GAS LIQUID CHROMATOGRAPHIC TECHNIQUE FOR THE QUANTITATIVE DETERMINATION OF "SOFT DRUGS" IN THE CLINICAL LABORATORY

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ABSTRACT

GAS LIQUID CHROMATOGRAPHIC TECHNIQUE FOR THE QUANTITATIVE DETERMINATION OF "SOFT DRUGS" IN THE CLINICAL LABORATORY

By

Mohinder Jerath

An extensive review of the structural, chemical, physiological and pharmacological properties relating to the analysis of barbiturates are discussed. Presently available qualitative (TLC and photometric) and quantitative (photometric and UV spectrophotometric) methods are discussed briefly to indicate their limitations and shortcomings. The need for a rapid and accurate quantitative procedure that can precisely separate and identify each barbiturate and the other commonly used "soft drugs" is presented.

After a brief discussion of the essentials of GLC pertaining to the techniques used in this study, three different GLC methods employing three separate columns packed with 3% SE-30 on 80/100 mesh Chromosorb G, 7% DC-200 on 80/100 mesh Gas Chrom Q and 1.5% OV-17 on 100/120 mesh Chromosorb G High Performance are presented. Attempts are made to quantitate barbiturates and other "soft drugs" by (1) extracting them into acidified chloroform at pHs between 6 and 7 and (2) methylating them to their corresponding derivatives. Utilizing these methods, unknown blood samples were successfully analyzed. Relative retention times and respective chromatograms are included.

Mohinder Jerath

The essentials of GLC/mass spectrometry including fragmentation, coding of spectrograms and identification utilizing a reference library are discussed. A 5 ft x 2 mm inside diameter U-shaped glass column packed with 3% OV-1 on 100/120 mesh Gas Chrom Q and a Finnigan GLC/MS coupled with a Finnigan computer were available for analyzing standard mixtures and some unknown blood samples. The drugs of interest were successfully analyzed. Fragmentation spectra of secobarbital, Hexetal, diphenylhydantoin and reconstructed chromatogram of commonly used barbiturates are listed.

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INTRODUCTION

Objectives

The purpose of this study was to review the literature and devise a rapid and accurate quantitative procedure for "soft drugs" (barbiturates, glutethimide, diphenylhydantoin, diazepan, chlordiazepoxide and quaalude) screening by gas liquid chromatography (GLC) that can be utilized in a hospital clinical laboratory at a reasonable cost.

Statement of the Problem

The current methods used in the clinical laboratories in most hospitals are relatively inadequate; they lack conformity, accuracy and reproducibility. Despite improvements of the colorimetric procedures, they are not sensitive enough. Reliable results are difficult to obtain and reproduce. Most of these procedures are nonspecific for individual barbiturates or barbiturates as a class of compounds. There are many sources of potential error, loss and contamination. Thin layer chromatography is reliable for identification with some accuracy. However, spotting with microliter quantities and then drying requires patience, since it is time consuming and tedious. Visual color identification of a large spectrum is often difficult. Fumigating and spraying are messy processes and one often loses the whole chromatogram by over-spraying or some other problems. Most solvents employed in the process for barbiturate separation and identification give inadequate separation of pentobarbital, secobarbital and amobarbital,

to name a few. This makes identification extremely difficult and, in most cases, interference masks the separation of other barbiturates. Attempts have been made for quantitation by thin layer chromatography but the process seems to be inaccurate.

Ultraviolet techniques have shown some promise but, as long as the compound is not known, analytical determination following or preceding identification is very difficult. The absorption curve only partially identifies the particular barbiturate. Hexobarbital, mephobarbital and pentothal do not show any change in absorption by the pH differential procedure. In other cases, interference by salicylates and metabolites of barbiturates may overshadow the actual compound and give false results.

Hypothesis

The facts regarding the physiological, pharmacological and chemical properties of barbiturates indicate the very complicated nature of these compounds. The onset of their action, absorption, distribution, and metabolism along with the duration of their action is dependent upon the basic group and type, which are discussed on pages 4 and 6. It is very important that their levels in blood and other body fluids be determined in terms of these basic types. In other words, a total concentration in mg% of barbiturates is of little value unless the compound is identified by name because 1 mg% of blood concentration of short-acting barbiturate will produce coma and 2 mg% of the same may produce death, while 4 to 8 mg% of a long-acting barbiturate in blood concentration may leave a patient conscious.

LITERATURE REVIEW

Use, Abuse and Related Problems

Barbiturate use is of significant occurrence and related in many of its characteristics to the use of alcohol. Approximately 10% of all drug prescriptions contain barbiturates. Production exceeds therapeutic needs by many tons each year. During the past few years drug abuse (including barbiturates) has increased alarmingly. With the use of LSD, marijuana and amphetamines as "uppers", barbiturates are frequently used as "downers".[1] With the availability of heroin on the decline, the hard drug addicts often use barbiturates intravenously as substitutes. Considerable abuse is frequently noted among housewives and young disappointed and depressed executive types.[2]

Realizing that the major medical use of these drugs is limited to sleeping pills, sedatives, hypnotics and anesthesia, one may reasonably presume that the major legal abuse is closely related to these functions. It is generally believed that one-third of all medication dispensed to patients in most hospitals includes barbiturates and other similar sedatives (Doriden, Dilantin, Librium, Valium and quaaludes). Among the non-hospital users, simultaneous ingestion of alcohol with these sedatives, hypnotics, tranquilizers or concurrent response to other toxic agents has been of the greatest concern to the medical profession. Under these conditions, an overdose may result in coma or abnormal behavior and the clinical picture is usually very complicated. The most up-to-date figures indicate that in our drug prone society,

acute barbiturate and related drug poisoning has steadily increased over the past few years. Reported hospital emergency room admissions indicate that over 70% of all overdoses in this country involve barbiturate and related drugs. This percentage closely correlates with the estimation in some of the Western European countries.[3]

The Structure and Naming of Barbiturates

Barbiturates are derivatives of barbituric acid, the structure of which is best understood as "malonyl urea" as shown in Figure 1. To date, approximately 2000 barbituric acid derivatives are known; about 50 of these are currently in commercial use. They are conveniently considered in three groups:

1. Those in which the 2 hydrogens at C_5 are substituted (-5,5-disubstituted barbiturates).

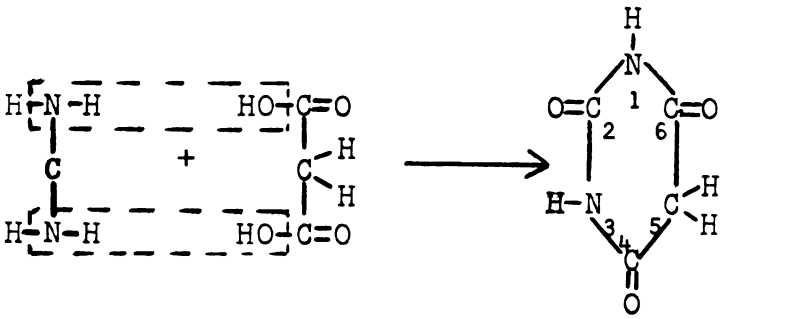
2. Those in which the oxygen on C_2 is replaced by sulfur-linked groups (the thio-barbiturates).

3. Those in which the hydrogens at N_1 and N_2 are substituted along with the hydrogen on C_5 (-1,5,5-trisubstituted derivatives).

However, the -5,5-disubstituted derivatives are by far the most commonly encountered. The substitutes may be cyclic, acyclic, saturated, unsaturated or halogenated among other arrangements. Generally the potency of barbiturates is proportional to the length of the alkyl side chain. Compounds with branching side chains often have anticonvulsant properties.

Physiological, Pharmacological Properties and Medical Importance

Barbituric acid itself has no pharmacological activity. However, for physiological purposes, some 50 or so commercially manufactured



Urea Malonic acid Barbituric acid

Fig. 1 Formation of barbituric acid from urea and malonic acid.

derivatives are classified according to the duration of their action. Four basic types are:

1. Long acting. These usually belong to the group of -5,5-disubstituted derivatives. They exert hypnotic effects for more than 6 hours and are useful as sedatives and anti-epileptics.[4] Examples of this class are barbital (Veronal) and phenobarbital (Luminal).

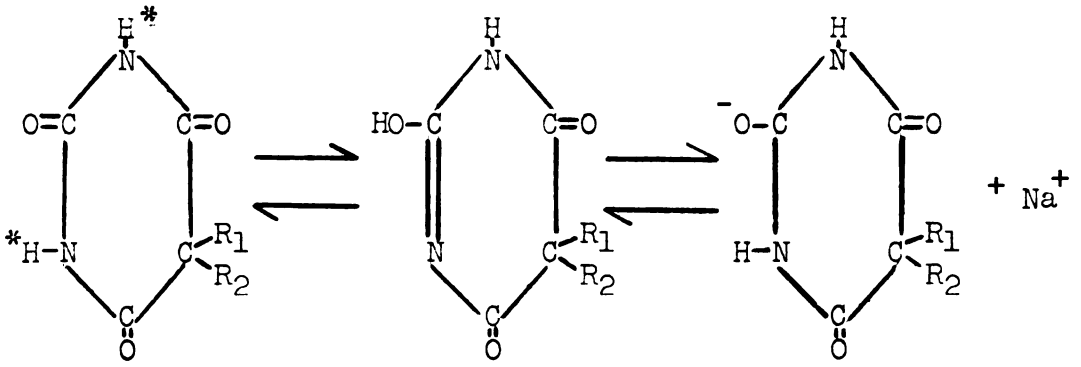
2. Medium or intermediate acting. These produce drowsiness and hangovers. The duration of action is from 3 to 6 hours. Examples are amobarbital (Amytal), butabarbital (Butisol) and pentobarbital (Nembutal).

3. Short acting. They usually act within an hour and are used as sleeping pills and in anesthesia. An example is seconal (secobarbital and Quinal).

4. Ultra short acting. These usually belong to the group in which the oxygen on C_2 is replaced by a sulfur-linked group. The sulfhydro group substitutes render the compound highly lipid-soluble, thus increasing their transport across membranes. Examples are hexobarbital (Evipal), pentothal (Thiopental) and surital (Thioamytal).[4]

Chemical Properties

While barbituric acid itself is a fairly strong acid, the derivatives (barbiturates) are weak acids or salts of weak acids with a pK of about 7.5 to 8.6. Depending on the nature of the substituents and the pH of the solution, barbiturates exist in equilibrium mixture as keto-enol or lactum-lactim tautomers as shown in Figure 2.[5] The keto and enol forms are important for understanding the solubility characteristics, absorption, distribution and excretion of these compounds.



Keto Enol Enol salt

At acid or neutral pH At alkaline pH

* One or both of these hydrogens may be partially ionized in the enol form.

Figure 2. Equilibrium mixtures in which barbiturates exist; R_1 and R_2 are the substitutes.

* One or both of these hydrogens may be partially ionized in the enol form.

Absorption

Most barbiturates are absorbed as nonionized salts and move by passive diffusion across membranes. However, in the case of thiobarbiturates, the sulfhydro group substituents render the compounds highly lipid-soluble thus increasing their transport across membranes. This results in a rapid onset of activity and short duration of action.[6] Barbiturate absorption as nonionized salts follows the scheme illustrated in Figure 3. The nonionized forms are highly lipid-soluble and hence have no impenetrable barrier to their diffusion in the body. The absorption into plasma from the gastrointestinal tract after oral administration is mostly dependent upon the chemical nature of the compound. The short-acting barbiturates are rapidly absorbed while the absorption of the long-acting barbiturates is much slower. A dilute solution of thiopental may be absorbed from the gastrointestinal tract at a much more rapid rate than ethanol.[7]

Distribution

The clinically useful barbiturates are all bound to albumin in varying degrees and transported in the body as a complex held together by Van der Waal's forces of physical attraction acting on their surfaces. The tissue proteins also bind barbiturates to about the same extent as the plasma protein and this contributes to the accumulation of drugs in the tissues throughout the body. Hence, the two factors in the barbiturate pharmacokinetics are the lipid solubility of the drugs and the concentration of the unbound and undissociated molecules at the site being examined.

Like alcohol, barbiturate absorption observes the zero order of kinetics, i.e., the rate of absorption is independent of the

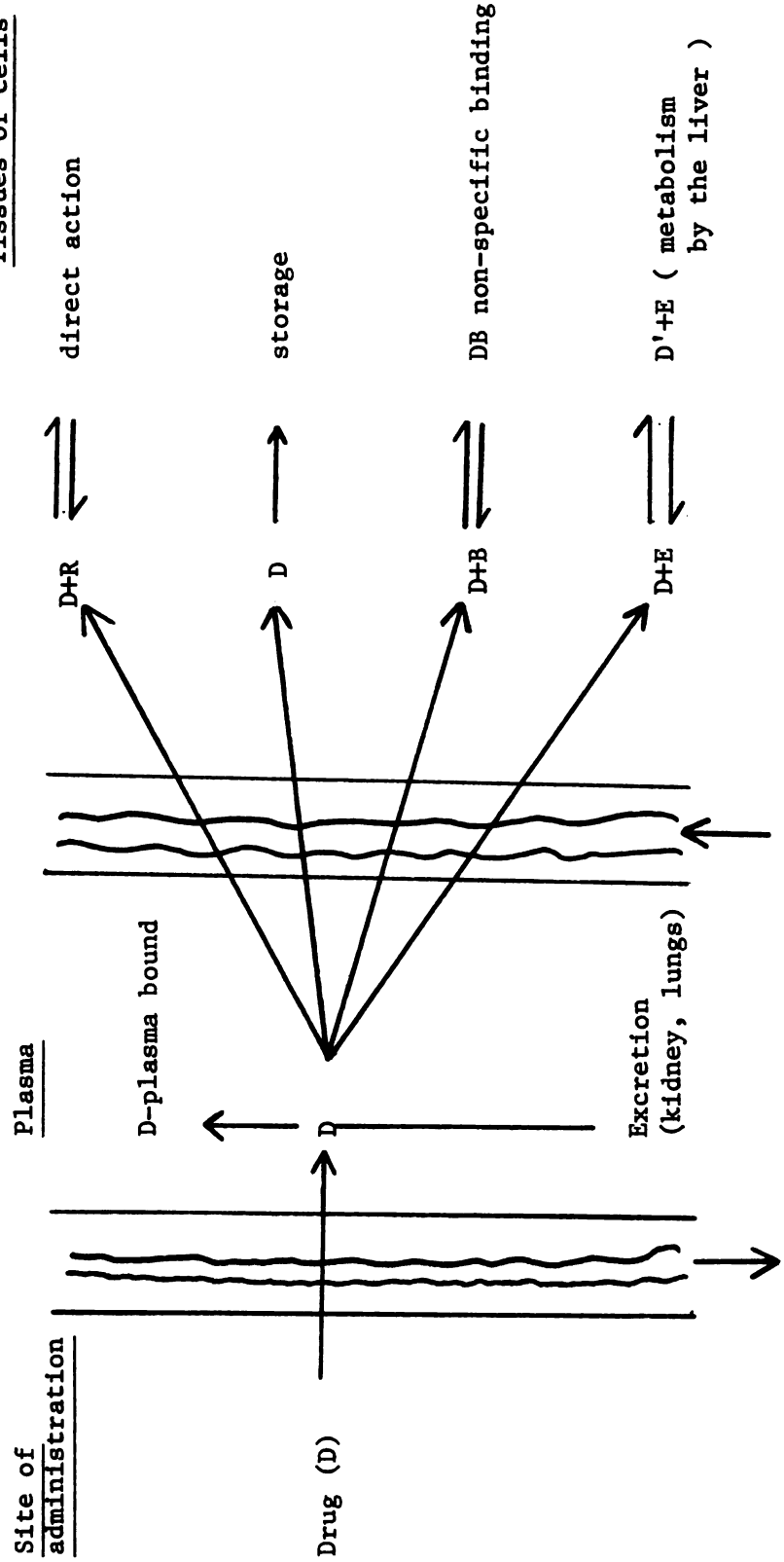


Figure 3. Illustrative scheme of drug absorption, distribution, metabolism and excretion.

concentration. The distribution of barbiturates (thiopental and phenobarbital) in various tissues of dog and mean distribution ratios as studied by Brodie *et al.*[8] are depicted in Table 1. For this distribution, lipid solubility (partition coefficient) is the dominant factor. However, protein binding and the extent of ionization are also important factors. In the undissociated forms, barbiturates have a rather high affinity for nonpolar solvents and there is a definite correlation with their pharmacological properties. Thus, highly lipid-soluble thiobarbiturates are short acting agents with a rapid onset of action.[7]

Metabolism and Excretion

Barbiturate metabolism is mediated by microsomal enzymes in the endoplasmic reticulum liver cells. Impaired hepatic function is an important critical factor in the duration of pharmacological activity. Duration of action appears to be increased in proportion to hepatic dysfunction. Normally, most barbiturates are metabolized organically; however, in some cases the initial pH of the compound may be altered in the renal system. This usually renders the barbiturates highly ionized. In this form, they are not reabsorbed by the renal tubules but excreted in the urine.

During oxidative metabolism in the liver, most barbiturates are transformed into both inactive and some active metabolites. A great deal of research has been devoted to the identification of barbiturate metabolites, but so far it has been almost impossible to account for all the end-products of any single barbiturate. The liver has primarily two roles in terminating the central depressant action of barbiturates. First, it transforms lipid-soluble agents into polar derivatives that can be excreted by the kidney. The second function

Table 1. Barbiturates in tissues (dog). Data from Brodie *et al.* (1950; 1953). The studies were made 2-1/2 hr (dog A), 3-1/2 hr (dog B) and 3 hr (dog C) after intravenous administration of barbiturates.

