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Simultaneous high pressure liquid chromatographic determination of twelve common sedatives and hypnotics in serum

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SIMULTANEOUS HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF TWELVE COMMON SEDATIVES AND HYPNOTICS IN SERUM

by
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THESIS

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ABSTRACT

A method for simultaneously determining 12 hypnotics and sedatives (primidone, methyprylon, phenobarbital, butabarbital, butalbital, ethchlorvynol, pentobarbital, amobarbital, phenytoin, glutethimide, secobarbital and methaqualone) in 200 µl of serum is presented. The proteins are precipitated with an acetonitrile solution containing 5-(4-methylphenyl)5-phenylhydantoin, the internal standard. The drugs are eluted from a reversed-phase column with a mobile phase consisting of an acetonitrile/phosphate buffer at a flow rate of 3.0 ml/min. The eluted drugs are detected by their absorption at 195 nm, their quantities are measured from their peak heights. Each analysis requires a maximum of 30 min at the optimum column temperature of 50°C. The lower limit of detection for all of these drugs is less than 10 ng for a drug standard. A sensitivity of 1.0 mg/liter of serum for each of the drugs is attained routinely. Analytical recoveries for the 12 drugs varied from 93 to 112%, with good day-to-day precision (CV= 3.8 - 10.4%). Of more than 35 drugs tested for possible interference, ethotoin interferes with the analysis of phenobarbital and mephobarbital interferes with the analysis of amobarbital.

PREFACE

Until recently, analytical toxicology has played a minor role in modern health care programs (1,2). Most clinical chemists were unsure of the role of analytical toxicology in the clinical laboratory. The main reasons for this insecurity were untrained personnels and a lack of sensitive and specific methods and instruments. The problem of specificity was often further complicated by the presence of active and/or inactive metabolites, as well as by the need for specific identification of drugs in patients who had ingested multiple drugs. Higgins and O'Brien (3) originally noted that overdoses usually consisted of a single drug, however, three years later, they found that overdoses with multiple drugs had risen to 13% and that they were on the rise (4). Law (5) recorded that out of 240 proven drug misuse cases reported in Suburban Hospital, Bethesda, Maryland, in a period of four years, 59.9% of the cases were single drug overdoses and 40.1% were multiple drug ingestions. Horwitz (6) recently reported that between June 1974 and May 1977, out of 1607 drug overdose cases, 39% were multiple drug overdoses.

Clinical chemists have recently had to assume a new and extremely important responsibility in the area of

analytical toxicology because of this ever increasing incidence of drug addiction and drug poisoning in a modern society (7,8). Improvements in existing instrumentation and the introduction of new techniques, and the increasing importance of therapeutic drug monitoring (9,10) has converted this responsibility into a reality. Keeping with this trend, we have developed a method for the rapid identification and quantitation of 12 of the most commonly abused sedatives - hypnotics (primidone, methyprylon, phenobarbital, butabarbital, butalbital, ethchlorvynol, pentobarbital, amobarbital, phenytoin, glutethimide, secobarbital and methaqualone) by liquid chromatography.

INTRODUCTION

In recent years, most large hospitals have observed a marked increase in the admission of patients suffering from drug overdose (7,8). Overdoses of the narcotic drugs such as the opiates represent less of a problem on a day-to-day basis than do overdoses of prescribed drugs, such as sedatives and hypnotics. Loomis (11) reported that the majority of fatal poisoning were due to one or a combination of four agents: barbiturates, carbon monoxide, ethyl alcohol, and salicylates. Berry (12) estimated that 5,5 disubstituted barbiturates were the second commonest cause of fatal poisoning in the United Kingdom and that the frequency of their use was increasing. Other drugs commonly involved in coma-producing incidents include glutethimide (Doriden^{R}), methyprylon (Noludar^{R}), and meprobamate (13,14). In the past five years, diazepam (Valium^{R}) has become one of the leading misused drugs (15). Between August 1974 and December 1976, the Drug Assay Laboratory of Stanford University Hospital reported that out of four thousand drugs found in positive toxicology screens, the ten most commonly found drugs were ethanol, barbiturates, salicylates, diazepam, phenothiazines, acetone, codiene, chlordiazepoxide, phencyclidine, and phenytoin (16). Other

hypnotics, such as methaqualone, glutethimide, methyprylon, and ethchlorvynol, comprised 3.0% of the total drugs recovered from blood and urine.

In 1977 the National Institute of Drug Abuse (17) estimated that barbiturates are associated with nearly five thousand deaths a year in the United States, and that users of barbiturates make about 25,000 visits to hospital emergency rooms each year. It is also reckoned in this report that in 1976 Americans received about twenty seven million sleeping pill prescriptions, including many commonly prescribed nonbarbiturates sleeping pills such as glutethimide, methyprylon, ethchlorvynol, or methaqualone.

Almost all of the coma producing drugs are acid or neutral compounds. Acidic and neutral drugs are defined by the acidic or neutral pH of the aqueous solution from which they are extracted by immiscible organic solvents. Common techniques for screening the acid-neutral class of drugs in biological fluids used for the past several years include the following analytical approaches: thin layer chromatography (47-53), ultra-violet spectrophotometry (54-60), gas liquid chromatography (69-79), gas chromatography/mass spectrometry (67,81,82), enzyme multiplied immunoassay techniques (99), and radioimmunoassay (100).

Toxicological Effects of Sedative-Hypnotics Poisoning

Barbiturates are still the most frequently abused drugs in adult poisoning. The therapeutic and lethal

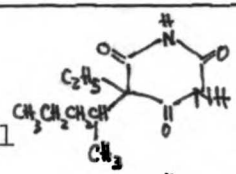
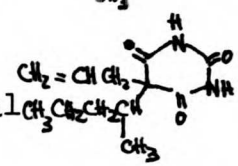
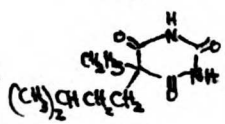
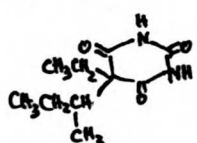
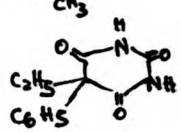
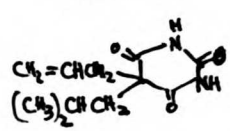
Barbiturates	Chemical structure	Type of barbiturates	Ave. conc. in blood produced by 600 mg oral doses (mg / liter)	Lethal Levels: Barbiturates alone (mg / liter)	Lethal Levels: Barbiturates plus ethanol (mg / liter)	References
Pentobarbital		Short acting	3.3	15-44	10-24	18, 19
Secobarbital		Short acting	4.8	15-24	10-24	18, 19
Amobarbital		Intermediate acting	9.6	30-54	10-29	18, 19
Butabarbital		Intermediate acting	14.0	30-39	15-19	18, 19
Phenobarbital		Long acting	23.0	105-134	10-54	18, 19
Butalbital		Intermediate acting	Not reported	13	Not reported	20

Table 1. Pharmacologic Properties and Toxic Levels of Barbiturates

levels for the short-acting and long-acting barbiturates are given in table 1. McBay (21) reported that when the blood concentration of ethanol is > 1g/liter (0.10%), as little as 5 mg of barbiturates/liter of blood is sufficient to cause death. Usually 1.2 g of a short-acting barbiturate can cause death; but only 600 mg may be sufficient when the blood alcohol concentration is approximately 1.5 g/liter.

Table 2 gives a summary of the pharmacologic properties and the lethal levels of non-barbiturate sedative-hypnotics. Glutethimide is lipid soluble and only slowly absorbed from the stomach and small intestine (22). As a consequence, in acute overdose coma may be dangerously prolonged unless the stomach and upper gastro-intestinal tract are promptly cleared of unabsorbed drug. In a comatose patient blood concentrations may reach a steady state at a relatively low level (10 to 15 mg/liter) and remain at that level for many hours (22). If a series of determinations made show relatively unchanged concentrations over 18 to 24 hours, the situation is considered to be extremely dangerous.

