found to be unsatisfactory since they would leak upon tightening. A single holed graphite ferrule was bored out slightly to accommodate the two columns and no leaking

was observed. Some concern was expressed with respect to the nature of the splitting since the inlet ends of the two columns were not positioned in identical locations in the injector. Nevertheless, this approach is under investigation by myself and Dr. Pandya.

Another approach might involve the replacement of the TLC confirmatory method with HPLC. This would allow both peak identity confirmation and quantitative confirmation. However, the technical skill needed to properly operate and maintain an HPLC is considerably higher.

The DB-5 column might also be used in TDM since many anti-arrythmics, anticonvulsants, and antidepressants separate and elute rapidly from the column. Van Brunt[51] has already published an extensive paper dealing with the analysis of tricyclic antidepressants using the DB-5 column and a NPD. Capillary analyses may even extend into clinical chemistry where methods for catecholamines, hormones and various lipids would be welcome.

The potential uses of the new fused silica capillary columns are virtually limitless. As with HPLC in the 1970's, applications of capillary columns to various separation problems are likely to be the thrust of much research in the 1980's. This work is on the forefront of such research in an effort to more efficiently use time, manpower and other limited resources in drug related analyses.

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