	THIOPENTAL		PENTOBARBITAL
Dog	A	B	C
Weight	8.4 kg	14.0 kg	10.7 kg
Dose	0.65 gm	0.65 gm	0.43 gm
Tissue	**Concentration**		**Concentration**
	mg/l	mg/l	mg/l
Plasma	14.7	19.0	34.4
Plasma water	3.4	4.8	18.7
Cerebrospinal fluid	2.9	---	18.2
	mg/kg	mg/kg	mg/kg
Red blood cells	---	---	36.0
Liver	27.8	---	64.4
Brain	23.9	---	42.3
Heart	18.4	---	38.4
Lung	13.6	---	20.8
Kidney	17.6	---	45.8
Spleen	13.0	---	41.4
Muscle	22.1	---	27.5
Lumbodorsal fat	---	119	37.3
Omental fat	---	192	---
Perirenal fat	---	222	---

is detoxification. However, not all barbiruates lose their pharmacological activity. To summarize, the barbiturate biotransformation follows the following pathways:[9]

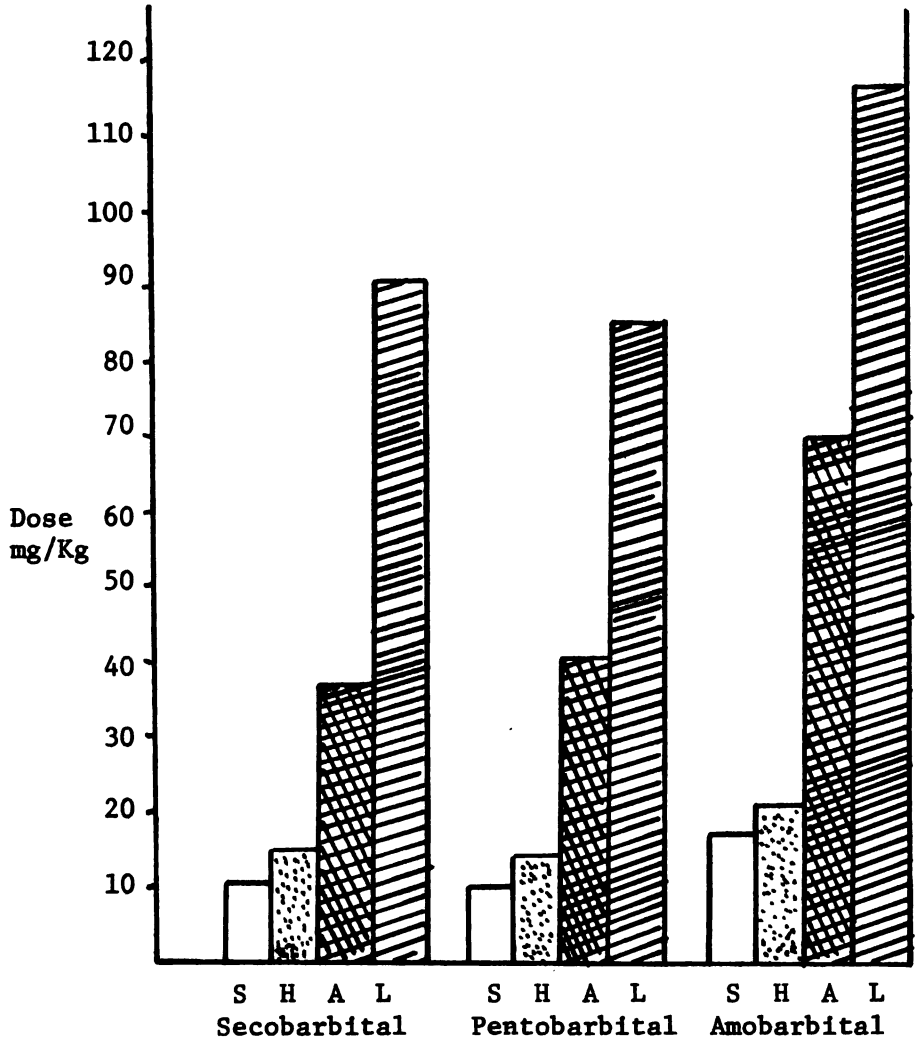
> "[1] Carbon 5 substituents may undergo oxygenation, oxidation or complete removal. The drugs are oxidized at the larger of the two 5 substituent groups, and the products are relatively polar alcohols, ketones, phenols, or carboxylic acids, which may appear in the urine both as free compounds or as conjugates of glucuronic acid. [2] Nitrogen 1 and 5 alkyl groups may be removed, unsubstituted nitrogen atoms may be methylated. This is essentially a N-dealkylation reaction by which N-alkyl barbiturates are converted to simpler but active metabolites. [3] The sulfhydro groups may be replaced by oxygen, converting thio-barbiturates to the corresponding oxy-barbiturates. [4] Scission of the barbiturate ring may occur at 1:6 bond to give substituted malonyl ureas."

Action

Barbiturates inhibit oxygen consumption of brain tissue. Depressant effects are produced by interference with respiratory enzymes. Liver cell function is affected by inhibition of an early step in the respiratory chain involving coenzyme I and flavoprotein. They also act preferentially on the synapse and inhibit ganglionic transmission in which total oxygen consumption is unaffected.[7] The degree of this depression (from mild sedation to complete anesthesia) is dose dependent and is illustrated in Figure 4, as reported by Brodie *et al.* During anesthesia, spinal cord reflexes which mediate muscular tone and the medullary center which controls blood pressure and respiration become depressed. In addition large doses depress the respiratory center and diminish responsiveness to carbon dioxide.[10]

Other Problems

As mentioned earlier, the simultaneous ingestion of alcohol with these sedatives, hypnotics and tranquilizers or concurrent response to



Comparative activities of three barbiturates with regard to sedative (S), hypnotic (H), anesthetic (A) and lethal (L) doses in dogs.

Figure 4. Dosage effects of barbiturates.

other toxic agents is of great concern. From physiological and pharmacological aspects a synergistic effect results. This normally confuses clinical as well as laboratory diagnosis and results in complex problems.

Important Chemical and Physical Properties Related to Chemical Analysis

The keto and enol forms (Figure 2) are important to the understanding of solubility characteristics and UV absorption patterns of barbiturates. Like most organic acids of medium or high molecular weight, barbituric acid derivatives are soluble in alcohol and organic solvents but insoluble in water. However, the salts of these derivatives, which are obtained by adding sodium hydroxide or sodium carbonate until the solution is distinctly alkaline, are soluble in water and insoluble in organic solvents. These properties are extremely important in the analysis by extraction procedures.

Ideally, these derivatives are easily extractable with chloroform or ether from an acidified solution, since the addition of hydrochloric acid suppresses the ionized water soluble form. In practice, however, extraction with chloroform is often made from blood at its normal pH (7.4). This has been found to be most satisfactory because the extraction is nearly complete yet other weak acids which may be coextracted from acidified solutions are left behind. Barbiturates cannot be extracted with organic solvents from strongly alkaline solutions. The keto structure in its acidic or neutral form does not absorb ultraviolet light. For this reason, UV measurements must be run in alkaline solutions and localization of barbiturates on thin layer chromatograms by ultraviolet light can be made usually only after exposure to NH_3, Na_2CO_3, or NaOH.

It should also be noted that hydrolysis of the ring structure in alkaline solutions varies with the nature of the substituents and has been made the basis for one method of barbiturate identification. Throughout determination, barbiturates should not be left in strong alkaline solutions for any prolonged time because they are very unstable under these conditions.[4]

MATERIALS, METHODS AND PRINCIPLES

Principles of Methods Currently Used in Clinical Hospital Laboratories

It should be noted that tablets or powder specimens for forensic purposes are usually submitted in sufficient quantities to prepare chemical derivatives which may then be identified by melting point or microscopic examination. Analytical quantities in biological specimens are, however, limited.

Qualitative analysis

1. The Koppany (cobalt acetate) test, or Parri's (cobalt nitrate) reaction. Cobalt acetate or nitrate in a suitable solvent will result in a purple or pink color in "presence" of barbiturates or other related compounds.

2. Zwikker's test employs the principle that copper pyridine in the presence of barbiturates and similar compounds forms violet complexes.

3. Millon's test. Mercury salts form insoluble white precipitates with all 5,5-disubstituted barbiturates but not with 1,5,5-trisubstituted barbiturates.* On the addition of diphenylcarbazone to the precipitates, a blue color results, the intensity of which follows the Beer's law. This method is also employed in thin layer chromatography because

*This is only a postulation by Millon; there is no evidence that all 5-5-disubstituted barbiturates adhere to this rule.

diphenylcarbazone spray yields pinkish-blue spots in the presence of barbiturates.

4. Potassium permanganate is decolorized by barbiturates with unsaturated substituents and by fully saturated thiobarbiturates. Phenyl substituents behave as saturated substitutes in this methodology.

5. Ultraviolet light and fluorescein may be used in a thin layer technique. Fluorescein serves as a suitable background for the ultraviolet examination of thin layer chromatograms. Barbiturates present in their salt forms, i.e., their enolized structure, will give purple spots against a yellow-green background of the fluorescein. On thin layer, barbiturates usually separate in the following order: (a) long acting--nearest to the point of application, (b) intermediate acting, (c) short acting, and (d) ultra short acting--farthest away from the point of application.

Acetic acid and hydrogen peroxide sprays serve to identify bromo barbiruates on fluorescein sheet by distinct pink spots. On the other hand, saturated barbital, barbitone, barbituric acid and sodium barbital will yield purple spots with diphenylcarbazone.

Quantitative analysis. Colorimetric, ultraviolet spectrophotometric and occasional fluorometric methods are most commonly used in clinical situations. Gas chromatographic and infrared methods are available, but their use at present is limited to forensic laboratories. In addition, infrared techniques require a relatively large and preferably pure specimen which is difficult to obtain from biological sources.

For colorimetric determinations, barbiturates are extracted into acidified chloroform, precipitated with mercuric ions, reacted with dithiazone or diphenylcarbazone and the resulting color measured photometrically.[11,12]

For UV spectrophotometric analysis, barbiturates are extracted into acidified chloroform at a pH 7.0 to 7.4. The chloroform extract containing the barbiturates is re-extracted with 0.45N NaOH which removes the barbiturates into the aqueous alkali solution (pH 13). A UV absorption curve from 320 mμ to 220 mμ is obtained. The pH of the solution is then adjusted to 10.2 with an amine buffer (usually ammonium chloride) and again a UV curve obtained. The second curve is superimposed on the first by rolling the chart back and starting from the original spot. To adjust for concentrations, an equal volume of 0.45N NaOH (i.e., the volume of amine buffer used) is added to the first cuvette for the reading at pH 13. The amount of barbiturate present is calculated by dividing the absorbance difference of the two curves at 260 mμ by the difference obtained from a standard solution treated under similar conditions and taking into account the sample size and dilution factors.[13] Two typical barbiturate curves at pH 13.0 and 10.2 obtained from a recent analysis are plotted in Figure 5.*

Ultraviolet absorbance curves of different barbiturates. The rationale of the UV procedure can be understood by inspecting the UV absorbance curves at different pH's as depicted in Figures 6a, 6b and 6c. It can be seen that disubstituted derivatives have different absorption curves at different pH's. Absorbance in acid is low because carbon #2 is present in the carbonyl structure; the absorbance in alkali is due to the enolized structures (which can be represented by different resonance structures). A peak is observed at 240 mμ at pH 10.0. The peak shifts to a higher wave length, about 255, at pH 13. The great

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*The procedure is modified after Goldbaum *et al.*

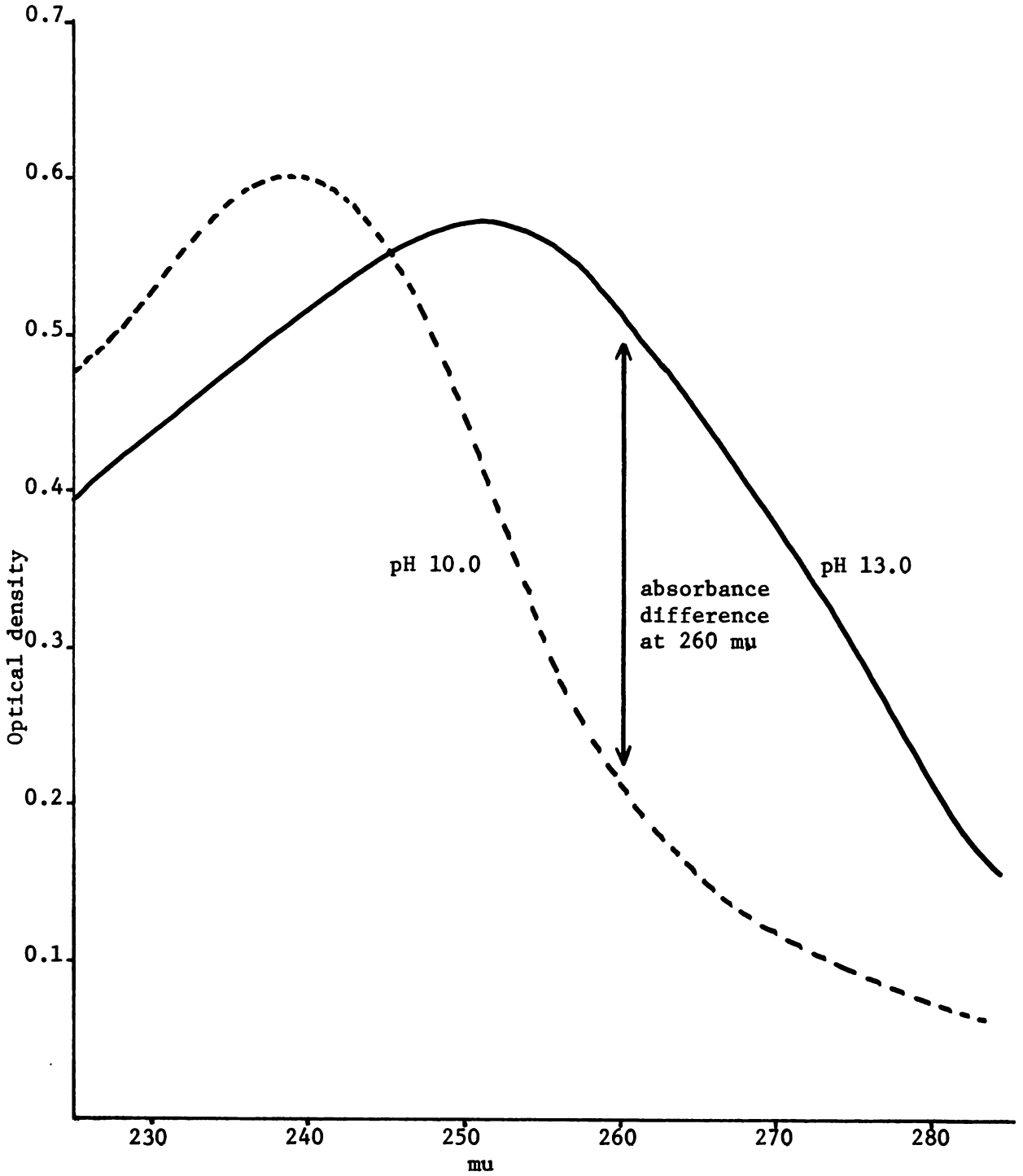

Figure 5. A typical barbiturate differential UV scan.

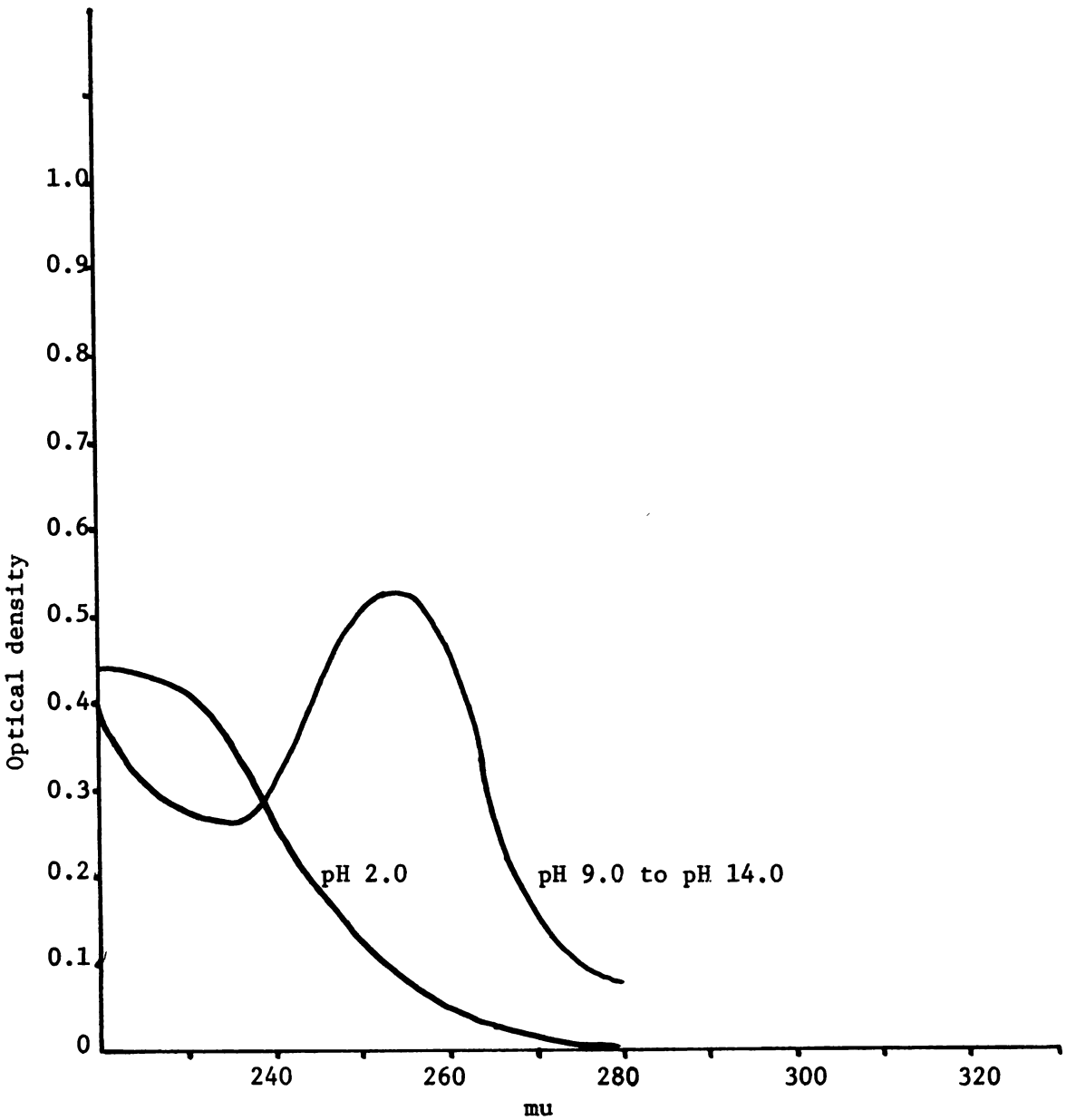

Figure 6a. UV absorbance differential curves of 1,5,5-trisubstituted barbiturates.