Although lethal blood concentrations of ethchlorvynol are usually greater than 60 mg/liter, ethchlorvynol concentrations as low as 25 mg/liter have been found to be lethal when small amounts of ethanol (0.05 to 0.15%) are also ingested concurrently (27). Unlike glutethimide methaqualone is readily absorbed from the gastro-intestinal

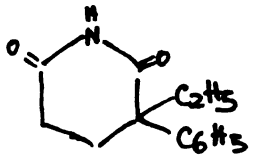
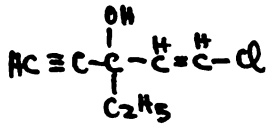
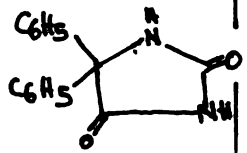
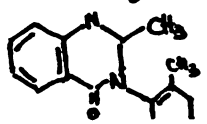
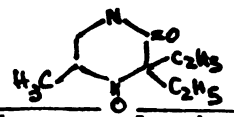
Drugs	Chemical structure	Ave. blood conc. (mg / liter) / Oral doses	Lethal blood levels (mg / liter)	References
Glute-thimide		7 / 1 g	30	22, 23
Ethchlor-vynol		< 2 / 200 mg	60	24
Phenytoin		10 / 600 mg	70	18
Metha-qualone		2 / 250 mg	5	25
Methypry-lon		10 / 650 mg	90	26

Table 2. Pharmacologic Properties and Lethal Levels of Some Sedatives and Hypnotics

tract. It is concentrated primarily in liver and fat. Lethal blood concentrations of methaqualone are usually greater than 5 mg/liter (25).

Methyprylon blood concentrations in excess of 90 mg/liter have been reported prior to death (26). Serum concentrations of 70 mg/liter produce coma. At concentrations of 30 mg/liter the patient is usually rousable and able to respond to questions.

Extraction of Acidic and Neutral Drugs

Many approaches have been described for the extraction of acidic and neutral drugs and their metabolites from blood. All these methods involved extractions with organic (non-polar) solvents followed by back extraction into aqueous (polar) solvents, such as sodium hydroxide or hydrochloric acid (28-32).

Other extraction methods often used for the analysis of drugs in urine primarily involve drug adsorption on cation exchange resin loaded paper (32), XAD-2 non-ionic adsorbent resin (33,34), and charcoal (35). Prantitis and his co-workers (34) reported the use of Amberlite^{R} XAD-2 resin for the extraction of drugs from biofluids other than urine. XAD-2 resin is a styrene-divinyl benzene copolymer which adsorbs drugs mainly by Van Der Waals forces, hydrophobic binding, and dipole-dipole interactions (36-38).

As an adsorptive agent, charcoal has several

advantages over other materials. Most drugs are completely bound to a small amount of charcoal (39) and are easily eluted from it by small amounts of solvents. Meola and Vanko (40-43) developed several adsorption procedures for drugs in blood and urine using charcoal. In all of these methods, however, recovery studies were not done.

Thin Layer Chromatography (TLC)

Thin layer chromatography is a technique for separating drugs by use of a thin layer of adsorbent material coated onto a supporting plate. The mixture of drugs to be separated is applied on one end of the plate and the plate is developed by a solvent passing through the adsorbent layer by capillary action. The competition for active adsorbent sites between solvent and drugs in the mixture produce separation of the drugs. As the process continues, the drugs in the mixture migrate different distances along the plates depending on their relative affinities for the adsorbent and the solvent.

Biological fluids can be prepared for TLC by a variety of methods (29,33-46). The concentrates of these extractions are applied on several TLC plates using different solvent systems. The drugs are then identified by employing chromogenic sprays which develop specific colors with individual drugs. Davidow et al. (47) described a simplified procedure by showing that a single chloroform extraction at pH 9.5 will recover sufficient amounts of basic,

neutral and acidic drugs. He also introduced a developing solvent system (ethyl acetate : methanol : ammonium hydroxide 17:2:1) which resolves the most frequently abused drugs in one development.

Cochin and Daly (48) developed a TLC method for the analysis of sixteen barbiturates and four nonbarbiturates hypnotics (methyprylon, glutethimide, ethinamate, and etchlorvynol) in urine, blood, and tissues. Aliquots of blood were adjusted to pH 5.0 and shaken with 3 volumes of methylene chloride. This extraction step gave recoveries of 90 to 100% for the barbiturates. The extracted residue was dissolved in a small volume of ethanol and spotted on chromatoplates with silica gel G 25g/50 ml. The total time from sample collection to completion of chromatography averaged about three hours. The mercurous nitrate spray allowed the detection of 1 to 5 µg for the barbiturates and glutethimide (white spot on light gray background), but was less sensitive for the other three nonbarbiturate hypnotics (10 to 20 µg). However, these nonbarbiturate hypnotics could be detected at 5 to 10 µg by using a potassium permanganate reagent. Caffeine and aspirin did not interfere. Amobarbital and pentobarbital did not separate even with two dimensional chromatography. Metabolites of some of the barbiturates were identified.

Dunlop and Curnow (49) modified the microscopic TLC slide preparation of Hofman (50) for the screening of some barbiturates and hypnotics drugs. Unlike Cochin et al.

(48), they separated neutral hypnotics from weak acidic barbiturates by back extraction. In their opinion, it was essential to separate the neutral and acidic drugs before chromatography to prevent any possible interference from metabolites. The sensitivity obtained was 1 μg for the barbiturates and glutethimide, 5 μg for meprobamate, and 10 μg for carbromal, using a NaOCl-KI-starch reaction. A recovery of 90% for diethyl barbituric acid at the level equivalent to 10 mg/100 ml plasma was obtained. The chromatographic time was considerably shortened when compared with the standard 20 cm plates used by Cochin et al. (48) and by Sunshine (29).

In addition to the above described TLC screening methods for sedative and hypnotics, several other TLC methods for the detection of barbiturates have been reported (51,52). Mule (53) suggested the following chromatogenic spray sequence for the detection of barbiturates. (1) 10% ammonium hydroxide followed by observation under ultraviolet light (no reaction is observed with non-fluorescent indicator plates. With indicator plates, the barbiturates appear blue on an orange background). (2) 0.1% aqueous $KMnO_4$ (all unsaturated substituted barbiturates, such as secobarbital, appear as light yellow spots). (3) 1.0% silver acetate spray (barbiturates appear as white spots). (4) 0.1% diphenylcarbazene (barbiturates appear blue on a yellow background).

Ultra-Violet (UV) Spectrophotometry

The major role of ultraviolet spectrophotometry is for the analysis of weak acids, neutrals, and certain weak bases in blood. The most common ultraviolet absorbing neutral drug is glutethimide (Dorinden^{R}). Certain other weak bases such as diazepam (Valium^{R}) and methaqualone are found in both the basic or neutral fractions depending upon the choice of solvent for extraction.

Although recording UV spectrophotometers are easy to use, they have several drawbacks. The main disadvantage is that drugs which do not have a sufficiently strong ultraviolet spectrum cannot be analyzed by absorption spectrophotometry. This eliminates drugs such as methyprylon and ethchlorvynol. The $E^{1\%}_{1cm}$ extinction coefficient is useful in evaluating this property. Extinction coefficient is defined as the absorbance of 1% (1gm/100ml) solution of a drug in a defined solvent in a 1 cm path length cuvette. Compounds with an $E^{1\%}_{1cm}$ of $\lesssim$200 cannot be effectively analyzed in blood (54). Even for drugs with relatively intense UV absorption spectra, a sufficiently high blood level must be reached for UV spectrophotometric analysis ($>$2μg/ml). Finally, UV spectrophotometry will not resolve mixtures of drugs with overlapping absorption spectra and cannot differentiate between different drugs which have similar spectra. This deficiency can only be overcome by separating drugs prior to spectrophotometric analysis.