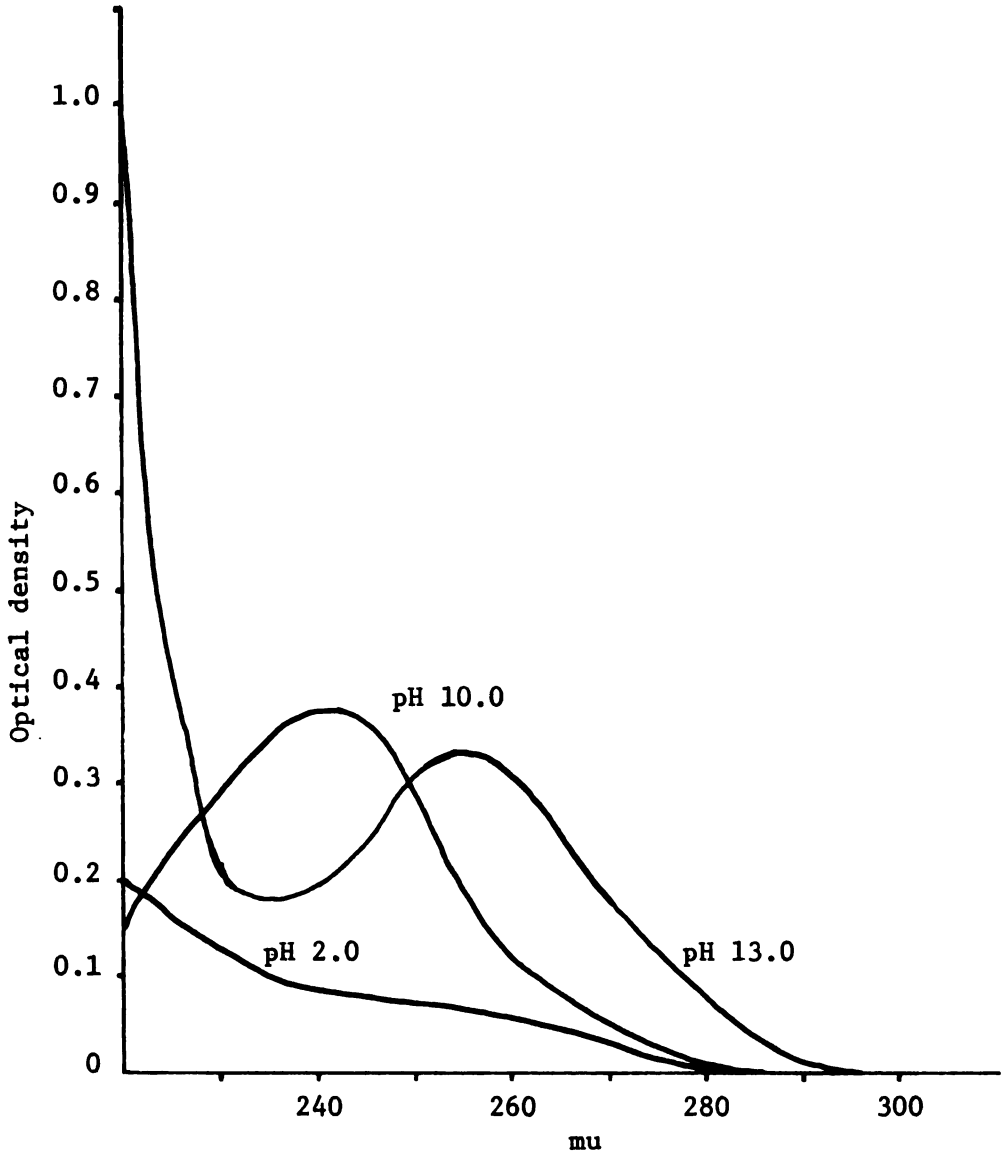

Figure 6b. UV absorbance differential curves of 5,5-disubstituted barbiturate.

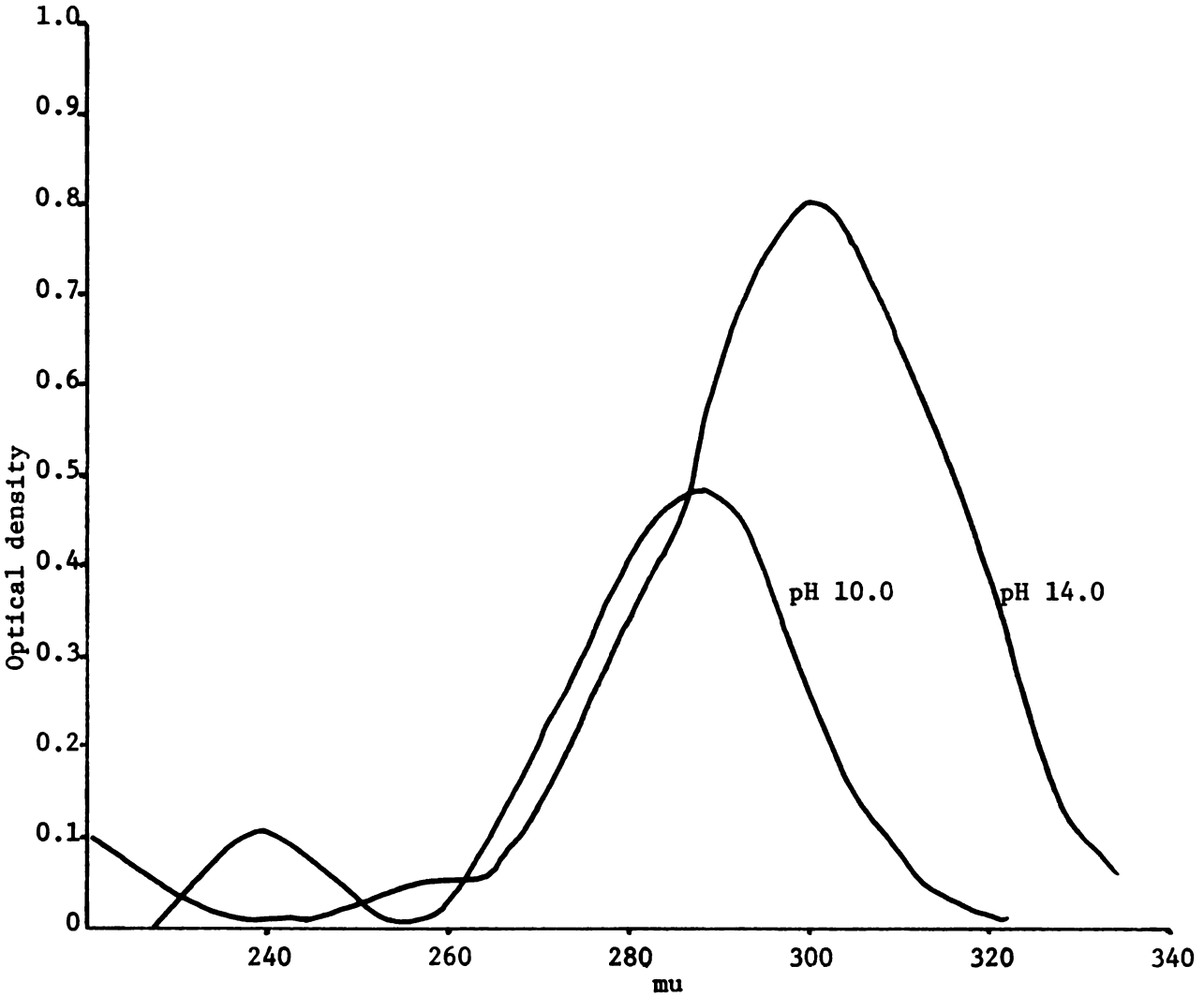

Figure 6c. UV absorbance differential curves of thiobarbiturates.

absorbance at 230 mμ at pH 13.0 is due to the alkali itself. These observations would suggest that determinations be made at the same wave length of maximum absorbance at a given pH. On plotting the concentrations of any one barbiturate, it is found that Beer's law is followed; however, each barbiturate has a different absorbance. Thus determination of absorbance would be meaningless unless one knows which barbiturate is present. In addition, impurities (interfering compounds and metabolites) would also be a source of difficulty in this type of analysis. However, it appears that the differences in absorbance at 260 mμ between pH 10 and pH 13 are nearly identical for most barbiturates. In other words, the decrease in absorbance at these two pH's is nearly identical for different barbiturates and dependent only on the concentration. However, it should be noted that at lower pH (i.e., <7.4), impurities such as sulfonamides, dilantin, dicoumarol and metabolites are usually extracted along with barbiturates. Decomposition products of chloroform absorb at 239 mμ. It is my personal experience that at pH 7.4 only about 90% of most barbiturates are extracted.

Limitations of the UV methods. Since absorbance difference at 260 mμ is not exactly the same for all barbiturates, this method is only semi-quantitative. Therefore, the level of concentration is only meaningful when the particular barbiturate is known before analysis. In some cases interfering substances and metabolites may affect the results. On the other hand, it is definitely known that Evipal, Meboral and Pentothal do not show any decrease in absorption due to pH change.[14]

With the advances in the field of gas liquid chromatography (GLC), toxicology in general has acquired some long needed improvements. During the past several years, many methods for separation, identification and

quantitative analysis of many drugs have been reported. However, for medical purposes they have been impractical, cumbersome and costly. Research and forensic science laboratories have some good procedures available for analysis of most drugs in pill and powder form. However, their applications for quantitation of widely used "hospital type drugs" in body fluids are in some respects inadequate. On the other hand, their application in routine clinical laboratories is difficult because of instrument cost and complicated operations.

Essentials of Gas Liquid Chromatography

Gas liquid chromatography (GLC) is a technique in which a carrier gas is passed over an immobile liquid phase coated on an inert support on the walls of a capillary tube. The basis for separation is the partitioning of the sample components between the gas and liquid phases.

Although the details of operation and the whole process of GLC are beyond the scope of this manuscript, it should be noted that in any quantitative analysis by this complicated technique, optimum conditions are necessary. During GLC analysis, sensitivity, resolution and quantitation are improved by the proper choice of the instrument.

Column. The column is the single most important part of the system. A good understanding of the process taking place in the column is essential in order to obtain maximum performance from the instrument. The rest of the instrument enables the column to perform its separation and detect the results. The column is composed of the tubing or the container, the solid support and the stationary phase. The column material (glass, etc.) does not enter into the chromatographic process except in an adverse way. The adverse process usually involves adsorption on the column or chemical interaction between the column material and the

sample to be analyzed. For this reason, glass columns are preferred over metal.

Solid support. The function of the solid support is to act as an inert platform for the liquid phase in the column. As such, the solid support must distribute the liquid phase in a thin film. The solid support is usually diatomaceous earth which has been flux calcined. It is then screened using standard wire mesh screens to provide a narrow range of particle size. The size of the particle is expressed in terms of the screens, i.e., 80/100 mesh means particles that will pass through an 80 mesh screen but be retained on a 100 mesh screen. The solid support is often acid-washed to remove trace metals and in many cases is silanized to reduce its activity. The activity being reduced by silanization is the interaction of the solid support with the sample causing peak tailing and distortion. For optimum conditions, the solid support should have a large surface area and a pore structure with uniform pore diameter in the range of 10 μ or less. It should be mechanically strong and inert, i.e., it should have a minimum of chemical and adsorptive interaction with the sample. For the purposes of my study, Chrom G and Q,* two very hard and polar solid supports, were the best available.

Liquid phase. The liquid phase is the active portion of the column; the separation process takes place between the carrier gas and the liquid phase. This process may be visualized as a series of partitions where the sample goes into solution in the liquid phase and is subsequently revaporized. The affinity of the sample for the liquid phase

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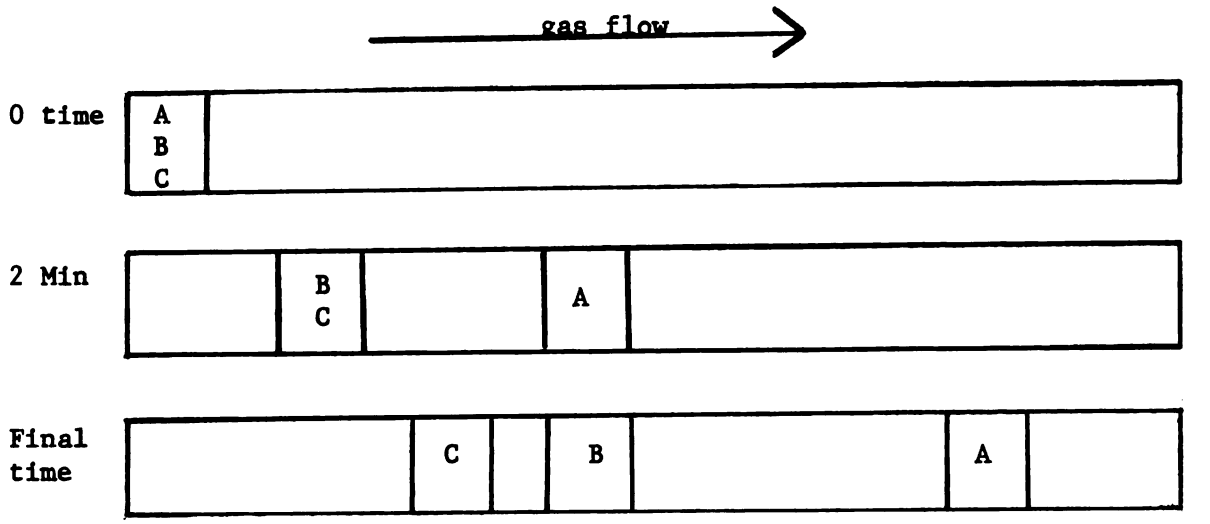
*Varian Aerograph, Walnut Creek, Calif.

determines the length of time the individual sample components will remain in the solution. Those compounds with the least affinity for the liquid phase emerge first and those with the greatest affinity emerge last.

The various components in the sample separate into discrete bands and travel at different velocities down the column. This is exemplified in Figure 7.

Liquid phase requirements. The right selection is based mainly on experience, trial and error. However, it must be a good absolute solvent for the sample components. If solubility is low, components elute rapidly, and separation is poor. It must also be a good differential solvent for the sample components, nonvolatile, thermally stable (instability may be promoted by catalytic influence of the support as the temperature increases) and chemically inert towards the solutes of interest at the column temperatures. If the components of the mixture to be separated are of different chemical classes, but close in boiling points, liquid phase of different polarity must be used. By varying the polarity of the solvent, interaction forces may be brought into play to effect a separation.

Percentage of liquid phase. Most recent literature indicates that from 0.3% to 30% concentration of liquid phase has been used successfully in various GLC analyses. However, as a general rule, high concentrations lead to high capacity and longer retention times while low concentrations lead to low capacity and short retention times.[15] A reduction in liquid phase may not only reduce retention time but also produce a reduction of resolution. In some cases it may result in an interaction between the solid support and sample, thus leading to "tailing" peaks.



Three molecules A,B and C with corresponding affinities for the liquid phase.

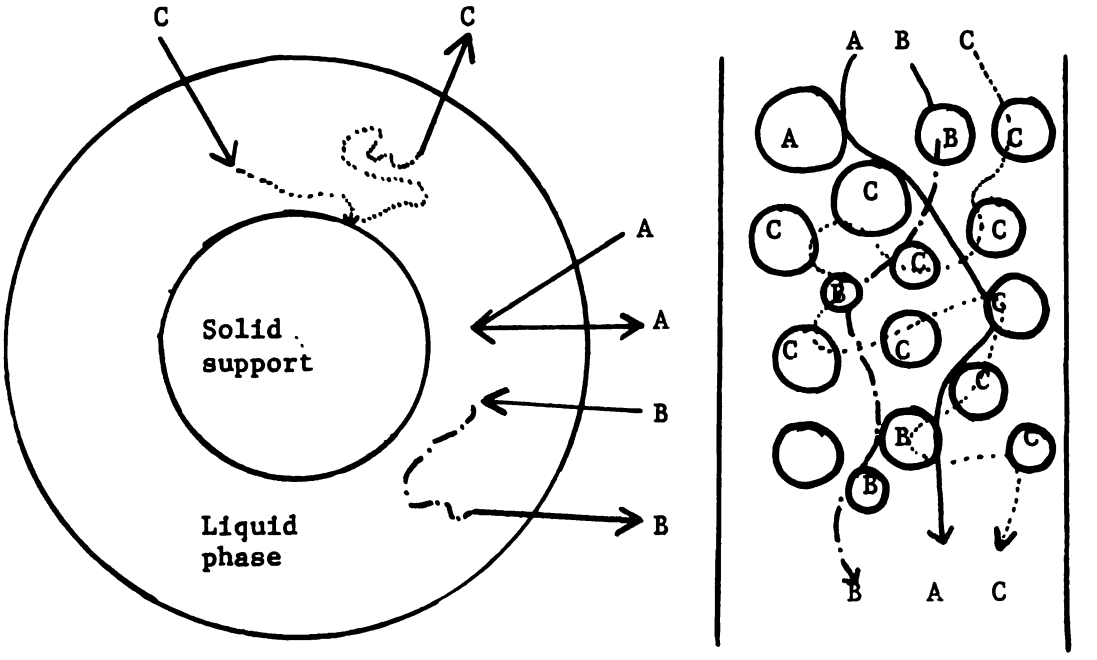


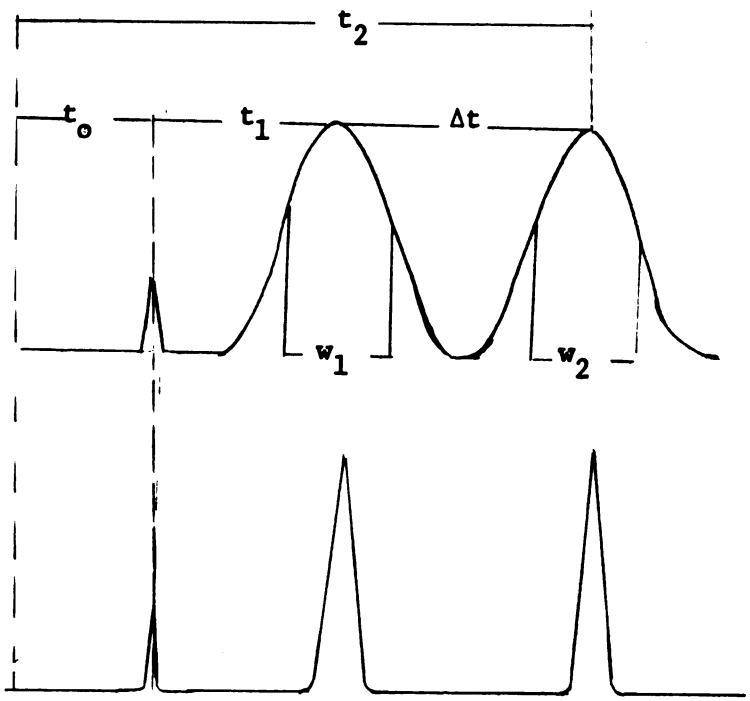
Figure 7. Sample bands separating on a column characterized by the affinity for the liquid phase.

Chromatographic Parameters

The resolution of the chromatographic peaks is related to two factors: column efficiency and solvent efficiency.

Column efficiency. Efficient columns keep peaks narrow. The chromatogram below with narrow peaks thus offers the best resolution (R) (see the illustration below).

$$R = \frac{2[t_2 - t_1]}{W_1 + W_2} = \frac{\Delta t}{W_2}$$



Column efficiency is concerned with the peak broadening of the initially compact band as it passes through the column. The broadening results both from the column design and operating conditions and can be quantitatively described by the height equivalent to a theoretical plate (HETP). The HETP is that length of column necessary for the

attainment of solute equilibrium between the moving gas phase and the stationary liquid phase.

Theoretical plates can easily be measured from the chromatogram. Tangents are drawn to the peak at the points of inflection as shown in the illustration. The number of the theoretical plates, N, is given by $16\ [\frac{t_1}{W_1}]^2$, where "t_1" is the distance from the point of injection to peak maximum and "W_1" is the length of the baseline cut by the two tangents.

Factors influencing column efficiency are: solvent, solute, temperature, flow rate of the carrier gas and sample size. Most of these factors are evaluated by their effect on N, or the height equivalent to a theoretical plate, HETP. This is related to N by:

$\text{HETP} = \frac{L}{N}$, where L is the length of the chromatographic column.

HETP calculation allows comparison between columns of different lengths and is the preferred measure of column efficiency. However, the number N is not a direct measure of the difference in separating power of a column but is useful in comparing similar columns and their packing needs.

Band broadening is a result of (A) multipath effect, (B) molecular diffusion and (C) resistance to mass transfer of gas and liquid. It has been found that $\text{HETP} = (A) + (B)/U + (C)\cdot U$, where U is the linear gas velocity (or flow rate) through the chromatographic column.[16]

$$U = \frac{\text{length of the column (cm)}}{\text{retention time of air (sec)}}$$

If HETP is plotted against U, a hyperbola with a minimum HETP is obtained. This minimum is the flow rate (U optimum) at which the column

is operating most efficiently. However, owing to the compressibility of the carrier gas, U is not constant over the entire length of the column, hence only a small section can operate at maximum efficiency. In other words, shorter columns under optimum conditions are more efficient.

Solvent efficiency. The liquid phase is one of the most interesting aspects of gas chromatography. It introduces a high degree of versatility to the method. Substances having the same vapor pressure can easily be separated by appropriate selection of liquid phases. In selecting a solvent, the following forces are very important:[17]

(a) Orientation forces resulting from the interaction between two permanent dipoles, e.g., "hydrogen bonds."

(b) Induced dipole forces resulting from interaction between a permanent dipole in one molecule and an induced dipole in a neighboring molecule.

(c) Dispersion of non-polar forces. These forces are present in all cases and are the only source of attraction energy between two non-polar substances.

(d) Specific interaction forces, i.e., chemical bonding, complex formation between solute and solvents.

These forces of interaction determine the separation achieved. Their combined effects are expressed by the partition coefficient K.

$$K = \frac{\text{amount of solute per unit volume of liquid phase}}{\text{amount of solute per unit volume of gas phase}}$$

The value of K is high when most of a substance is retained in the liquid phase. This means that the substance moves slowly down the column because only a small fraction will be in the carrier gas.

Transport is negligible in the liquid phase and only the fraction in the gas phase is carried through the column. Thus, separation between two compounds is possible if their partition coefficients are dissimilar. The greater the difference in their K values, the fewer plates or shorter column length is required for separation. Also, by altering the liquid phase, it is possible to alter its interaction with the components being separated. By this technique, change in the relative volatility is effected, making difficult separations very easy.

The separation as given by the equation below is a property of the specific liquid phase and is referred to as the selectivity. It is the ratio of the partition coefficients K_1 and K_2, respectively, of the two compounds in the liquid phase at the column temperature. If α is small for two compounds, it may not be possible to effect a separation even with very efficient columns; a change of liquid phase is required. The selectivity (α) is temperature dependent and lowering the temperature often improves separation.

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0} = \frac{K_2}{K_1}$$

Adjustments of peak retention. The distribution coefficient decreases with increasing temperature. However, peak retention can be adjusted by adjusting the temperature, carrier gas flow rate, column length, column inside diameter, percentage (quantity) of the liquid phase and the type of the liquid phase.

Temperature. Peak migration as a function of temperature can be approximated by a general rule that an increase of 30°C will decrease the retention time by one-half. A decrease of 30°C will double the retention time.[18] Resolution is generally improved by lowering the

temperature; however, the efficiency of the column is generally unaffected.

Temperature programming. For best analysis, column compartment or oven temperature control of ± 1°C is preferred over others. The controlled change of column temperature or what is otherwise called temperature programming during an analysis is used to improve, simplify or accelerate the separation, identification and determination of sample components. Isothermal operations limit gas chromatographic analysis to a narrow boiling sample. At constant temperature the early peaks, representing low boiling components, emerge so rapidly that sharp overlapping peaks result while higher boiling materials emerge as flat, immeasurable peaks. In some cases high boiling components are not eluted and may appear in later analyses as baseline noise or "ghost" peaks which cannot be explained.

With temperature programming, a lower initial temperature is used and early peaks are well resolved. As the temperature increases, each higher boiling component is "pushed" out by the rising temperature. High boiling compounds are eluted earlier as sharp peaks, similar in shape to the early peaks. Trace compounds emerge as sharp peaks which can be distinguished more easily from the base line. Total analysis time is much shorter. However, temperature programming instruments are more expensive because they need separate heaters for injection port, column compartment and the detector. In addition, a suitable temperature resistant liquid phase, pure, dry, inert carrier gas and a differential flow controller for carrier gas makes operation more complicated.

Flow rate. The behavior of peak migration as a function of flow rate can be approximated by the rule that doubling or halving the

flow rate will halve or double the retention time.[19] Studies indicate that, between 20 and 40 ml/min of carrier gas flow, a 1/8" column has at least 600 plates/foot or a HETP of less than 0.5 mm. Beyond these flow limits, the column efficiency is seriously affected with a resulting loss of resolution. This then becomes the limiting factor in flow rate changes. The peak retention could be halved by increasing the flow rate from 20 to 40 ml/min. Any further reduction in retention time would have to be accomplished by other means.

Column length. The behavior of peak migration as a function of column length follows the rule that if column length is halved or doubled, the retention time is halved or doubled.[15] However, changing the column length is the least desirable variable. For simplicity, it may be added here that the inside column diameter is another factor that will influence both the flow rate and the retention time. Capillary columns in general take a longer time; 1/2" columns are too fast, while 2-4 mm columns give better separation and resolution.

Detector System

In GLC analysis, the detector is the second most important factor in the systematic operations of this complicated but precise technique. It is desirable that the detector system respond within practical limits of sensitivity and the required limits of detection. It must be stable and show repeatability. Its need for frequent calibration must be small and show a long term stability with negligible base-line drift. With respect to its versatility, it must be operable over a wide range of compounds and have a reasonable linear response over a practical range of concentrations. In addition, its material of construction should be

chemically inert and it should be able to withstand normal laboratory vibrations.

After having utilized several detectors, it was found that, for the purpose of this study, the flame ionization detector proved best. It is rugged, has a high sensitivity response with a minimum detection limit of 1-10 x 10^{-12} gm/sec. It is highly selective, stable and sensitive to organic compounds. It has a linearity of 10^6 to 10^7 (the widest of commonly used detectors). Nitrogen and helium are good carrier gases for it. It can withstand extreme temperature changes with a maximum temperature limit of 400°C. In addition, it is simple, and insensitive to fixed gases and water. The disadvantages are (1) that it requires an amplifier and (2) that it is destructive to components eluted off the column. Percentage recovery of compounds cannot be determined because of the destruction.

In general, ionization detectors operate on the principle that the electrical conductivity of a gas is directly proportional to the concentration of charged particles within the gas. In case of flame ionization detectors (FID), organic compounds yield ions when burned in a flame (hydrogen and oxygen with air preferred). If two electrodes with a potential of 200 to 350 volts are inserted in the flame, the conductivity of the flame can be measured as the components emerge from the chromatographic column.[20] The diagrammatic illustration of a flame ionization detector is depicted in Figure 8. It should be noted that the background current is reduced to zero by opposing it with a "bucking voltage." Therefore, with only carrier gas in the electrode gap, the recorder draws a baseline.

Response. The FID responds to all organic compounds except for single carbon molecules containing a carbonyl, such as CO, CO_2, and fixed

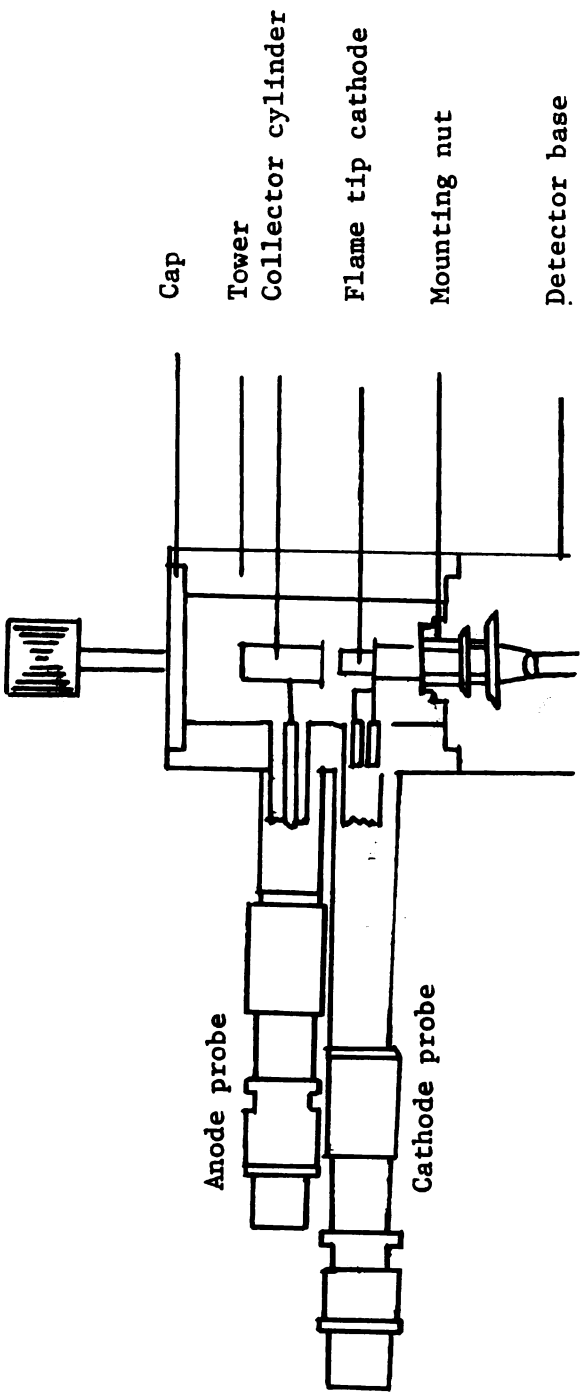


Figure 8. Flame ionization detector.

gases such as H_2S and SO_2. The detector does not respond to inorganic compounds. The response to organic compounds increases as the number of carbon atoms increases. As a precaution, it is very important that the carrier gas lines be free of all organic matter.

Analog to digital conversion. Assuming that all of the sample reaches the detector, the first requirement is that the detector will not only sense the presence of the material of interest but will also produce an instantaneous response for the material that is directly proportional to its concentration. However, the FID as mentioned earlier has certain advantages. It excludes all inorganic compounds, single carbon molecules containing carbonyl structures and fixed gases. The instantaneous response is reflected as an analog voltage drop across a resistor.

The second basic step in the quantitation process consists of conversion of the analog voltage (generated by the chromatographic detector) to digital data. This is commonly done by either of the following techniques:

1. A graphic recorder may be used to obtain the chromatogram and the chromatogram converted to digital data by measurement of peak heights or areas.

2. An electronic apparatus may be used to automatically perform peak integration resulting in printed digital output.

In addition, a third very elaborate and expensive system of GLC/mass spectrometer peak identifier coupled with a computer has been utilized for separation, identification, reconstruction of chromatographic peaks and quantitation.

For the purpose of my study, analog digital conversion was done using a strip chart Beckman recorder and a Finnigan Model 1015C* gas chromatograph-mass spectrometer equipped with a Finnigan System 150* computer.

Use of the Internal Standard

Throughout this discussion on quantitative GLC, it is assumed that all of the sample introduced into the column passes through to the detector. However, possible inaccuracies in this assumption are syringe errors, loss in the injection port, loss on the column, sample decomposition and loss due to leaks. These errors can be eliminated by the use of an internal standard. If the internal standard is added before the extraction process, it may also compensate for errors during the preparation of the sample. Some of the requirements for the internal standard are: (1) it must be completely resolved, (2) it must elute near the peaks of interest, (3) it must be similar in concentration to the peaks of interest, (4) it must be chemically similar but not present in the original sample.

Results with an internal standard may be interpreted either graphically or analytically using the formula:

$$\% x = \frac{A_x}{A_{is}} \times \frac{W_{is}}{W_s} \times 100$$

where Wis and Ws are the weight (or volume) of the internal standard and sample, respectively, and A is the area or may be substituted as height of the particular peak.

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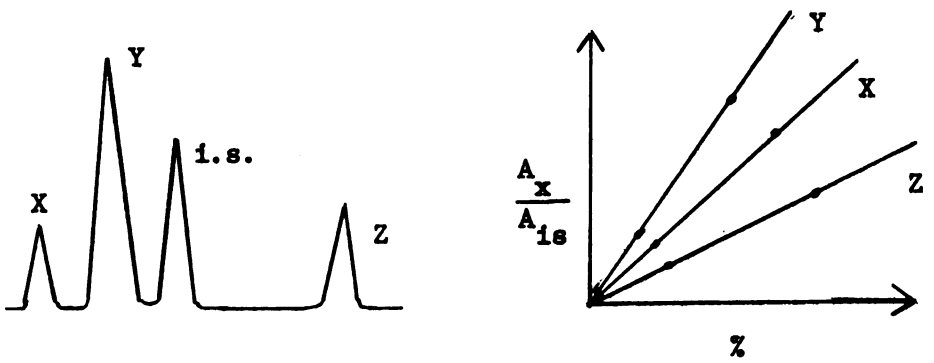
*Finnigan Corporation, Sunnyvale, California.



However, taking into account that the internal standard and the unknowns throughout the process of sample preparation and GLC analysis are subjected to the same parameters and that the column efficiency is at its best, the integration can be done either by comparing peak area determinations or peak height measurements. Furthermore, adding the internal standard to the sample before extraction and using a standard mixture as a control sample, respectively, will compensate for losses and for the individual differences in the distribution coefficients of the compounds under consideration.

EXPERIMENTS

As indicated earlier, several GLC methods for several types of drug analyses are available in forensic toxicology laboratories. It is my personal experience that most of these procedures are adapted for identification of drugs in pill or powder form. Most recently, a simple GLC system utilizing four columns and three liquid phases, complemented by a direct solvent extraction scheme designed to detect common poisons, drugs and their human metabolites to a sensitivity limit of 2 μg/ml in blood, urine and tissue specimens has been developed by B. S. Finkle *et al.* of the Laboratory of Criminalistics, County of Santa Clara, San Jose, California. Relative retention data for almost 600 different compounds are available utilizing this extensive procedure.[21] However, no attempt was made to quantitate the drugs by this procedure.