Most of UV spectrophotometric method for barbiturates analysis have been adapted from the method described by Goldbaum and his co-workers (55). Differential spectrophotometry described by Williams and Zak (56) provides a spectrum which is characteristic of 5,5 disubstituted barbiturates and eliminates most interfering substances. However, the UV spectrophotometric method does not allow individual barbiturate identification. After barbiturates are back extracted into 0.45 N NaOH, one aliquot is adjusted to pH 10 by the addition of 10.7% NH_4Cl while the other is maintained at 13. The pH 13 aliquot is placed in the sample beam and the pH 10 in the reference beam of a recording spectrophotometer and a differential spectrum is obtained. Jatlow (57) has tested a large number of other potentially interfering weakly acidic drugs and found that none of them (including salicylates) interfere because they are either not extracted, or they do not have differential spectra similar to barbiturates.

There are several reports in the literature concerning the determination of glutethimide by UV spectrophotometry (58,59). The general principle of all these methods is that the products of alkaline hydrolysis of glutethimide have a distinct absorbance at 230 nm. Glutethimide is extracted from the biological matrix into non-polar organic solvent. The non-polar solvent is then evaporated and the residue is dissolved in a mixture of hexane and ethanol.

An aliquot of ethanol is removed and alkalinized. The alkalinized sample is immediately analyzed by UV spectrophotometric analysis. The absorbance is then measured 10 minutes later and the concentration of glutethimide is calculated from the absorbance change ($Absorbance_{10}$ - $Absorbance_{0}$).

Methaqualone is generally analyzed by extraction of serum in hexane and back extraction in HCl (60). Methaqualone is then identified and quantitated from its characteristic spectrum at 235 nm.

Gas Liquid Chromatography (GLC)

Gas chromatography is a process by which a mixture of volatiles compounds are separated by the interaction between a carrier gas phase and a stationary phase, which is either solid or liquid. Under controlled conditions the individual components of the sample, in accordance with their vapor pressure, will be present partially in the stationary phase and partially in the carrier gas phase. A compound with a high vapor pressure will be present to a greater extent in the gas phase. Thus, this compound will be eluted more rapidly than compounds with low vapor pressure. If there is selective interaction between a compound in the sample and the stationary phase, the order of elutions from the column may be different. Gas chromatography is divided into two major categories: (1) gas-solid

chromatography (GSC), in which the sorbent is a solid of large surface area, and (2) gas-liquid chromatography (GLC), in which a nonvolatile liquid (stationary phase) is coated on an inert solid support.

Most GLC methods for the analysis of barbiturates and other acidic and neutral drugs of interests utilize OV-17, OV-1, Dexil 300, and SE-30 liquid phase columns (61). The adaptation of derivatization procedures employing either alkylation or silyation are also often used in such analyses (62-63). Other factors being equal, the derivatized barbiturates can usually be analyzed with less peak-tailing than non derivatized method. In addition, derivatized compounds often yield less irreversible absorption on the column resulting in greater accuracy and reproducibility than is the case for underivatized compounds.

OV-1 and SE-30 possess similar selectivity and polarities and will, therefore, give comparable separation with similar retention time. OV-17 possesses a higher selectivity and polarity than OV-1 and may resolve some drugs better than OV-1, but will generally give longer retention times (61). Berry (64) has made a critical evaluation of some of the commonly used GC columns in order to find a single column able to give reliable, accurate, and specific identification of barbiturates at both therapeutic and toxic concentrations. Out of 12 columns tested, Berry determined that cyclohexanedimethanol succinate (CDMS)

was the best column for his purpose, and that OV-225 proved to be the second most useful column.

The availability of a wide variety of columns allows one to choose a second chemically different column to confirm the identity of unknown peaks eluted by the first column. The need for such practice is to prevent any false positive identification of compounds (65). A typical illustration of the unreliability of determining an unknown peak solely based on the information derived from one column was described by Sine and his co-workers (66). In a suspected case of poisoning a single symmetrical peak corresponding to glutethimide was subsequently discovered by them to be an organo-phosphorus insecticide. Law et al. (67) also reported the unreliability of using one column in gas chromatography.

One of the earliest attempts to separate barbiturates in biological fluids by GLC was that of Janak (68). He separated the pyrolytic products of barbiturates by heating the sample to 800°C. Prior to 1970's, GLC methods were developed for barbiturates only. MacGee (69) in 1971 introduced a GLC method for the separation of five common barbiturates and glutethimide. The blood was acidified and the sedatives extracted into toluene. The drugs were derivitized to ethyl derivatives by treating with tetra-ethylammonium hydroxide.

Fiereck and Tietz (70) described a GC method for the

quantitative determination of eleven barbiturates and glutethimide in blood. This method was based on the technique of on-column methylation described by Brochmann-Hanssen and Oke (71). A 7% DC-200 column was used for the separation, and methohexital was utilized as an internal standard. Although good accuracy and precision was achieved, methyl derivatives of hexobarbital and glutethimide could not be separated. In addition, phenobarbital and mephobarbital formed identical 1,3-dimethyl derivatives.

Flanagan and Withers (72) described a rapid micro method for the screening and measurement of some barbiturates, glutethimide and methaqualone using a column packed with 4% cyclohexanedimethanol succinate (CDMS). They extracted 50 µl of plasma with 50 µl of chloroform containing 10 µg/ml tetra-phenylethylene (TPE), the internal standard, in the presence of 5 µl of 4.0 M/liter sodium dihydrogen orthophosphate buffer. After thorough mixing and centrifugation, the organic layer extract was directly injected into the GLC.

Sine et al. (73) developed a simultaneous GLC determination of six barbiturates, phenytoin, glutethimide, methyprylon, and some antidepressants. 3 ml of acidified serum was extracted with 15 ml of chloroform. After the chloroform layer was evaporated to dryness, the residue was dissolved in exactly 200 µl of 250 mg/l caffeine (internal standard). The sample was then injected into a

column packed with 3.8% SE-30 on acid washed dimethyldichlorosilane (AW-DMCS) treated chromosorb W (80/100 mesh). A temperature program at a rate of 12°C per minute from 170°C to 240°C was used. The analysis time was approximately 12 minutes and recovery of drugs ranged from 68% to 100%.

The above procedure was later modified by Rice and Wilson (74). As little as 0.5 ml of acidified plasma could be successfully extracted into diethyl ether instead of chloroform (73) and separation was accomplished at two different columns temperatures, 160°C and 180°C, on a column packed with 2% SE-30 chromosorb W (80/100 mesh). The low temperature, 160°C, was necessary for adequate separation of secobarbital from meprobamate. Once the identification was made, the column temperature was raised to 180°C to speed the analysis and also to decrease peak tailing especially with phenobarbital and meprobamate. No internal standard was used in this method. Automatic integrating equipment was necessary for peak area calculation in this procedure because resolution between amobarbital and phenobarbital as well as secobarbital and meprobamate were not complete to enable quantitation by peak height measurements.

Kaufman (75) published a simultaneous method for the screening of six commonly used barbiturates and two similar acid extractable drugs (glutethimide and methyprylon) by

GLC using a 5% OV-1 column with temperature programming from 130°C to 185°C. Sample preparation time was 20 to 30 minutes and the analysis time was 8 minutes. No internal standard was incorporated.

Levy and Schwartz (76) developed a mixed bed column for rapid gas-liquid chromatographic determination of sedatives and phenytoin concentrations in serum. A mixed bed column composed of 6% SE-30 (non-polar methylsilicone) and 4% XE-60 (moderately polar cyanoethylmethylsilicone gum) liquid phase on chromosorb W-AW solid support was used to separate eight barbiturates, glutethimide, and phenytoin. Due to the polarity of the mixed bed column it was not necessary to convert the free barbiturates to their dimethyl derivatives to achieve a shorter elution time. However, phenytoin could not be run simultaneously, and has to be derivatized according to McGee's flash pyrolytic trans-methylation (77).

A comprehensive dual temperature program and dual column (3% OV 1 and 3% OV 17) GLC analysis for sedative drugs was developed by Thoma and Bonda (78). The procedure provided for the identification and quantitation of barbiturates, glutethimide, meprobamate, methyprylon, benzodiazepines, methaqualone, and propoxyphene as the free drug in serum. The drugs were first identified by injecting into an OV-17 column using a temperature program from 165°C to 280°C at a rate of 32°C per minute. Confirmation of the

identified peak was then followed by derivitizing the same sample with trimethyaniline hydroxide (TMAH) solution on an OV-1 column, and using a second temperature program from 135°C to 250°C at a rate of 20°C per minute. The time to the last peak, 5-(4-methylphenyl)5 phenylhydantoin (internal standard) was less than 6.5 minutes for both columns. With the relative retention times from two columns that had different characteristics of separation, significant improvement in identification of compounds of toxicological importance was achieved (fig. 1 and 2).