My first study was conducted following the modifications of this procedure.

Study Number I

Apparatus. Varian Aerograph Model 2700 Gas Chromatograph* fitted with FID; nitrogen carrier gas; inlet flow rate 27 ml/min; 1.0 mv strip chart recorder; speed 0.5 inch/min.

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*Varian Aerograph, Walnut Creek, California.

Column. Five feet by 4 millimeters inside diameter coiled glass column packed with 3.0% SE-30* on 80/100 mesh Chromosorb G. The solid support was acid washed and the packed column heated overnight at 250°C for stabilization. The column temperature was programmed from 160°C to 220°C. The injection port was kept at 240°C and the detector temperature controlled to 220°C.

Sample preparation. Known pure standards in 95% ethanol were obtained from The Anspec Company, Incorporated, Ann Arbor, Michigan. Each sample was prepared by adding 1 ml of 1 mg/dl of working standard to 2 ml of drug-free whole blood. To each mixture were added 5 drops of 10% acetic acid and 10 ml of GC grade chloroform. The mixtures were vigorously shaken for 2 minutes and the accumulated chloroform vapor pressure slowly released. Blood plugs were removed by filtration and the chloroform extracts evaporated just to dryness** by flameless heating and by blowing a gentle stream of nitrogen at the surfaces of the extracts. After cooling to room temperature, the residues were redissolved in 0.1 ml aliquots of chloroform and 5 µl of the final extract were injected into the column. Before each extract was singly injected, a blank drug free blood sample was also prepared. The chromatogram of the blank was allowed to run for 20 minutes, at the end of which a cholesterol peak was identified. At the end of each standard run each peak was identified by determining its retention time.

For the second stage of this study, a standard mixture of 1 mg/dl in chloroform of each of the barbiturates, Librium, Valium, Dilantin, Doriden and methaqualone, was prepared. One milliliter of this mixture

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*See Appendix A for further details.

**Prolonged dryness and heating may cause decomposition and loss of specimen.

was added to 2 ml of drug-free whole blood. The extract was prepared as indicated earlier. Five microliters of the final extract were injected into the column.

Results. After emergence of all peaks, the retention times relative to pentobarbital were calculated. The results are listed in Table 2. Peak height and area of each peak of equal concentration were compared to the others. It was discovered that they lacked conformity due to "tailing." Broadening of the peaks with increase in temperature and occurrence of dead bands* were observed.

Further modifications of the procedure were made by reducing the attenuation from 265 to 128, using 0.5 mg/dl concentration of each known standard, decreasing the span of temperature change, increasing the gas flow rate and checking all the fittings for leaks. Peak heights were improved to some extent but broadening was still a problem.

After discussing this problem with faculty members in the Department of Chemistry and Dr. E. Kivla of the Michigan Department of Public Health, Crime Laboratory, it was concluded that SE-30 was a poor choice of a liquid phase and that the gas chromatograph and the recorder needed some repairs. Since no other instrument was available at this particular laboratory, it was necessary to change instruments and make use of another column under a different set of conditions.

Study Number II

The second study was conducted by converting the free barbiturates to their 1,3-dimethyl derivatives before the sample was injected into the column.[22]

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*See Appendix B for explanation.

Table 2. Relative retention times of acid extractable drugs eluted off a 5 ft x 4 mm inside diameter coiled glass column packed with 3% SE-30 on 80/100 mesh Chromosorb G.

Compound	Relative Retention Time*	Duration of Action
Barbital	0.38	long
Probarbital	0.54	intermediate
Aprobarbital	0.65	intermediate
Butabarbital	0.74	intermediate
Amobarbital	0.91	intermediate
Pentobarbital	1.00	short
Vinbarbital	1.09	intermediate
Methohexital	1.15	ultrashort
Secobarbital	1.21	short
Methaqualone	1.23	
Valium	1.26	
Dilantin	1.43	
Doriden	1.50	
Hexobarbital	1.62	short
Librium	1.75	
Phenobarbital		long

*Retention times are relative to the retention time of Phenobarbital.

Apparatus. A Model GC-45 Gas Chromatograph with a hydrogen FID* was used and operated with a carrier gas (helium) flow rate of 50 ml/min and column temperature of 230°C. A 12 ft by 4 mm inside diameter U-shaped glass column packed with 7% DC-200 on 80/100 mesh Gas Chrom Q** and conditioned by heating overnight at 250°C was used. The reagents and the modified procedure used in this analysis are those used by Fiereck *et al.*[23]

Sample preparation. Standard samples were prepared by adding 1 ml of 1 mg/dl standard of each compound to 2 ml of drug-free whole blood. Sample extraction into chloroform was continued as described in the first study. The chloroform extracts were dried with 1 gm aliquots of anhydrous Na_2SO_4 and the dried extracts evaporated to about 0.1 ml concentrates. One milliliter aliquots of methanol reagent 3*** and 0.1 ml of dimethyl sulfate were added. The solutions were then evaporated and 1.0 ml aliquots of distilled water added to the residues. After vigorous mixing and separation of the layers by centrifugation, the aqueous layers were aspirated and discarded. The solvent layers were dried with 1 gm aliquots of anhydrous Na_2SO_4 and, after separation, the final extracts (chloroform) were concentrated to approximately 0.1 ml as described in the first study. Approximately 1 µl of each concentrate was injected into the column separately for individual runs. The location of each peak in relation to the air peak was determined as described in the first study.

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*Beckman Instruments, Inc., Palo Alto, California.

**Applied Science Laboratories, Inc., Philadelphia, Pennsylvania.

***See Appendix A for further details.

For the second stage, a mixture of each of the barbiturates and other drugs was treated as a single sample. To this sample 1 ml of 1 mg/dl internal standard Methohexital was added. This is an ultrashort-acting barbiturate which is not used orally. The peak of this barbiturate by this method is centrally located in the chromatogram. The mixture was treated as a single sample and processed as described earlier.

Results. After injection of 5 µl of the concentrate, a chromatogram as shown in Figure 9 was obtained in 20 minutes. Compounds other than glutethimide peak #12 are not shown because they were not available during this analysis and they cannot be eluted by this particular method. The other relative retention times for 11 barbiturates and glutethimide are listed in Table 3.

Calculations. Barbiturate in mg/dl of the sample equals

$$\frac{\text{peak area of the barbiturate in sample}}{\text{peak area of the I. std. in sample}} \times \frac{\text{peak area of I. std. in Standard}}{\text{peak area of corresp. barb. in Standard}}$$

x conc. Internal standard

Many practical applications of this method were made by using unknown blood samples from hospitalized and emergency room patients. Two example chromatograms obtained are depicted in Figures 10 and 11. Figure 10 is a chromatogram of a blood specimen from a conscious patient with a blood phenobarbital concentration of 4.3 mg/100 ml.

It should be noted that phenobarbital and mephobarbital (1-methyl-phenobarbital) form identical 1,3-dimethyl derivatives and therefore cannot be separated by this technique.

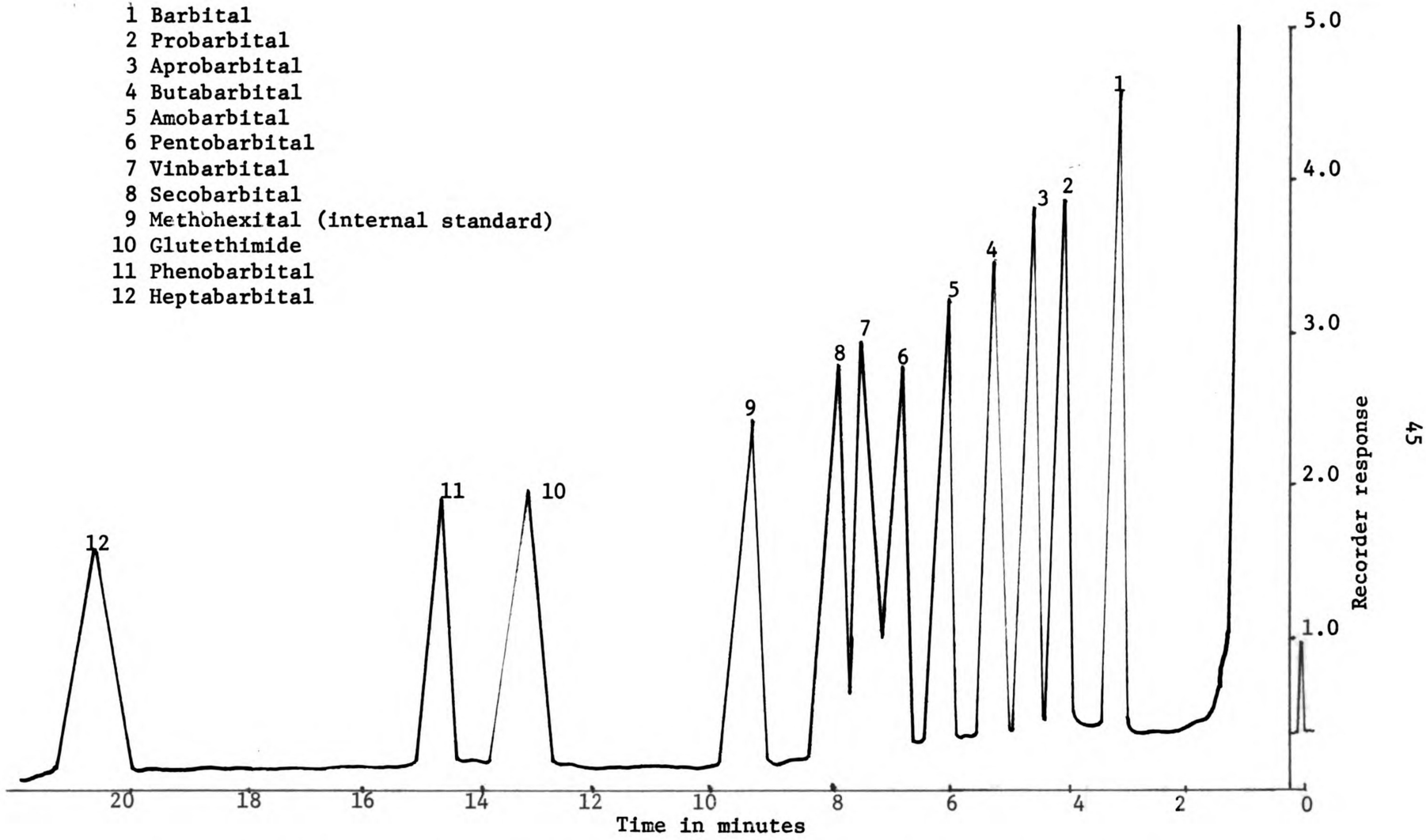

Figure 9. Gas chromatogram of common barbiturates and glutethimide (2mg/dl of each) as their methyl derivatives.

Table 3. Relative retention times of methyl derivatives of barbiturates and glutethimide eluted off a 12 ft x 4 mm inside diameter U-shaped glass column packed with 7% DC-200 on 80/100 mesh Gas Chrom Q

Compound	Relative Retention Time*	Duration of Action
Barbital	0.31	long
Probarbital	0.40	intermediate
Aprobarbital	0.47	intermediate
Butabarbital	0.56	intermediate
Amobarbital	0.64	intermediate
Pentobarbital	0.72	short
Vinbarbital	0.77	intermediate
Secobarbital	0.83	short
Methohexital**	1.00	ultrashort
Hexobarbital	1.38	short
Glutethimide	1.41	short
Phenobarbital, Mephobarbital	1.53	long
Heptabarbital	2.22	short

*Retention times are relative to Methohexital.

**Internal standard. Time taken to merge from the column after the solvent peak = about 11 minutes.

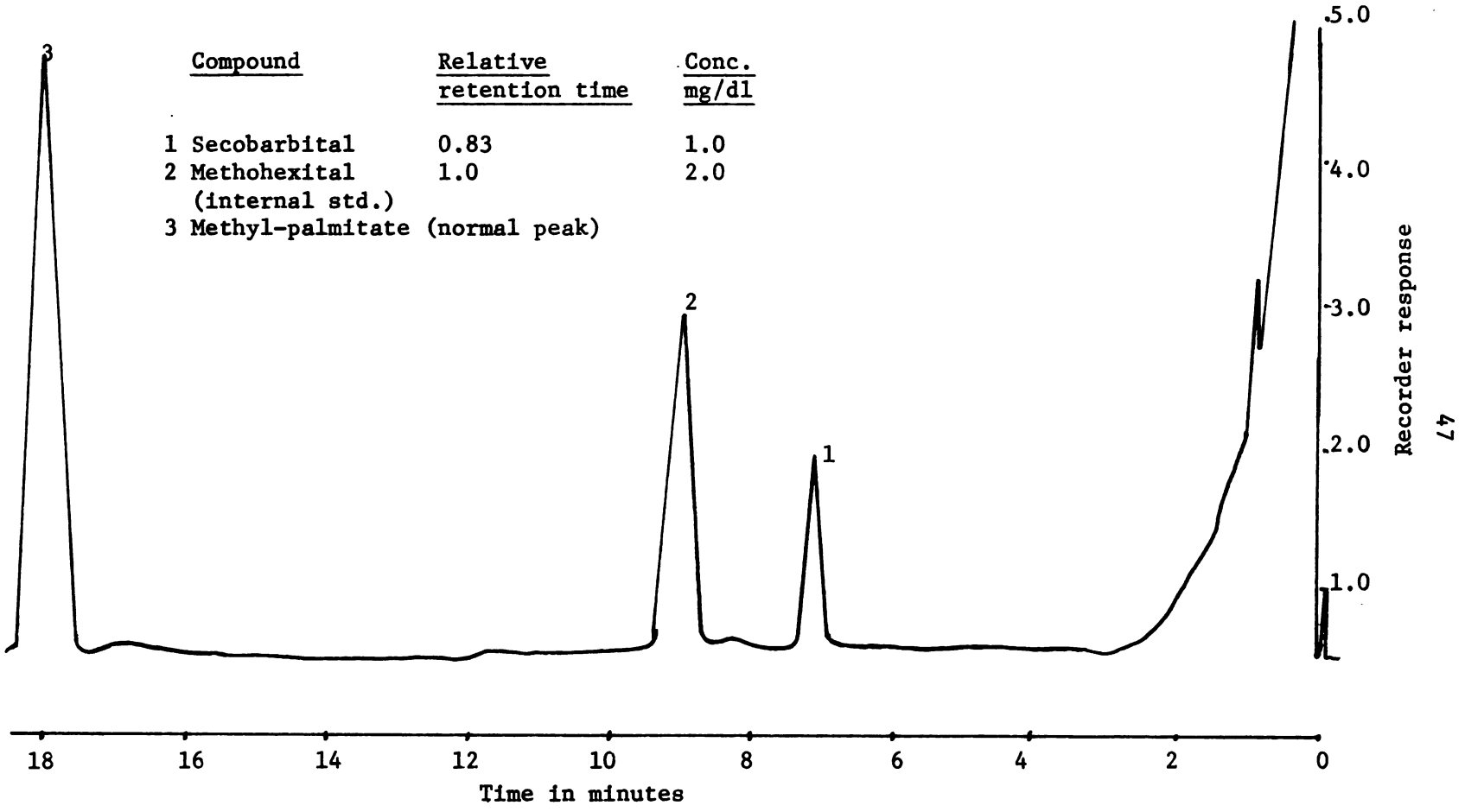

Figure 10. Gas chromatogram of a blood specimen from an unconscious patient (barbiturates analysed as their methyl derivatives)

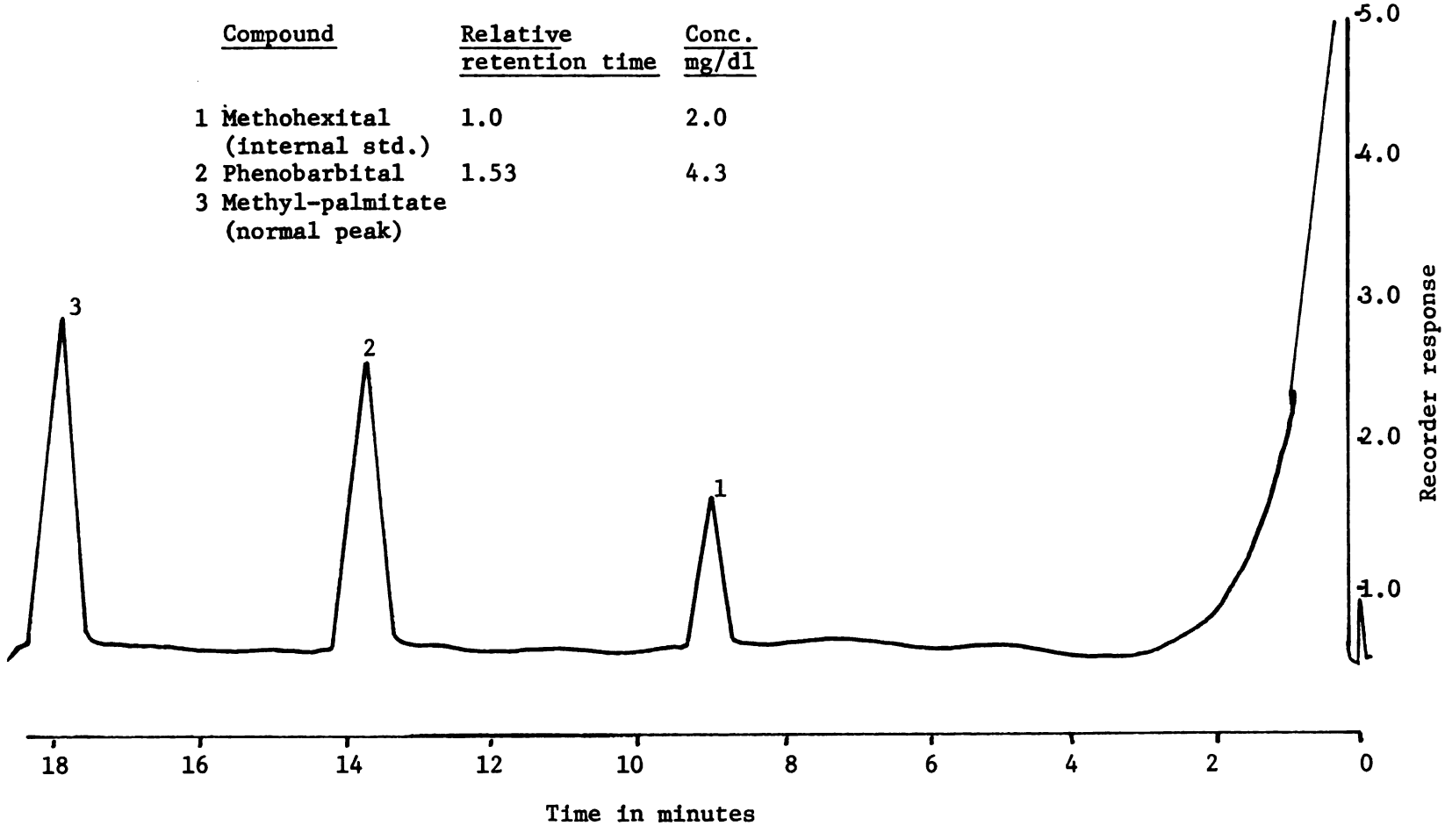

Figure 11. Gas chromatogram of a blood specimen from a conscious patient (barbiturates analysed as their methyl derivatives)

Since the procedure of the second study is quite long and requires the additional complicated and cumbersome steps of methylation, a much simpler and more rapid method was sought.

Study Number III

Apparatus. A Model 600 Varian Aerograph Gas Chromatograph equipped with a hydrogen FID, dual differential electrometer and temperature programmer was operated with nitrogen carrier gas; flow rate 30 ml/min; strip chart recorder* with 5 mv span; speed 0.5 in/min.

Column. A 6 ft by 4 mm inside diameter U-shaped glass column packed with 1.5% OV-17** on 100/120 mesh Chromosorb G high performance*** was conditioned overnight by heating at 250°C. The column was extremely stable and was routinely used thereafter for drug screening.

Temperatures. Injection port: 240°C; detector: 220°C; column compartment temperature programmed from 125°C to 250°C at 6°C/min.

Sample preparation. Pure standard compounds in 95% ethanol were obtained from The Anspec Company, Incorporated, Ann Arbor, Michigan. One milliliter of 1 mg/dl working standard of each compound was added to 2 ml of drug-free whole blood samples. Five drops of 10% acetic acid were added to each standard sample. After addition of 10 ml of chloroform to each sample the mixtures were vigorously shaken for 2 min. The chloroform vapor pressure was slowly released and the blood plugs

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*Beckman Instruments, Palo Alto, California.

**See Appendix A for further information.

***Varian Aerograph, Walnut Creek, California.

removed by filtration. The chloroform extracts were concentrated to dryness by flameless heat and a slow stream of nitrogen. Each residue was redissolved in 0.1 ml aliquot of chloroform and 5 µl of each injected into the column for temperature programmed individual runs. Prior to each sample injection, a sample blank prepared in the similar fashion without any drug, was injected in order to adjust and establish a steady base line to zero recorder and bucking adjustments that must correlate.

For the second stage, a similar 1 mg/dl standard mixture sample of all the compounds was prepared and processed in a similar fashion. Five microliters of the final concentration were injected into the column for an individual temperature programmed run.

Results. During the blank run, a cholesterol peak was observed and identified at the end of 20 minutes chromatogram time. A smooth, steady and noiseless base line was also obtained. The location of each peak for each sample standard was recorded and the retention time of each compound as a single entity in a sample was calculated.

A chromatogram of the standard mixture was recorded and relative retention times (RRT) relative to internal standard Methohexital calculated to identify each compound. These RRTs are listed in Table 4. It should be noted that these RRTs are only relative to the Methohexital under the conditions of instrument operation as indicated in this study.

Many practical applications of this particular procedure were routinely made under the conditions listed in the study. Within a period of three months approximately 50 unknown blood samples were analyzed. An example of this is depicted in Figure 12, a chromatogram

Table 4. Relative retention times of acid-extractable drugs eluted off a 6 ft x 4 mm inside diameter U-shaped glass column packed with 1.5% OV-17 on 100/120 Chromosorb G high performance

Compound	Relative Retention Time*	Duration of Action
Barbital	0.09	long
Probarbital	0.22	intermediate
Aprobarbital	0.26	intermediate
Butabarbital	0.35	intermediate
Amobarbital	0.39	intermediate
Pentobarbital	0.42	short
Vinbarbital	0.45	intermediate
Secobarbital	0.49	short
Glutethimide	0.66	
Phenobarbital	0.90	long
Methohexital**	1.00	ultrashort
Methaqualone	1.23	
Hexobarbital	1.30	short
Dilantin	1.67	
Valium	1.70	
Heptabarbital	1.81	short
Librium	1.90	

*Retention times relative to Methohexital.

**Internal standard.

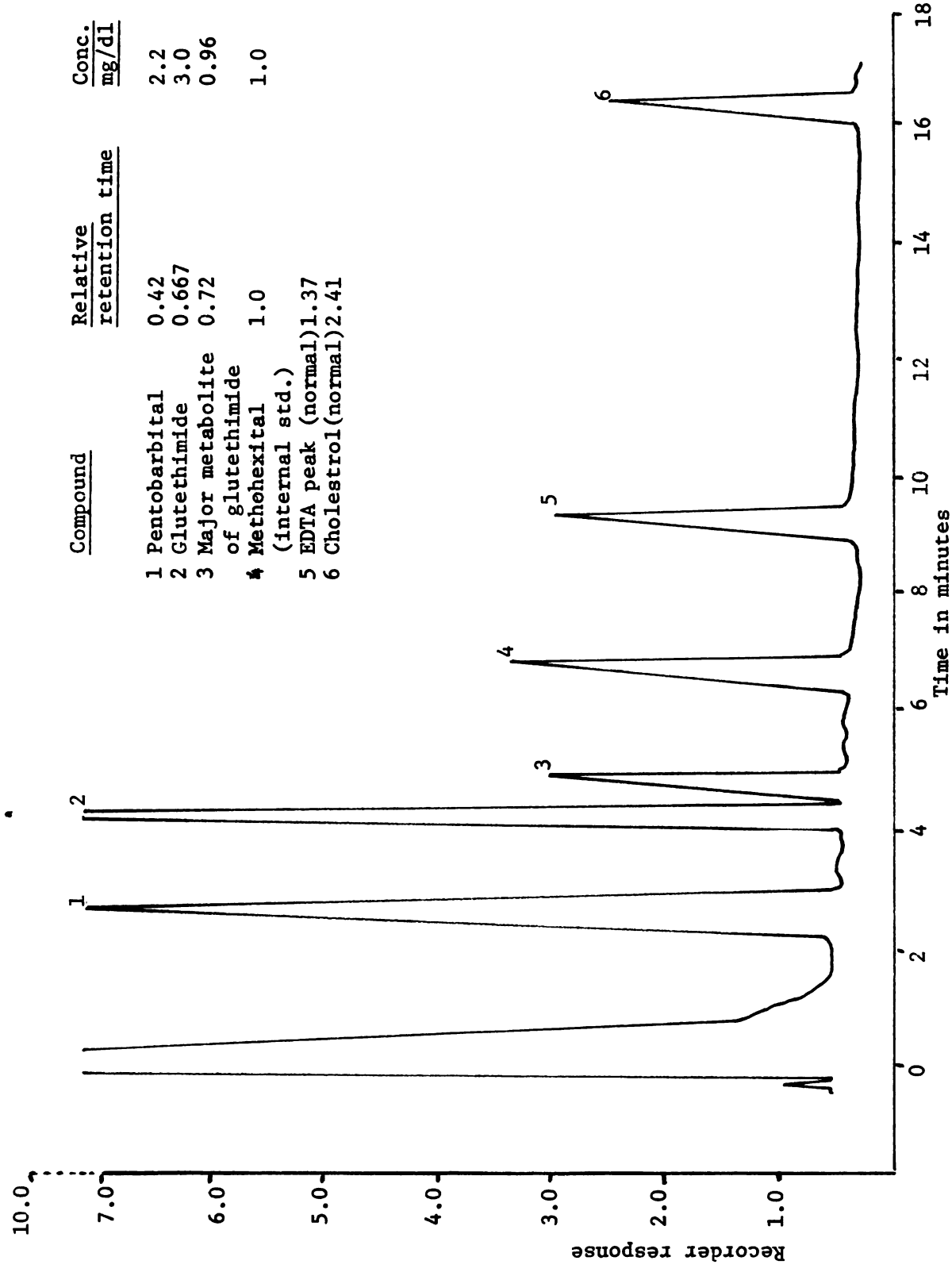

Figure 12. Gas chromatogram of an acidified chloroform extract of the blood specimen of a comatose patient. (EDTA preservative)

of a blood sample from a comatose patient. The unknown sample from the patient was prepared in the same way as described earlier, except that 1 ml of 1 mg/dl internal standard in chloroform was added before pH adjustment.

Identification and calculation. The unknown peaks were identified by comparing them to the list of RRTs obtained earlier from the standard mixture. Noting that the peaks appear quite narrow and symmetrical, calculations were made by just comparing the peak heights. For this particular case, the standard peak height measured 8 cm. Since the standard concentration was 1 mg/100 ml, the concentrations of the unknown peaks were obtained by dividing each peak height (in centimeters) by 8. The results are listed along with the chromatogram in Figure 12. This particular specimen was sent out to a commercial laboratory in California where it was analyzed by another GLC procedure. The results indicate a 99% correlation.

This procedure was sent to the Sacramento Medical Center, Sacramento, California, where the clinical laboratory utilizes a similar instrument and column under the parameters indicated earlier. I am informed that the list of drugs that can be quantitatively analyzed by this procedure has been expanded to include other drugs that can be extracted into organic solvents at weakly acidic or neutral pH. Some of the drugs that have been successfully quantitated are listed in Appendix C.

The 6 ft by 4 mm inside diameter U-shaped glass column packed with 1.5% OV-17 on 100/120 mesh Chromosorb G High Performance* is very stable over a period of time. However, with daily continuous usage some of the

*Varian Aerograph, Walnut Creek, California.

peaks may tend to drift a little. To correct for this, new RRTs may be established by running a standard mixture on a weekly basis. A quality control pattern may also be established by analyzing a random known specimen of known concentration on a daily basis.

Study Number IV

In the past few months, several GLC/mass spectrometers have become available in forensic laboratories. My fourth study was conducted by employing this complicated but very precise and accurate technique of unmistakable separation and identification.

Although mass spectrometry is a highly sophisticated and more extensive technique than GLC, for the purpose of this study, it will be considered as a universal detector which provides structural and molecular weight information of high sensitivity and specificity.

Principle. The complicated operational aspects and parameters affecting the whole process of mass spectrometry are beyond the scope of this manuscript. However, this detector works on the principle that molecules in a gas sample as they are eluted off the gas chromatographic column are ionized upon entering the ion chamber of the mass spectrometer (MS). The resulting ions are introduced into the quadrupole mass filter where they are separated according to their mass-to-charge ratio (m/e). As they emerge from the mass filter, they are detected by an electron multiplier detector producing a signal which is fed to a preamplifier and to a suitable output device. The output can be displayed on an oscilloscope, recorded on an oscillographic recorder or stored on a magnetic tape via a computer interface.[24] The various processes that occur in the MS during operation are displayed in Figure 13.

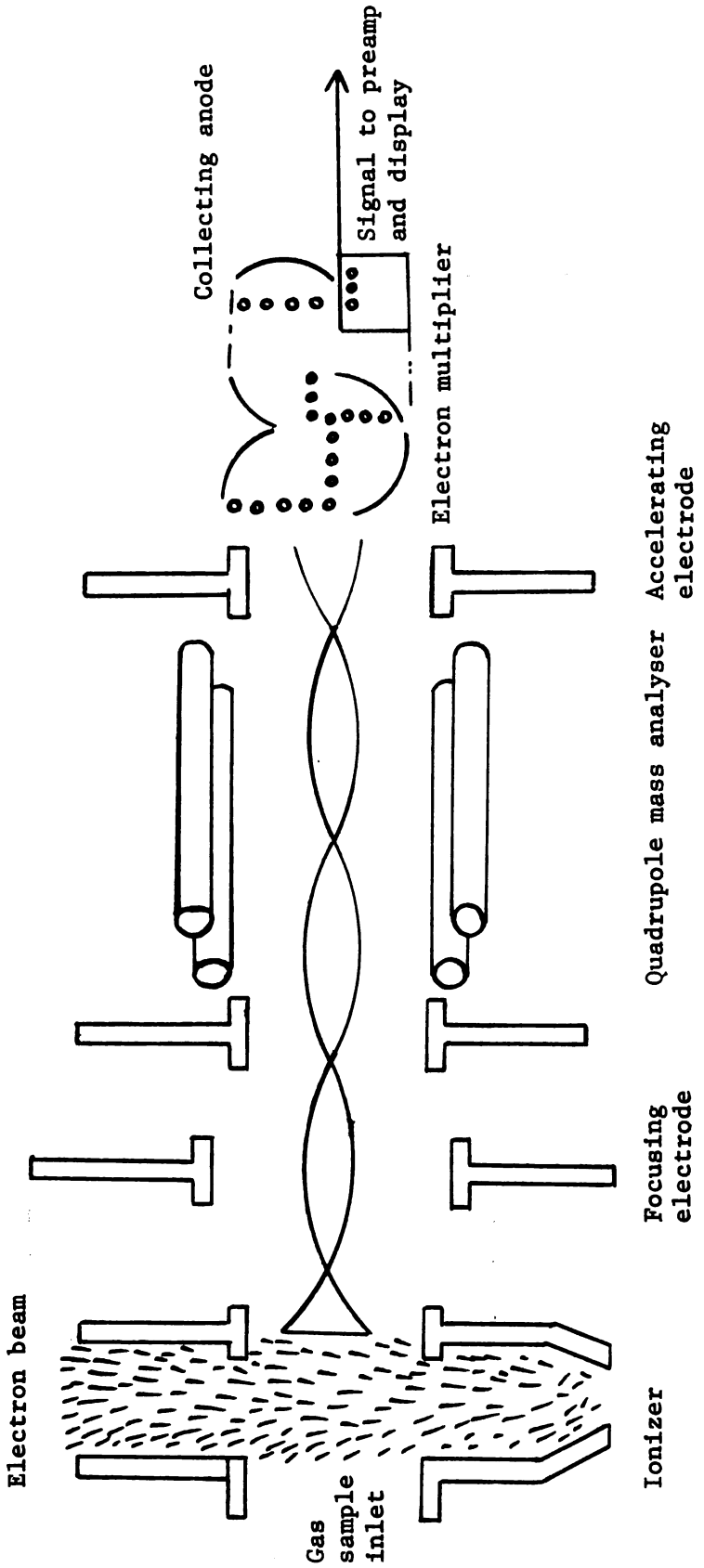

Figure 13. Quadrupole mass analyzer assembly.

The most important aspects in understanding the whole process of further integration are based on mass analysis or mass separation of ions as is performed in the quadrupole mass filter. It should be noted that as the molecules are ionized, they fragment; each fragment has a separate m/e. The mass analysis or mass separation of ions or fragments is performed in the quadrupole mass filter. The quadrupole mass filter consists of four metal electrodes or rods in a square array as shown in Figure 14. To each diagonally paired set of rods a combination radio frequency (RF) and DC voltage of increasing amplitude is applied. One pair (X rods) receives an RF voltage and a positive DC voltage and the other pair (Y rods) receives an RF voltage with 180° phase shift and a negative DC voltage.

By applying a suitable RF/DC voltage ratio, it becomes possible to transmit through the mass filter only ions or fragments of specific m/e. The ions which are not transmitted strike one of the rods where they are neutralized and pumped away by the vacuum system. By scanning the RF and DC voltages from zero to the maximum values, ions or fragments will be transmitted in a sequential order to their m/e, beginning at low mass and proceeding to higher masses. For each voltage sweep, the entire mass range is scanned. This scan is accomplished electrically at an extremely fast scanning speed so that any specific portion of the mass range or any one or more specific mass units can be continuously monitored or have their signals integrated.