Flanagan and Berry (79) published a modified GLC method for the determination of some common sedative-hypnotics. The major improvement had been the simultaneous use of a second GLC system containing Poly A 103 liquid phase to supplement the information derived from the extract analysis performed upon the CDMS system of their original method (72). The second column was isothermally operated in a dual detector chromatograph simultaneously with the primary CDMS column, and the outputs were monitored independently. The original micro-extraction technique was again emphasized over conventional bulk extraction methods in drug analysis (79). The major advantages of the adaptation of the second column system were: (1) to confirm the drug identification and to differentiate between the few drugs which co-chromatographed on the CDMS column; (2) to eliminate the need for complementary TLC

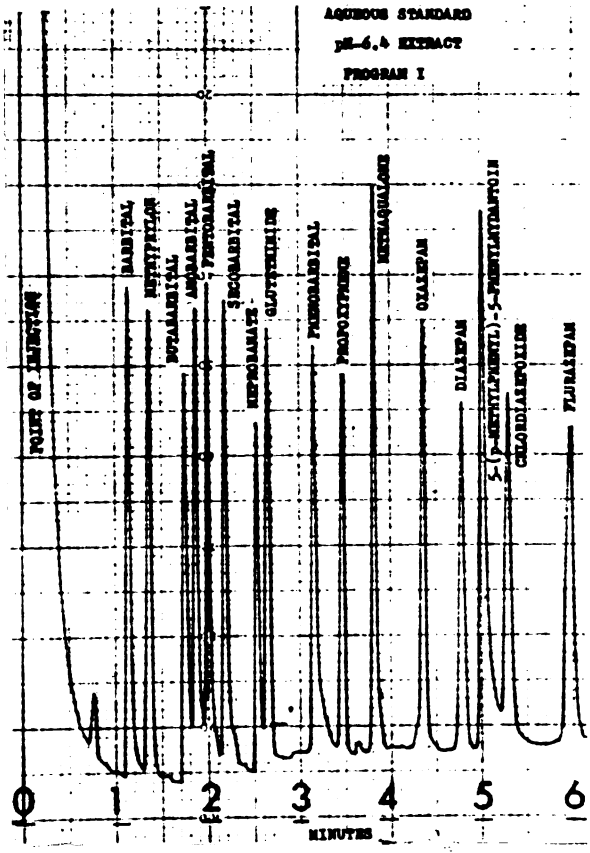


Figure 1. Chromatogram of underivatized drugs

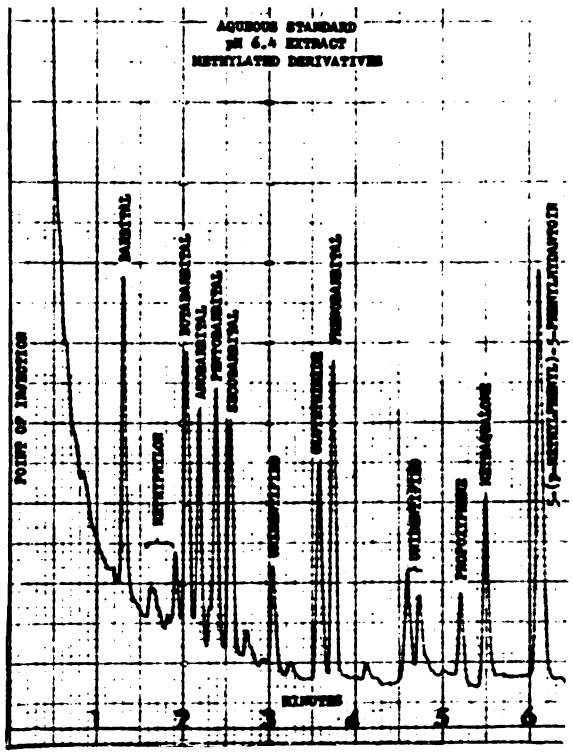


Figure 2. Chromatogram of TMAH derivatives of drugs

analysis for confirmation; and (3) to quantitate meprobamate, which was fully resolved from TPE (internal standard) on Poly A 103 system, but not on the CDMS system. Chromatograms from these two columns are illustrated in fig. 3 and fig. 4.

Gas Chromatography/Mass Spectrometry (GC/MS)

The combination of gas chromatography and mass spectrometry has given the analyst a most powerful and versatile tool. The recent increasing use of this technique has largely been due to the introduction of smaller, lower priced medium-resolution mass spectrometers designed to be linked to a gas chromatograph.

Mass spectrometry functions by producing ions whose mass to charge ratios are quantitated. Methods for the analysis of barbiturates and other drugs of interest have been developed by a number of investigators (67, 80-82). Reference mass spectral data have been accumulated by spectroscopists (80-82) and can be conveniently used manually or computerized for rapid identification of drugs and metabolites.

Fales et al. (82) presented a method for the mass spectrometric identification of barbiturates using chemical ionization techniques. They emphasized that utilizing electron impact techniques unstable molecular ions of the barbiturates were formed which created detection problems.

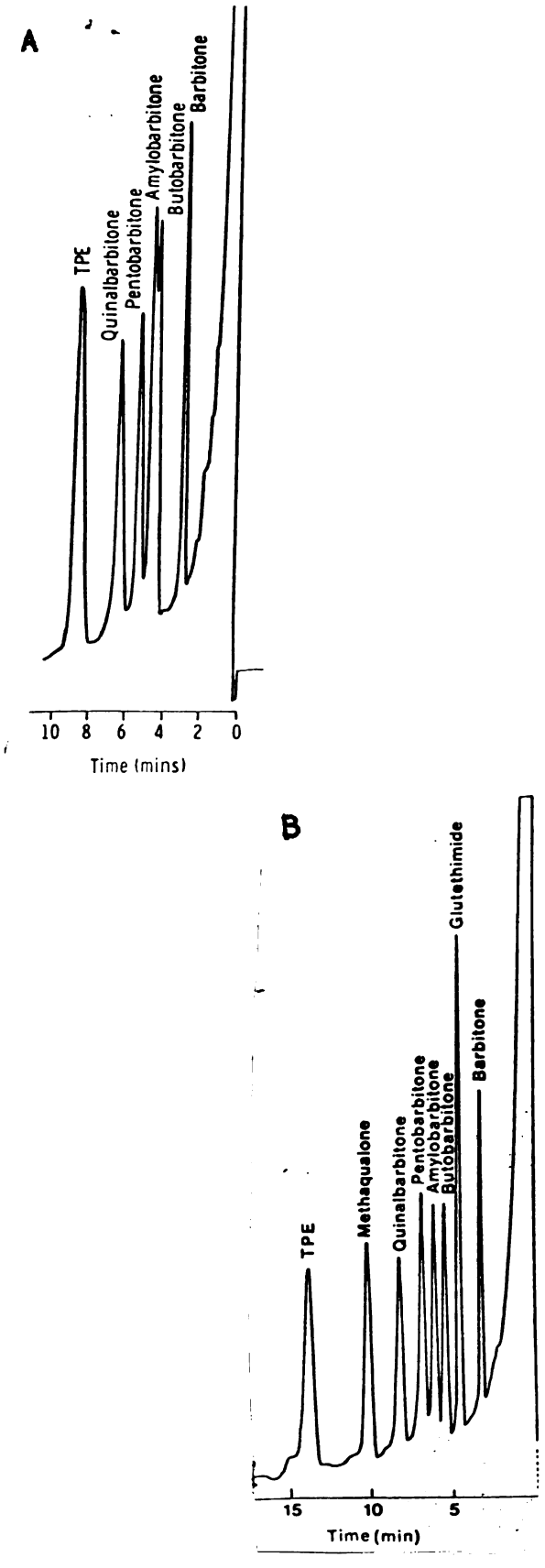


Figure 3.
A. Analysis of the barbiturate standard mixture on the CDMS system
B. Analysis of glutethimide and methaqualone together with some barbiturates on the Poly A 103 system