Coding the mass spectrogram. The mass spectrogram of the GC peak is drawn by the oscillographic recorder. It is numerically coded by identifying the water (m/e 18) and air (m/e 28,32) groups. Then the m/e of the most intense peak in each group of 14 amu (atomic mass units)

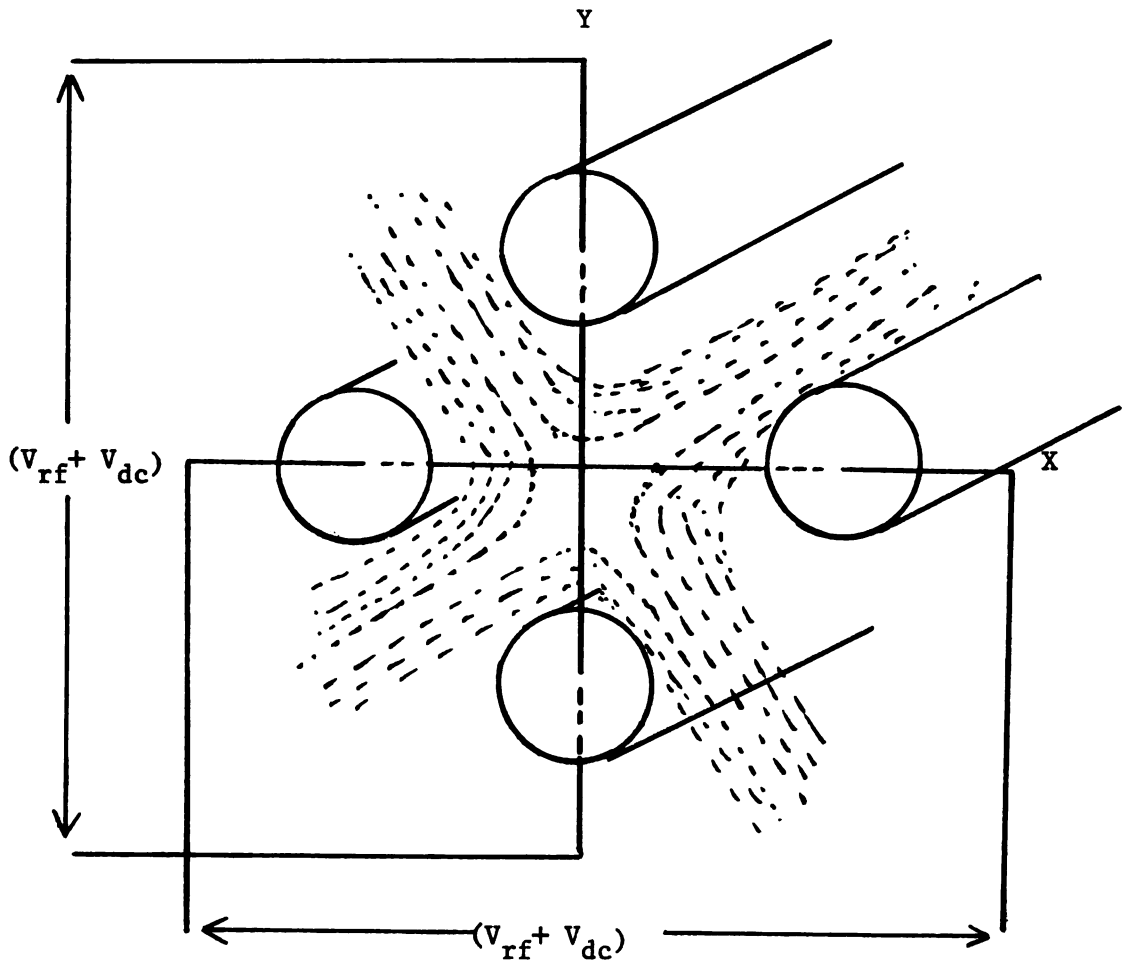

Figure 14. Quadrupole mass filter.

beginning at Mass 34 and continuing through Mass 453 are recorded in sequence. This procedure generates a maximum of 30 peaks which can be used to identify any compound which has a molecular weight not greater than Mass 453 (this being the limit of the instrument that was used during this study). Figures 15, 16 and 17 show typical mass spectrograms of ionized fragments of secobarbital (Spectrum number 99), Hexethal (Spectrum number 116) and diphenylhydantoin (Spectrum number 200), respectively. These are the results obtained during this study. It should be noted that each spectrum is generated from the ascending side of the GC peak to ensure uniformity and any background that need be subtracted.

Identification. After all the standards have been categorized by determining the most intense peak in each group of 14 amu, 3 tables are compiled by (1) reference codes, (2) base peak (largest mass fragment in the mass spectrum), and (3) molecular weights. Entries in the base peak and molecular weight tables are listed in numerical order (see Table 5). These tables can be used as a first search routine when attempting to identify an unknown spectrum by manual methods. Molecular weights can be used only if the molecular ion is present and recognized. The indicated drug can be compared in detail by matching its code to the reference codes in the alphabetized master index. Alternatively, the computer will help in a search by comparing the 30 number codes for the unknown with the coded library and printing a selected number of best matches, ranked in order of similarity to the unknown.

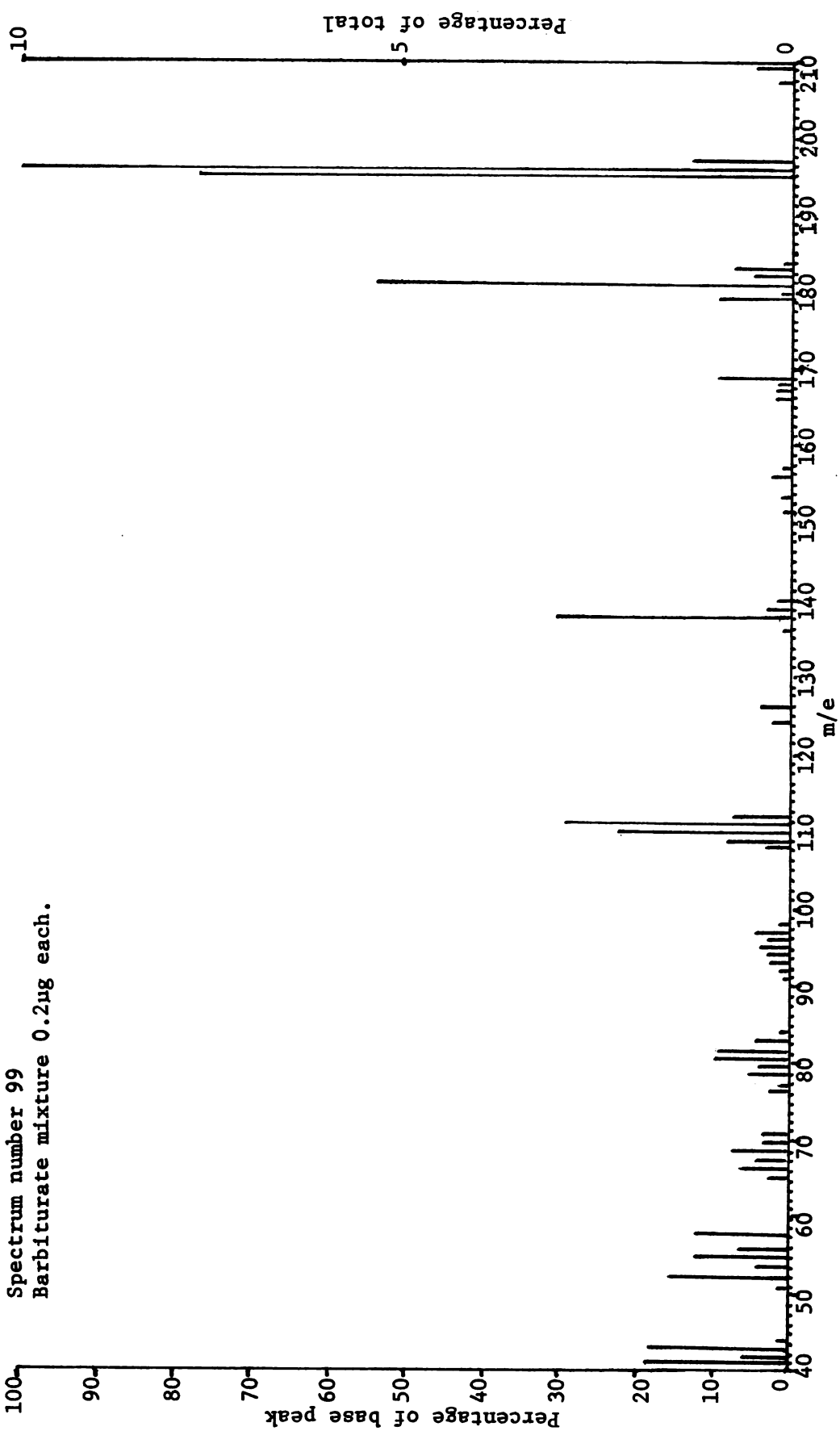

Figure 15. Standard spectrum of secobarbital.

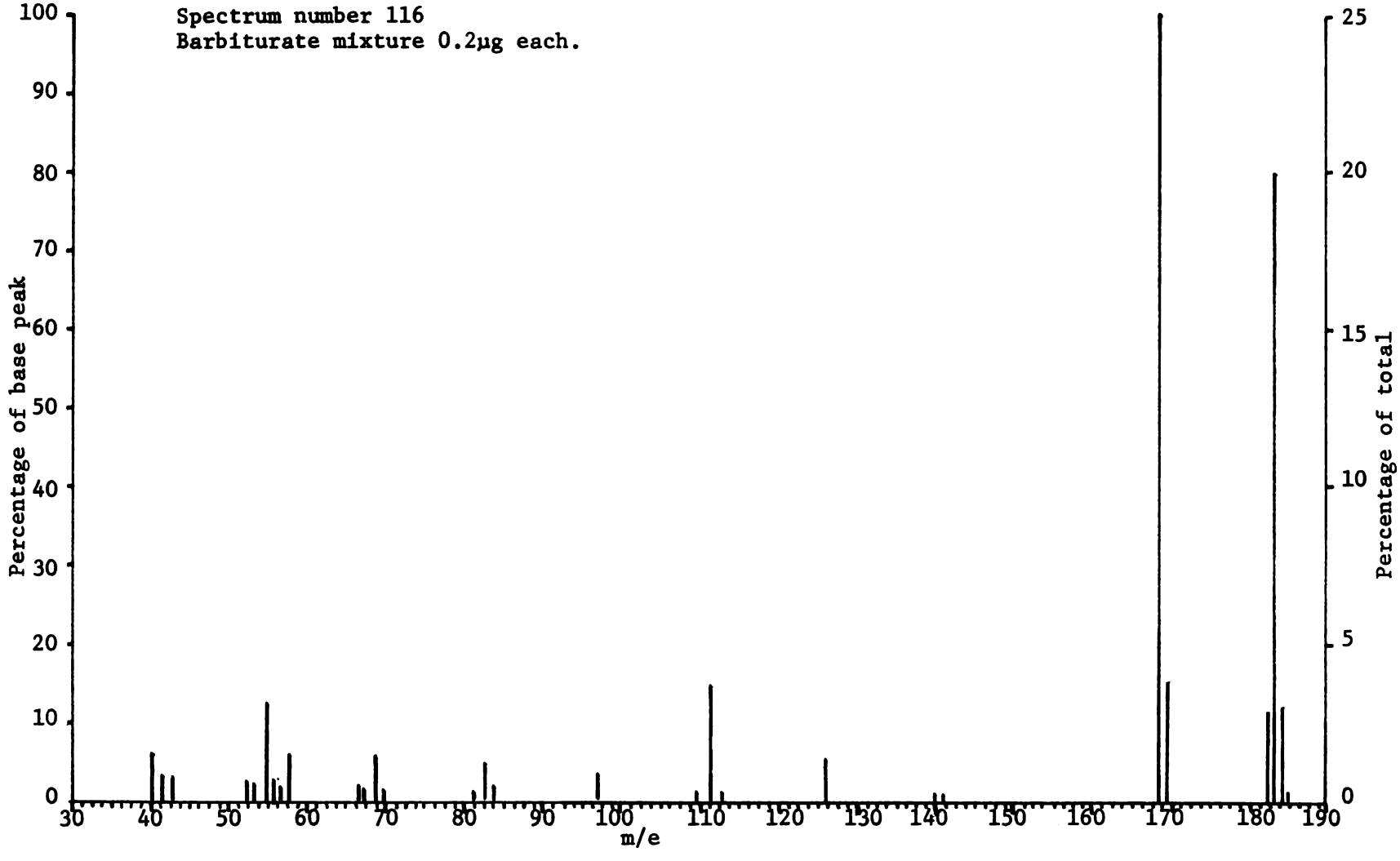

Figure 16. Standard spectrum of Hexetal.

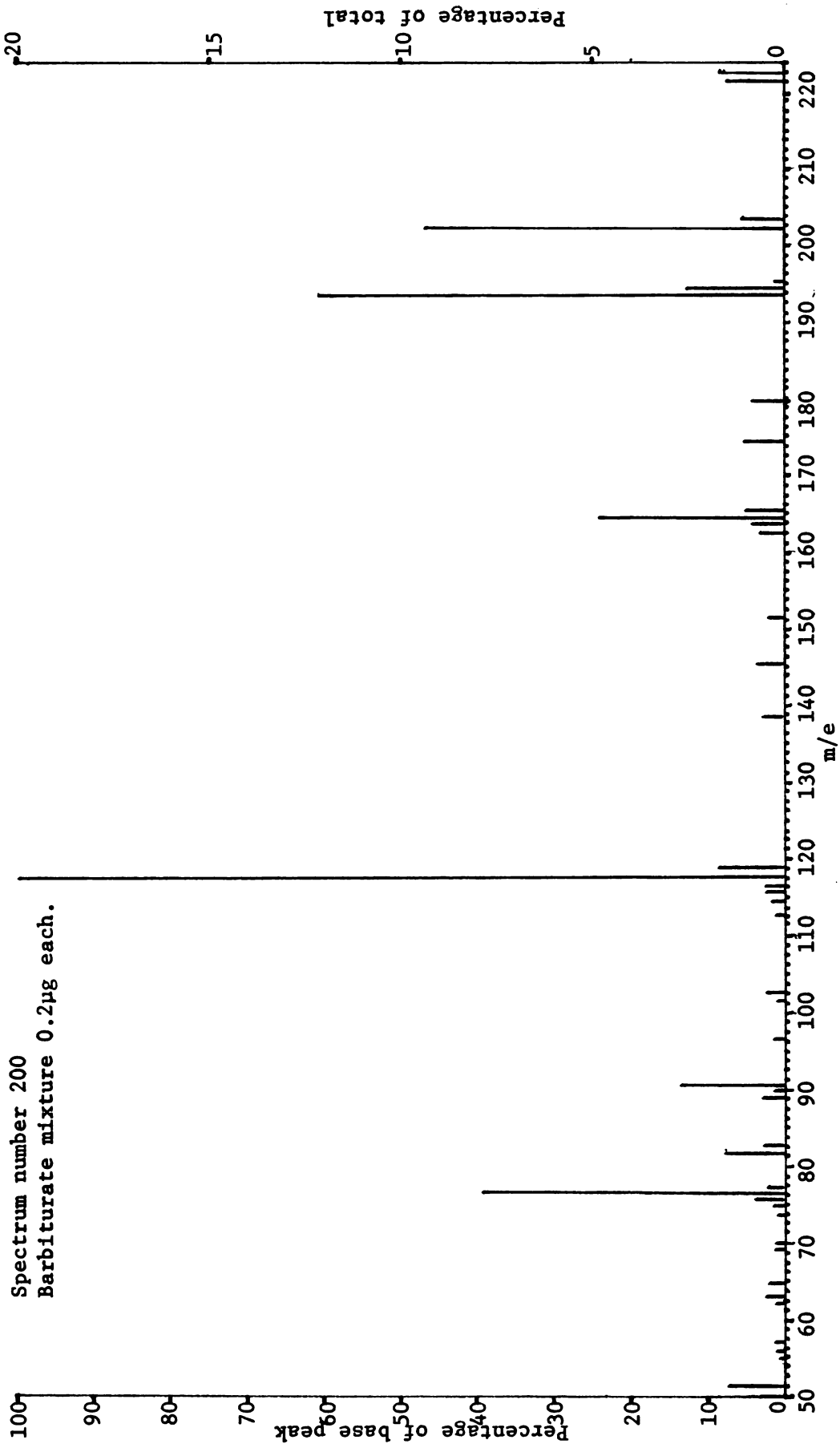

Figure 17. Standard spectrum of diphenylhydantoin.

Table 5. Mass spectrum index of ten barbiturates and glutethimide

Barbital											
Mol. wt. 184	Base peak 141										
41	55	69	83	98	112	126	141	156	161	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Probarbital											
Mol. wt. 198	Base peak 141										
41	55	69	83	98	112	126	141	156	169	183	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Butabarbital											
Mol. wt. 212	Base peak 141										
41	57	69	85	98	112	123	141	156	167	183	197
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Butethal											
Mol. wt. 212	Base peak 141										
41	55	69	83	98	112	126	141	156	167	184	197
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Amobarbital											
Mol. wt. 226	Base peak 141										
41	55	69	83	98	112	124	141	156	167	183	197
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Pentobarbital											
Mol. wt. 226	Base peak 141										
43	55	69	85	98	112	126	141	156	169	183	197
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Secobarbital											
Mol. wt. 238	Base peak 168										
41	55	69	79	97	108	124	141	153	168	181	195
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Glutethimide											
Mol. wt. 217	Base peak 117										
39	51	65	77	91	117	118	132	157	160	174	189
202	217	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						

Table 5 (cont'd.)

Hexobarbital											
Mol. wt. 236			Base peak 81								
41	53	67	81	91	108	123	136	157	164	178	195
0	221	236	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Phenobarbital											
Mol. wt. 232			Base peak 204								
39	51	63	77	103	117	118	133	146	161	174	189
204	217	232	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						
Vinbarbital											
Mol. wt. 224			Base peak 195								
41	53	69	79	98	109	124	141	152	167	181	195
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0						

Apparatus. A Finnigan Model 3000* gas chromatograph peak identifier and a Finnigan Model 1015C* gas chromatograph-mass spectrometer equipped with a Finnigan system 150* computer were used to obtain the data. Both instruments were equipped with a Gohlke all-glass separator interface and operated with helium as gas carrier at a flow rate of 26 ml/min.

Column. A 5 ft x 2 mm inside diameter U-shaped glass column packed with 3% OV-1 on 100/120 mesh Gas Chrom Q was used for barbiturates, glutethimide and diphenylhydantoin separation.

Temperature. The injector port was kept at 200°C and the column programmed from 120°C to 220°C at the rate of 8°C/min.

Sample preparation. A standard mixture of 2 mg/dl in ethanol was prepared from the standards received from The Anspec Company, Incorporated; 1 ml of this mixture was added to 2 ml whole blood. The pH of the mixture was adjusted by adding 8 drops of 10% acetic acid and extraction made with 10 ml of an equal mixture of chloroform and trimethylanilinium hydroxide.** After removal of the blood plugs by filtration, the extract was concentrated as described earlier. Five microliters of the methyl derivatives were injected into the column.