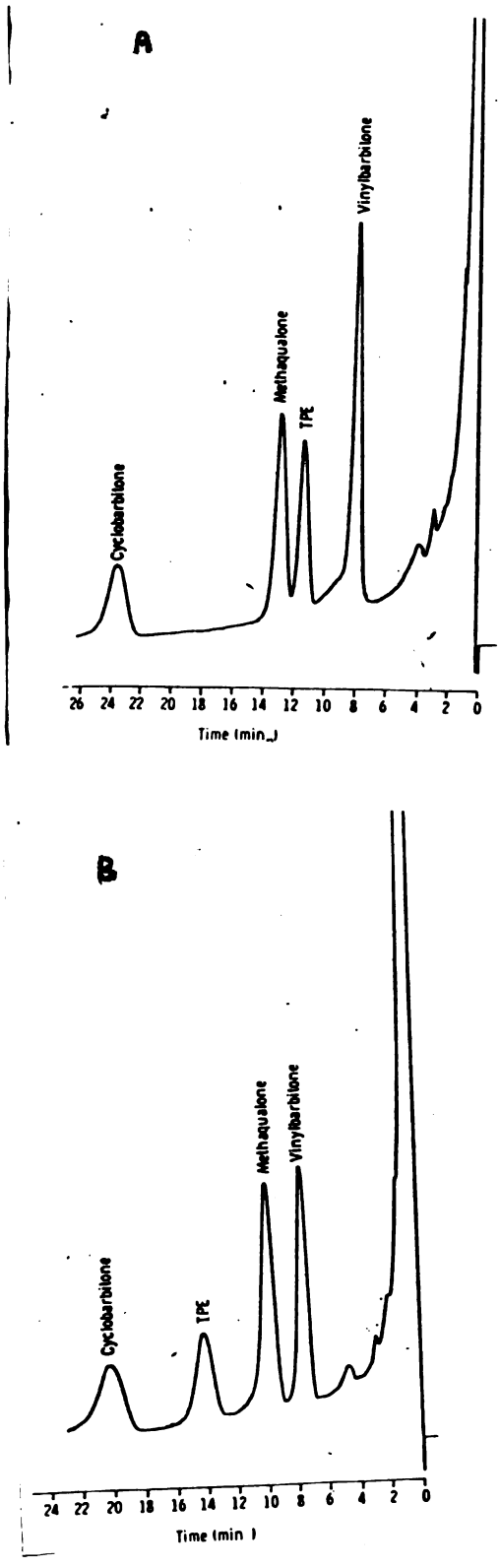


Figure 4.
Analysis of sample extracts from a patient after a multiple drug overdose. (A) CDMS system; (B) Poly A 103 system.

Fragmentation usually occurs by loss of CO and one of the two side chains. However, many barbiturates possess similar side chain so that loss of the other substituents may cause confusion. False et al. demonstrated that chemical ionization mass spectrometry with methane provided intense quasi-molecular (QM^+) ions at m/e $(M+1)^+$ in all eight representative barbiturates. Isomerics barbiturates such as amobarbital and pentobarbital could not be distinguished in this way but could be identified from their electron impact mass spectra which could be obtained by closing the methane valve.

Law (67) reported the use of the chemically more energetic H^+_3 ion generated by hydrogen gas as a reactant ion. He noted that although the formation of quasimolecular ion by chemical ionization technique was a specific method for determining molecular weight, there could be more than one metabolite and/or artifact which could give rise to the same "quasimolecular" ion in biological samples. However, it was shown that the use of H^+_3 ion could solve this problem. Usually, but not always, the molecular ion was present in the greatest abundance (i.e., the largest peak) but some cleavage occurred and the minor peaks helped to differentiate between similar drugs (e.g., amobarbital and pentobarbital).

High Pressure Liquid Chromatography (HPLC)

Liquid chromatography is one of the oldest and may be the most powerful form of chromatography. In 1906, a Russian botanist, Michael Tsweet (84) invented a technique for plant pigments separation and called this technique adsorption chromatography. Reichstein, in 1938, was the first chemist to develop flow chromatography; and in 1967, Huber and Hulsman introduced high speed (pressure) liquid chromatography (85).

Although liquid chromatography and gas chromatography share the same fundamental principles concerning the process of separation, there are several major differences between these two analytical tools. Substances of molecular weight >500 cannot be analyzed by GC, and many compounds of molecular weights of 300 or more have to be derivatized to form volatile compounds. In addition, many thermally labile compounds cannot be analyzed by GC. GC separations depend on vapor pressure while LC separations are based on solubility. The choice of the carrier gas used as the mobile phase in GC is predominantly dictated by the types of detector or sensor being used to monitor the column effluent. The separation will be similar whether hydrogen, helium, argon or nitrogen is used as the carrier. With liquid chromatography, the mobile phase is of prime importance in the separation.

One of the most significant differences between GC

and LC is the much lower (by a factor of 10^4) diffusivity of a liquid compared to a gaseous mobile phase. As a result, a Van Deemter plot of efficiency versus flow velocity for an HPLC column shows a much smaller efficiency optimum and this occurs at a much lower flow velocity optimum. Furthermore, efficiency decreases as the velocity is increased from the optimum. Current HPLC practice uses flow velocities considerably higher than the optimum. The small loss in efficiency is offset by a large reduction in separation time. However, high flow velocity produces higher column back pressure. In addition, the high pressure caused by the low diffusivity of the liquid mobile phase demands special attention in the design of the sample injection port.

There are several mechanisms of separation in liquid chromatography, namely, liquid-solid chromatography (adsorption and ion exchange) and liquid-liquid chromatography (normal phase partition and reverse phase partition). In liquid-solid adsorption chromatography, the stationary phase is the liquid-solid interface. Molecules are reversibly bound to this surface by dipole-dipole interaction. Liquid-solid adsorption chromatography is most often used for polar, non-ionic organic compounds. Ionic compounds are often better separated by ion-exchange chromatography. In this case, the stationary phase consists of acidic or basic functional groups bound to the surface of a polymer

matrix. Charged species in the mobile phase are attracted to appropriate functional groups on the ion exchanger.

The basic distribution mechanism in liquid-liquid chromatography is partition. Distribution is based on the relative solubility of the sample in the two phases. In normal partition, the stationary phase is more polar than the mobile phase. In reverse partition, the mobile phase is more polar than the stationary phase. Normal phase partition is used for polar compounds, while reverse phase is commonly used for non-polar compounds. The stationary phase may be either coated onto a support or chemically bonded to that support. In addition, exclusion (gel filtration or gel permeation) chromatography can be classified as liquid-liquid or liquid-solid chromatography. The stationary phase is usually a porous matrix permeable to mobile phase. Sample molecules small enough to enter the pore structure are retarded, while large molecules are excluded and are therefore rapidly eluted through the column.

Twitchett and Moffatt (85) gave a detailed evaluation of an octadecysilane (ODS) stationary phase column, which is also commonly called a reverse phase column. Over 30 drugs were evaluated on the ODS column as representatives of a wide variety of drugs, namely, strongly acidic, weakly acidic, neutral and basic drugs, and of varying chemical structure, molecular weight, lipid solubility and

pharmacological action. Chromatographic behavior was found to be highly predictable on the basis of pKa and lipid solubility measured in terms of the n-octanol/water partition coefficient. The ODS stationary phase was found to be especially valuable for the separation of acidic and neutral drugs but less so for basic ones.

The same investigators later gave another report on the evaluation of other HPLC columns for the identification and quantitation of drugs and metabolites (86). The results of their evaluation are as follows: (1) On cation exchange (Partisil-SCX) columns, retention depends on ionic strength of the eluent, while on reverse phase partition (ODS) column retentions depend on the eluent pH and organic solvent content. (2) HPLC has the ability to handle aqueous samples directly without the need for preliminary extraction procedures. (3) The use of reverse phase separation allows more polar drug metabolites to be eluted before the parent drug. (4) The use of specific detectors allows for the detection of trace quantities of drugs or metabolites in the presence of a large background of other components. (5) Preparative HPLC is simple and the separated components of a mixture can be collected for further analysis. This evaluation, especially the first three findings, is further substantiated in this presentation.