The computer utilized in this study was pre-programmed for coding identification, quantitation, and providing data for chromatogram reconstruction and elution temperature of each peak relative to phenobarbital.

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*Finnigan Corporation, Sunnyvale, California.

**Methalude manufactured by Pierce Chemical Company, P.O. Box 117, Rockford, Illinois 61105.

Results. The relative elution temperatures of the compounds obtained from this study are listed in Table 6. Three spectrums, Numbers 99, 116, and 200 of secobarbital, Hexethal and diphenylhydantoin, respectively, are shown in Figures 15, 16 and 17.

The reconstructed gas chromatogram, a result of mass analysis, computer integration and output and drawn by the recorder, is shown in Figure 18. The peaks appear narrow and symmetrical, indicating good column efficiency. The final results are accurate and specific. If the instrument is used on standby basis, the whole procedure from the time a sample is received in the laboratory to the time when a complete computer print-out is available takes a total of 15 minutes.

A pure compound in minute quantity can be directly identified by the use of a solid probe. The sample is directly introduced into the ionization chamber where vaporization is achieved by heating the tip of the solid probe containing the sample in a small glass capillary to the desired temperature and identification made by the process explained earlier. In cases where it is not possible to obtain a complete spectrum (e.g., metabolites), a dramatic increase in sensitivity and specificity can be obtained by using the technique of mass fragmentography.[25,26]

Table 6. Chromatographic properties of methylated derivatives of acid-extractable drugs obtained off a Finnigan GLC/MS system

Compound	Molecular Weights of Methylated Derivatives	Relative Elution Temperatures*
Barbital	212.2	0.790
Probarbital	226.2	0.814
Aprobarbital	238.2	0.833
Butabarbital	240.2	0.854
Butethal	240.2	0.855
Amobarbital	254.3	0.876
Pentobarbital	254.3	0.890
Vinbarbital	252.2	0.904
Secobarbital	266.3	0.912
Hexethal	268.3	0.953
Glutethimide	245.3	0.983
Hexobarbital	264.3	0.984
Phenobarbital	260.2	1.000
Heptabarbital	278.3	1.060
Diphenylhydantoin	280.3	1.183

*Relative elution temperature = elution temperature in degrees Centigrade of drug/elution temperature in degrees Centigrade of Phenobarbital.

Relative elution temperatures provide a reliable retention index for temperature-programmed GLC. Temperature programmed runs were from 120°C to 220°C at a controlled rate of 8°C/min.

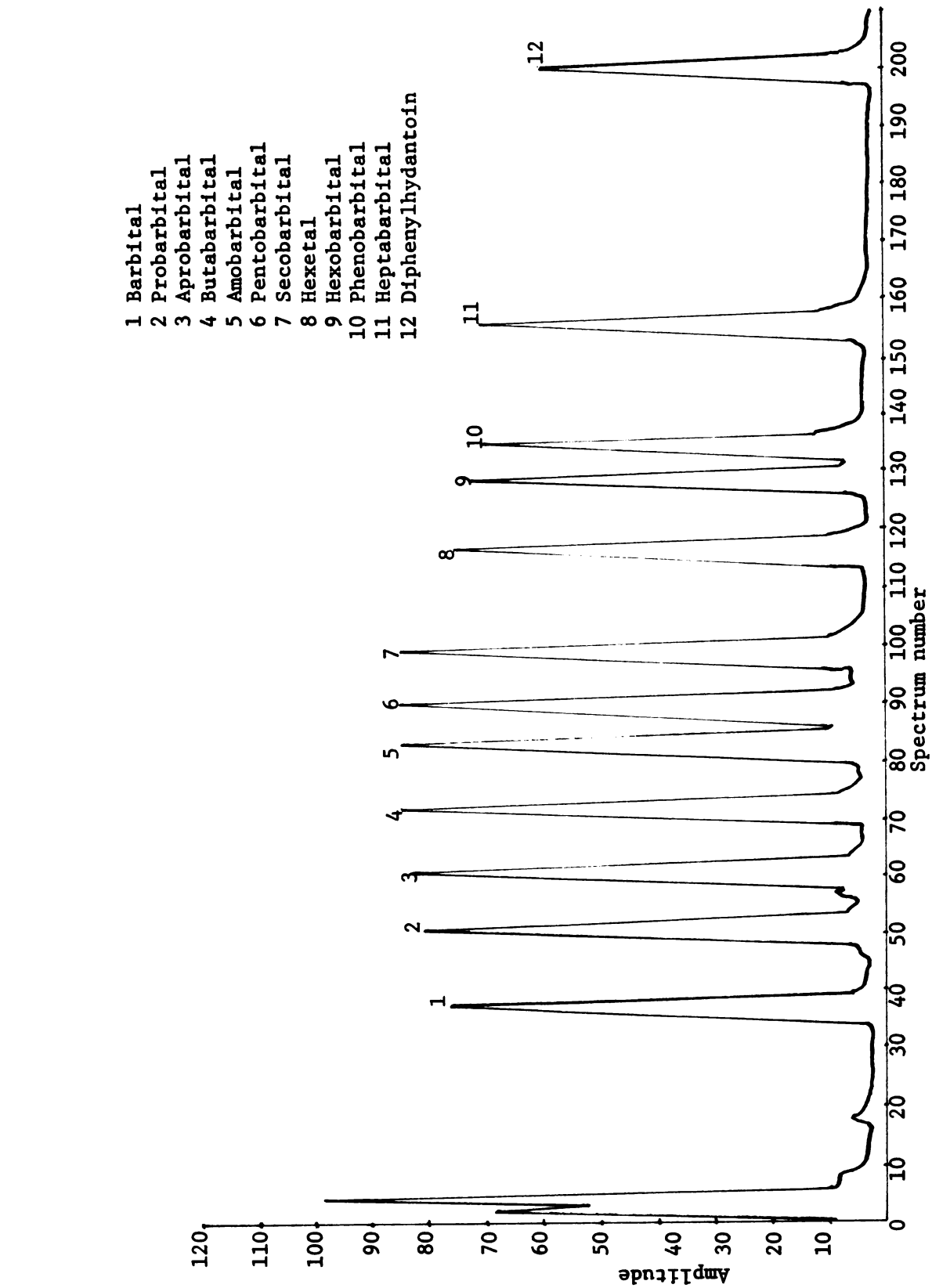

Figure 18. Reconstructed gas chromatogram of methylated barbiturate standard mixture, 0.2µg each.

DISCUSSION

The use of the aforementioned drugs is so widespread that cases of overdose involving them have become very common. Knowledge of the particular derivative in the case of structurally similar drugs may be necessary for treatment, as the potencies of these drugs may differ widely at a given concentration. In the case of barbiturates, the large number of photometric and ultraviolet spectrophotometric procedures available provide quantitative data of some reliability but they definitely fail to identify the compound. It has already been shown that the level of concentration is not meaningful unless the particular barbiturate is known. On the other hand, several barbiturates are completely overlooked because they cannot be analyzed by these procedures.

Three relatively simple GLC procedures have been presented. In the first case compounds were separated, definitely identified, and their RRTs determined. However, the desired quantitation was not achieved. This appeared to have been due to improper choice of the liquid phase and mechanical difficulties related to the GC and the detector.

During the second study, the drugs of interest were methylated and their corresponding methyl derivatives analyzed by GLC. Methylation in this procedure appears to be a relatively long and undesirable additional step. Barbiturates and glutethimide were the only drugs that were successfully analyzed. Phenobarbital and mephobarbital

(1-methyl phenobarbital) that form identical 1,3 dimethyl derivatives could not be separated. Furthermore, the GC lacked a temperature programmer, a very useful asset which could have improved and accelerated the separation considerably. Peaks 10, 11 and 12 in Figure 9, that appear towards the end of the chromatogram, appear to broaden. This could have been eliminated and the efficiency of the column improved by temperature programming.

The results of the third procedure indicate a highly efficient separation. The peaks appear narrow and symmetrical. The column is stable over a long period of time with continuous daily use. Provisions are made for any peak drift that may occur and an apparopriate quality control is suggested. The entire procedure has been utilized and tested by another laboratory which is operating a similar instrument on a daily basis. The application of the procedure reported here indicates that, besides barbiturates, other commonly abused drugs (Methaqualone, Valium, Dilantin, Doriden and Librium) can be successfully quantitated. The Sacramento Medical Center Laboratory has expanded the list to other drugs which have been analyzed by this procedure under the same operating conditions. The list is included in Appendix C.

With experience, one is easily able to recognize the interfering metabolites that may be encountered. The additional step of methylating the drugs is eliminated. Adding the internal standard to the sample before extraction and utilizing a standard mixture and/or a random known standard as a control compensate for losses and for individual differences in the distribution coefficients of each drug. The use of the internal standard under the listed conditions, the choice of the liquid phase and the solid support, temperature programming and

utilization of the hydrogen FID are the major factors that contribute to the high degree of accuracy of this method.

The Sacramento Laboratory and the Michigan Department of Public Health Crime Laboratory indicate that such a procedure can be made operable on a continuous basis at a cost of about $150/month. The cost of the instrument itself may be as much as $8000.

The three GLC methods presented here indicate that the drugs in question can be definitely separated, identified and quantitated after extraction. However, recognition and identification of metabolites and naturally occurring artifacts are essential to the toxicologist if urine, blood and tissue analyses are to be interpreted correctly. The combination of GLC/mass spectrometry as studied has proven satisfactory in providing rapid and positive identification both quantitatively and qualitatively within 15 minutes after receipt of the sample in the laboratory, providing the instrumentation is on a continuous basis. The fragmentation technique and mass spectrogram coding can definitely identify any metabolite and naturally occurring artifact. In addition, any suspected compound can be extracted in pure form and rapid identification within a matter of minutes can be made by utilizing the solid probe of the mass spectrograph.

However, it should be noted that GLC/mass spectrometric system requires the availability of a large reference library which involves extensive research and many man hours. The initial cost of the instrument, including the computer, is over $100,000.

SUMMARY AND CONCLUSION

Three different GLC methods employing three different columns are used to devise a rapid and accurate quantitative procedure for acidic drug analysis. The drugs of interest are analyzed by (1) extracting them into acidified chloroform at pH's between 6 and 7, and (2) methylating them to their corresponding derivatives. A highly sophisticated and extensive technique of GLC/MS for unmistakable separation, identification and quantitation is also presented. The initial cost of the GLC and GLC/MS instruments and the respective continuous operational costs are also listed in the hope that the clinical medical laboratories would seriously consider the application of GLC on a routine basis.

It should be emphasized that, with the widespread use of "soft drugs", the cases of overdose involving them have become very common. Knowledge of the particular derivative in the case of structurally similar drugs is necessary for treatment, as the potencies of these drugs may differ widely at a given concentration. Level of concentration is not meaningful unless the particular barbiturate is known.

The GLC procedure using a Model 600 Varian Aerograph GC, equipped with a hydrogen FID, dual differential electrometer and temperature programmer with a 6 ft x 4 mm inside diameter U-shaped column packed with 1.5% OV-17 on 100/120 mesh Chromosorb G high performance is ideal for "soft drug" analysis. The procedure and method as indicated in Study Number III is routinely used at the Sacramento Medical Center.

The author himself analyzed over 50 unknown blood samples. An example is cited in Study Number III.

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REFERENCES

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APPENDICES

APPENDIX A

REAGENTS

1. Chloroform, GC quality (AR or spectroanalyzed). This reagent must be checked for the presence of any interfering substances by using it as a blank.

2. Dimethyl sulfate, Eastman Kodak Co., Rochester, N.Y.

3. Methonal:water (90:10, by vol.) saturated with K_2CO_3.

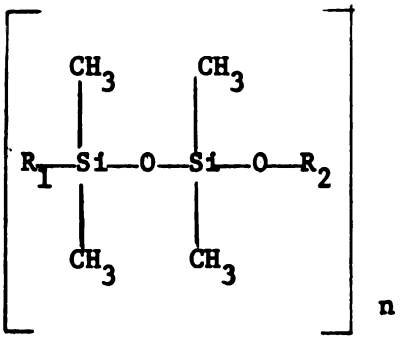
4. Sodium sulfate, GC quality, anhydrous.

5. Compressed gases helium, nitrogen, hydrogen and air were GC grade and products of Union Carbide.

6. Blood samples used in the studies were from selective patients who were not on any medication or drugs.

7. The blood sample from the comatose patient in Study Number II was whole blood with EDTA used as a preservative. The normal peak indicated at RRT 1.34 is due to EDTA and was identified by running a blank EDTA blood sample.

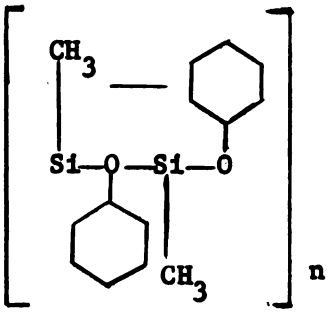
8. SE-30.



Dimethylsilicone polymer

This non-polar compound has been extensively used for bile and urinary acids, alkaloids, C_5-C_{10} aliphatic hydrocarbons, organo metals, phosphorus, steroids, pesticides and acid extractable drug GLC analyses.

9. OV-17.



Methylphenylsilicone polymer

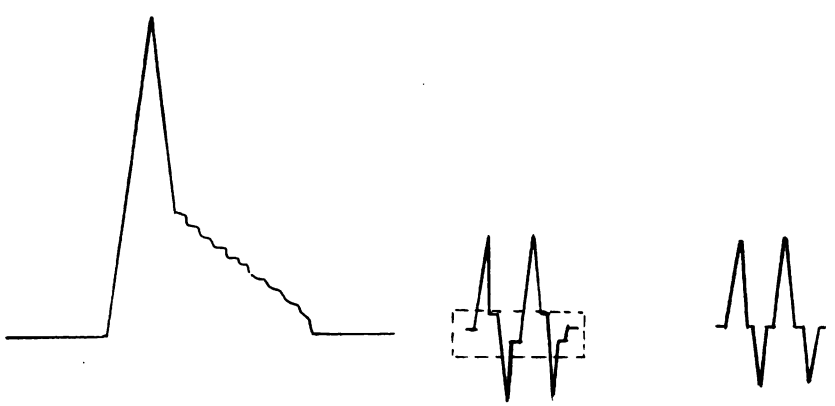
This is a relatively new liquid phase. Its use in the past has been limited to the separation of sugar derivatives, trimethylsilylethers and steroids.

APPENDIX B

EXPLANATIONS

1. Dead-band is a range through which the measured quantity can be varied without causing a readable graphic response. It is usually expressed as a percentage of the full scale. The Figure A below shows the step-like decay of the peak backside which results when using a recorder with a large dead-band. Mechanical load and amplifier gain are the two factors which cause dead-bands.

   A check for dead-band can be made by shunting the recorder input. This is done physically by forcing the servo-drive by turning the motor gear so that the pen is displaced a few percent from the balance point. The force is then released and the pen allowed to rebalance. The procedure is then followed by displacing the pen in the opposite direction. If there is a significant dead-band, a trace as shown in Figure B below will be observed. A properly damped amplifier (correct gain) on the other hand will result in Figure C as shown below.



2. Peak number 3 in Figures 10 and 11 was identified as methyl palmitate by using this pure compound in chloroform and injecting 5 µl of the 1 mg/dl concentration of it into the column.

APPENDIX C

EXPANDED LIST OF THE DRUGS THAT CAN BE ANALYZED BY THE PROCEDURE LISTED IN STUDY NUMBER II

Cyclobarbital
Hexetal
Hydroxybarbital
Metharbital
Mebutamate
Thiamylal
Thiopental
Chlorothymal
Chlorpropamide
Cyclopal
Digitalin
Digitoxin
Dihydrocodeinone
Histamine
Hydroxyamobarbital
Hydroxyphenamate
Mescaline
Methoaqualone
Methacarbomol
4,Methyl,2,5 dimethyoxy-amphetamine (S.T.P.)
3,4 Methylenedioxy 2 Methoxy-amphetamine (MMDA3)
3,4 Methylenedioxy 4 Methoxy-amphetamine (MMDA1)
p-Nitromethamphetamine
Oxazpam
Phenacetin
Phencyclidine
Phenyl Salicylate
Piperocaine
Vinbarbital

VITA

The author was born in Nairobi, Kenya, on August 6, 1939. He attended Ripon College, Ripon, Wisconsin, from September 1961 to June 1965 and received a B.A. degree in biology and chemistry. He completed his professional training in medical technology at St. Agnes Hospital, Fond du Lac, Wisconsin, in September 1966 and concurrently attended graduate school at Marian College, Fond du Lac, Wisconsin, during 1965 and 1966.

In September 1966 he acquired an affiliate membership of the American Society of Clinical Pathologists after passing the ASCP board examination. Subsequently he was employed as a medical technologist and a research specialist at the University of Wisconsin, Madison, Wisconsin, until May 1968. During this time he also attended graduate school on a part-time basis.

In May 1968, he moved to the Chicago area and worked as a senior medical technologist and supervisor at Lutheran General Hospital, Park Ridge, Illinois, Resurrection Hospital, Chicago, Illinois, Bethesda Hospital, Chicago, Illinois, and North Western University Hospital, Evanston, Illinois.

In October 1969 he moved to California and worked as a senior medical technologist at the University of California Medical Center in Sacramento, California, until April 1971. During this time he passed the California Medical Technology Board Examination and gained membership to the California Association of Medical Technologists.

For the spring and summer of 1971 he worked as a chief technologist at the Diagnostic Laboratory in Phoenix, Arizona. In September 1971 he was admitted to the graduate program in Clinical Laboratory Science at Michigan State University with the support of an Allied Health Traineeship.

In addition, the author is a member of the American Society of Medical Technologists, American Society of Clinical Chemists, American Forensic Association, and Rocky Mountain Biological Laboratory.