Although the use of HPLC has been extensively reported

upon for the analysis of various therapeutic drugs (87-91), there are only a very few reports concerning the utilization of HPLC for sedative and hypnotic drug screening (92-96). None of these methods include the major hypnotics presented in this paper. Dixon and Stoll (92) developed an HPLC system for the detection of 6 barbiturates (barbitone, phenobarbital, butabarbital, amobarbital, pentobarbital, and quinalbarbitone) using a C18 reverse phase column. They used a single extraction procedure, similar to that of common GC methods, and detected the drugs at 216 nm. Recovery varied from 68% for barbitone to 100% for amobarbital.

During the course of Dixon and Stoll's work other workers have reported on barbiturate analysis by HPLC (97, 98). These studies were either for pharmaceutical preparation analysis, or used ion-exchange chromatography of dansyl derivatives with fluorencense detection (99).

In 1977 Kabra et al. (91) developed a rapid, simultaneous analysis of five anticonvulsant drugs by reverse phase liquid partition chromatography. These anticonvulsant drugs could be analyzed simultaneously in less than 14 minutes. This system was found to be applicable to the analysis of some common weak acidic and neutral sedatives and hypnotics. Based on these earlier observations the present method was developed to analyze the commonly used barbiturates and hypnotics.

There are three distinctive features of the previous study. (1) The sample preparation is minimal and solvent extraction is not mandatory. (2) The utilization of UV "end absorption" phenomenon to detect drugs that show great absorption at 195 nm but little absorption at 254 nm, the common wavelength used in HPLC detection system. (3) The use of greater than ambient temperature (50°C) to increase the speed of elution, obtain better resolution, and decrease column back pressure. Sample size can be as low as 25 ul. The lower limit of detection for all five anticonvulsant drugs was less than 10 ng, and sensitivity was 1.0 mg per liter for serum. Recoveries ranged from 97% to 107% with excellent day to day precision.

The method presented in this study can also be used with as little as 25 ul of serum or plasma. The sample preparation is simple (acetonitrile precipitation of serum proteins), and analytical recovery is excellent for all drugs. The procedure is sensitive enough to analyze 1.0 mg/liter concentrations of these drugs, therefore the method can be adapted to both therapeutic and toxic monitoring. Analysis time is less than 30 minutes.

MATERIALS AND METHODS

Apparatus

A model 601 (Perkin Elmer Corp., Norwalk, Conn., 06856) high pressure liquid chromatograph equipped with a variable wavelength detector (Perkin-Elmer LC 55) and a temperature controlled oven was used. The reversed-phase column "μ-Bondapak C-18" 30 cm x 4 mm (Waters Associates, Inc., Milford, Mass. 07157) was mounted in the oven. The recorder was a Honeywell Electronic Model 194 (Honeywell, Inc., Fort Washington, Pa. 19036). Samples were injected into a Rheodyne 7105 valve (Rheodyne, Berkeley, California 94710) mounted on the chromatograph. The column was eluted with acetonitrile/phosphate buffer (21.5/78.5 by vol) at the rate of 3.0 ml/min at 50°C, and the column effluent was monitored at 195 nm. A model 5412 Eppendorf centrifuge (Brinkmann Instruments, Westbury, N.Y. 11590) and Brinkmann 1.5 ml Eppendorf polypropylene microtest tubes were used for sample preparation.

Reagents

Acetonitrile: Acetonitrile (ultraviolet grade) distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442).

Mobile phase: A solution of 215 ml of acetonitrile in 785 ml of phosphate buffer (pH 4.4).

Phosphate: Add 300 µl of 1 mol/liter KH_2PO_4 and 50 µl of 0.9 mol/liter phosphoric acid to 1800 ml of distilled water.

Ethyl acetate: Analytical grade, Mallinckrodt Chemical Works, St. Louis, Mo. 63147.

Drug Standards

Amobarbital, pentobarbital and Secobarbital were obtained from Sigma Chemical Co., St. Louis, Mo. 63178; butabarbital and phenobarbital were gifts from the University Hospital Pharmacy; butalbital was obtained from Gane's Chemical Inc., Carlsdalt, N.J.; phenytoin from Eastman Kodak Co., Rochester, N.Y. 14650; methyprylon from Hoffman-LaRoche Inc., Nutley, N.J. 07110; ethchlorvynol from Abbott Laboratories, N. Chicago, Ill. 60064; methaqualone from William H. Rorder Inc., Fort Washington, Pa. 19036; primidone from Ayerst Laboratories, Inc., New York, N.Y. 10017; glutethimide from USV Pharmaceutical Corp., Tuckahoe, N.Y. 10707 and the internal standard, 5-(4-methylphenyl)-5-phenyhydantoin, from Aldrich Chemical Co., Milwaukee, Wis. 53233. A standard mixture was prepared as follows: 25 mg each of primidone, methyprylon, phenobarbital, butabarbital, butalbital, ethchlorvynol, pentobarbital, amobarbital, phenytoin, glutethimide, secobarbital and methaqualone,

and 50 mg of 5-(4-methylphenyl)-5-phenyhydantoin were dissolved in 100 ml of methanol. This solution is stable at 4°C for at least three months. Working internal standard was prepared by dissolving 5 mg of 5-(4-methylphenyl)-5-phenylhydantoin in 100 ml of acetonitrile.

Procedure

Method A: Acetonitrile precipitation

Add 200 µl of acetonitrile containing 10 µg of 5-(4-methylphenyl)-5-4-phenylhydantoin (internal standard) to 200 µl of serum or plasma in a Brinkmann Eppendorf 1.5 ml polypropylene microtube (this method can be used with as little as 25 µl of serum or plasma, however, 200 µl is routinely used for pipetting accuracy and convenience). Vortex mix the mixture for 10 seconds, then centrifuge for 1 min. in an Eppendorf 5312 centrifuge. Inject approximately 20 µl of the supernatant into the chromatograph, and elute with the mobile phase at a flow rate of 3.0 ml/min. Column head pressure is approximately 11 MPa (1500 psi). (**Figures 5 and 6 illustrate some representative chromatograms obtained by this procedure.**)

Method B: Ethylacetate extractions

Transfer 300 µl of supernatant from Method A, along with 50 µl of glacial acid to a 12 ml stoppered glass tube.

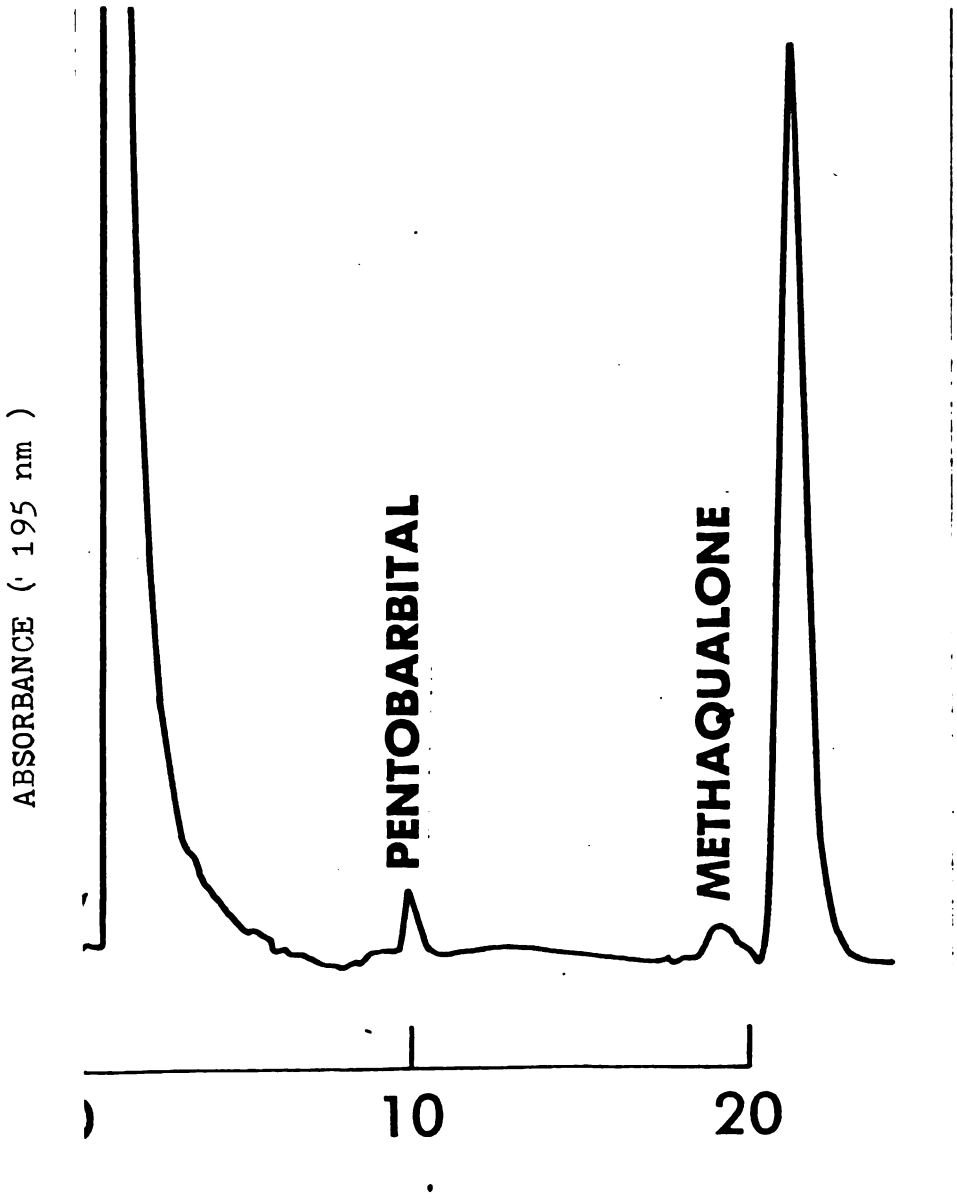


Figure 5. Chromatogram of serum containing 5.6 mg/liter of pentobarbital and 3.6 mg/liter of methaqualone. Sample was prepared by acetonitrile precipitation (method A).

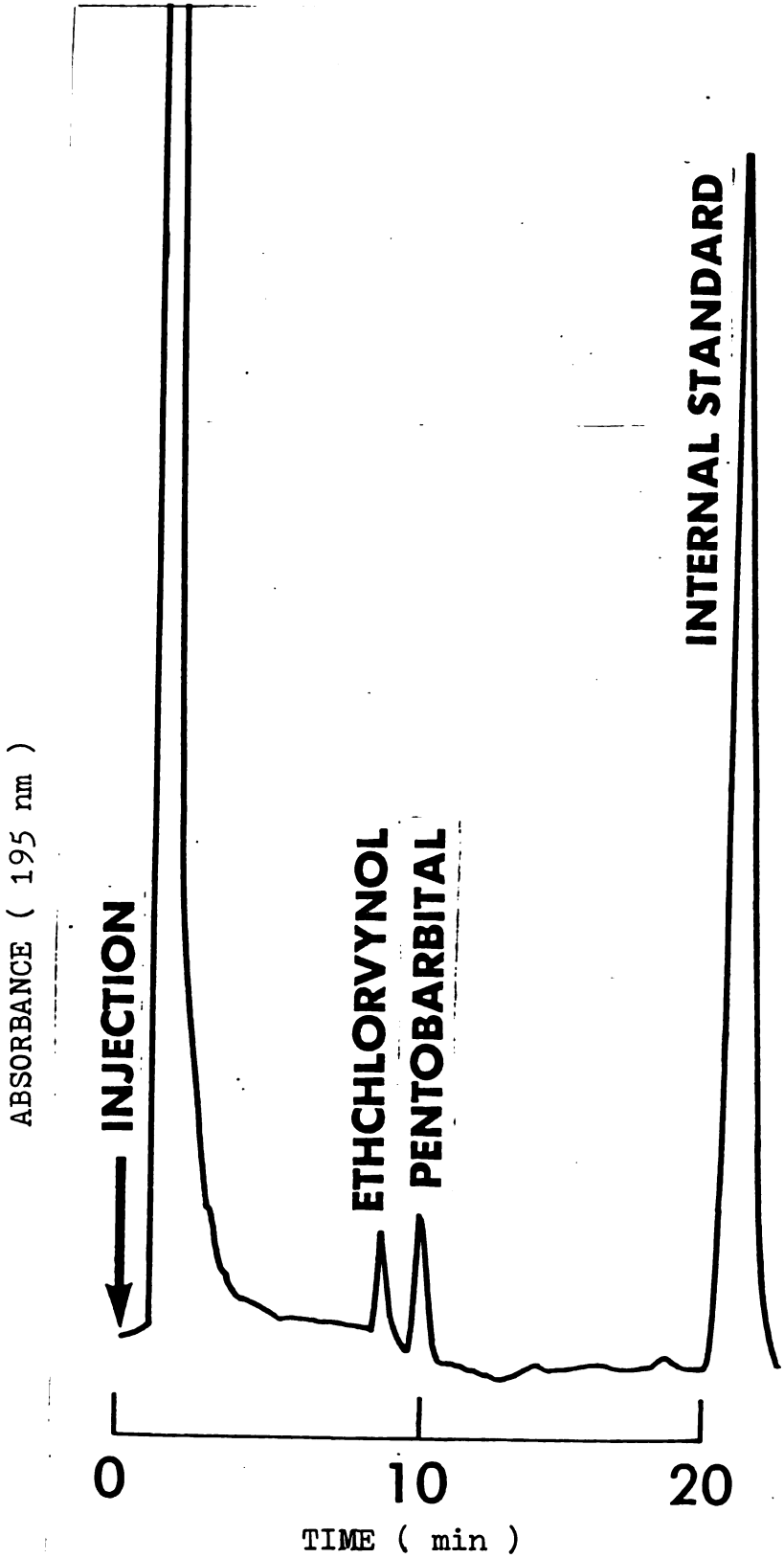


Figure 6. Chromatogram of serum containing 11.8 mg/liter of ethchlorvynol and 9.9 mg/liter of pentobarbital. Sample was prepared by acetonitrile precipitation (method A).

Add 3 ml of ethylacetate and vortex mix for 20 seconds. Centrifuge the tube for 5 min. Decant the ethylacetate and evaporate it at 70°C under reduced pressure in a rotary evaporator. Dissolve the residue in 10 µl acetonitrile. Inject 10-20 µl of the solution into the chromatograph and elute under the same conditions as in Method A. (Figures 7 and 8 are chromatograms obtained by this procedure.)

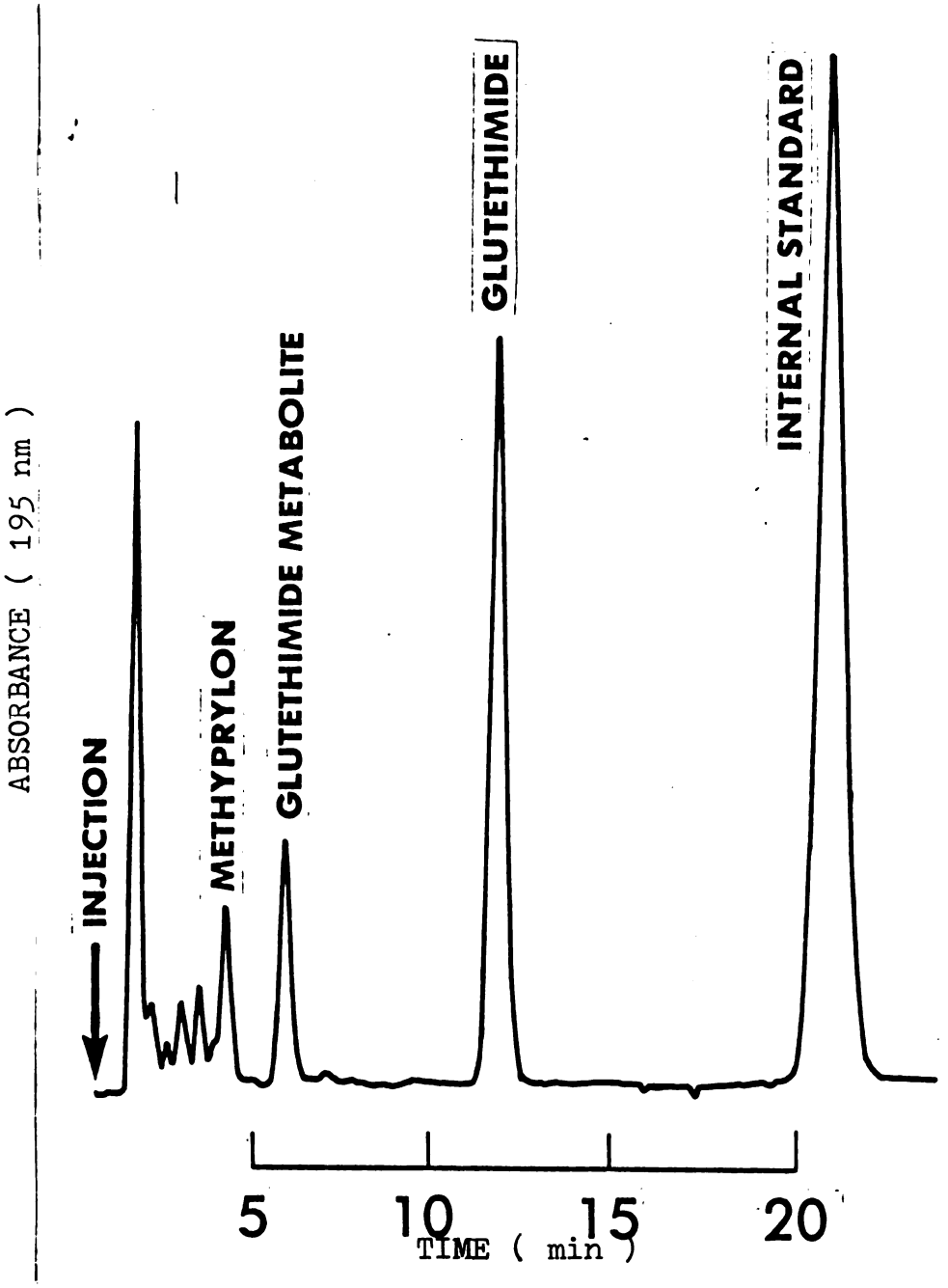


Figure 7. Chromatogram of serum containing 13.5 mg/liter methyprylon and 44.0 mg/liter of glutethimide. The peak which elutes at 6 min. corresponds to a glutethimide metabolite. The sample was prepared by the ethylacetate extraction (method B).

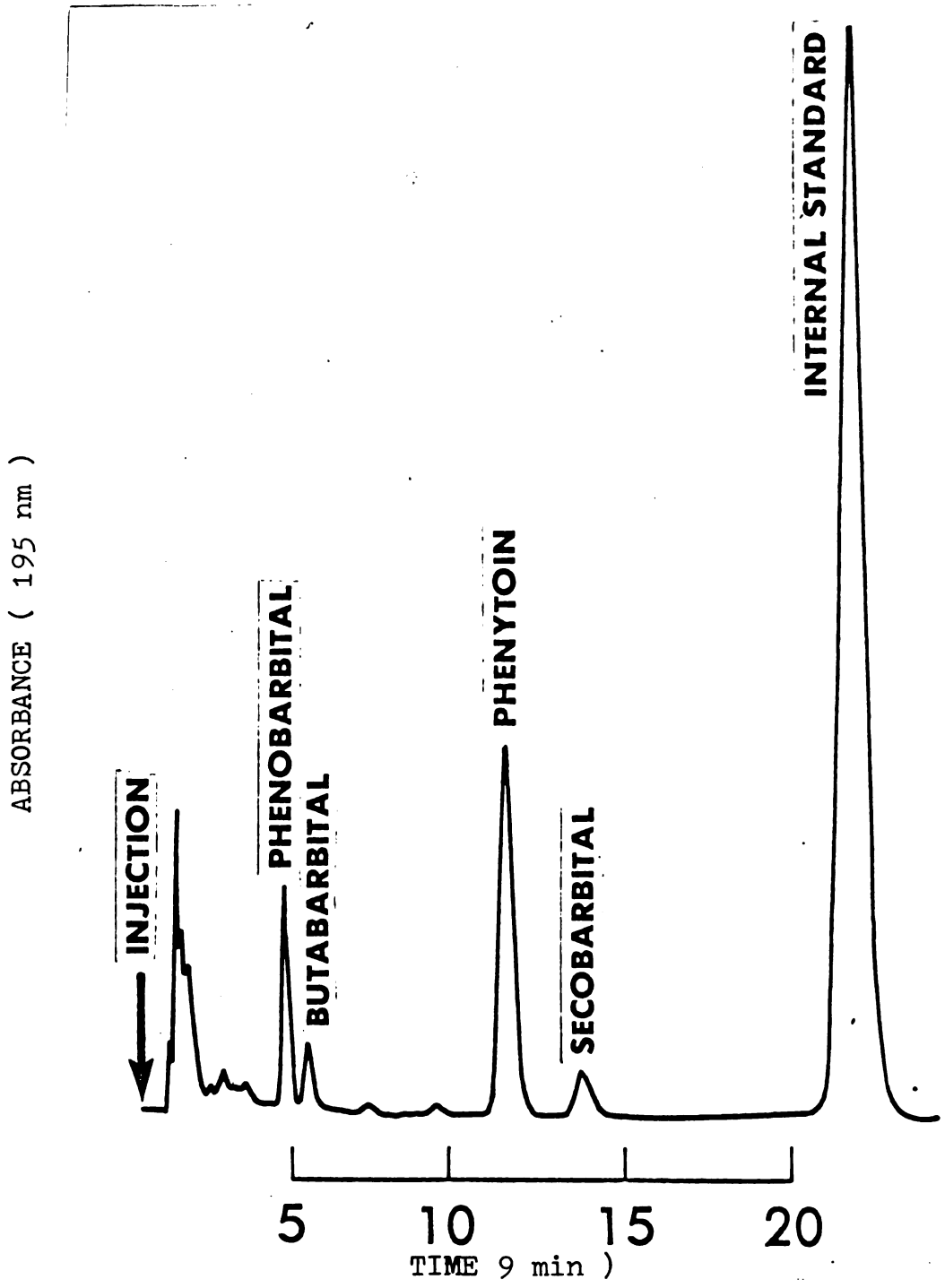


Figure 8. Chromatogram of serum containing 4.0 mg/liter of phenobarbital, 2.5 mg/liter of butabarbital, 2.5 mg/liter secobarbital and 8.7 mg of phenytoin. The sample was prepared by the ethylacetate extraction (method B).

RESULTS AND DISCUSSION

Various chromatographic conditions were evaluated by injecting 250 ng of each drug in 5 µl of methanol. The composition of the mobile phase, the pH of the mobile phase, and the column temperature were varied to achieve optimal chromatographic conditions (figures 9 and 10).

Initially, 50 µg/ml hexobarbital was used as an internal standard and the following chromatographic conditions were tried: (1) mobile phase = 21% acetonitrile, 79% phosphate buffer pH 4.4. (2) Oven temperature = 50°C. (3) Flow rate = 3 ml/min. There was adequate separation between the twelve drugs and hexobarbital, however, hexobarbital elutes between ethchlorvynol and pentobarbital and may interfere with their quantitation. Figures 11 and 12 illustrate two representative chromatograms, one with hexobarbital and one without.

The mobile phase composition was varied to achieve optimal separation. Various ratios of acetonitrile to phosphate buffer (15/85, 17/83, 19/81, 20.5/79.5, 21/79, 21.5/78.5, 22/78, and 23/77 parts by volume) were tried. The elution order of the drugs were unaffected with the change in acetonitrile concentration, however, a definite loss of resolution between pentobarbital and amobarbital was

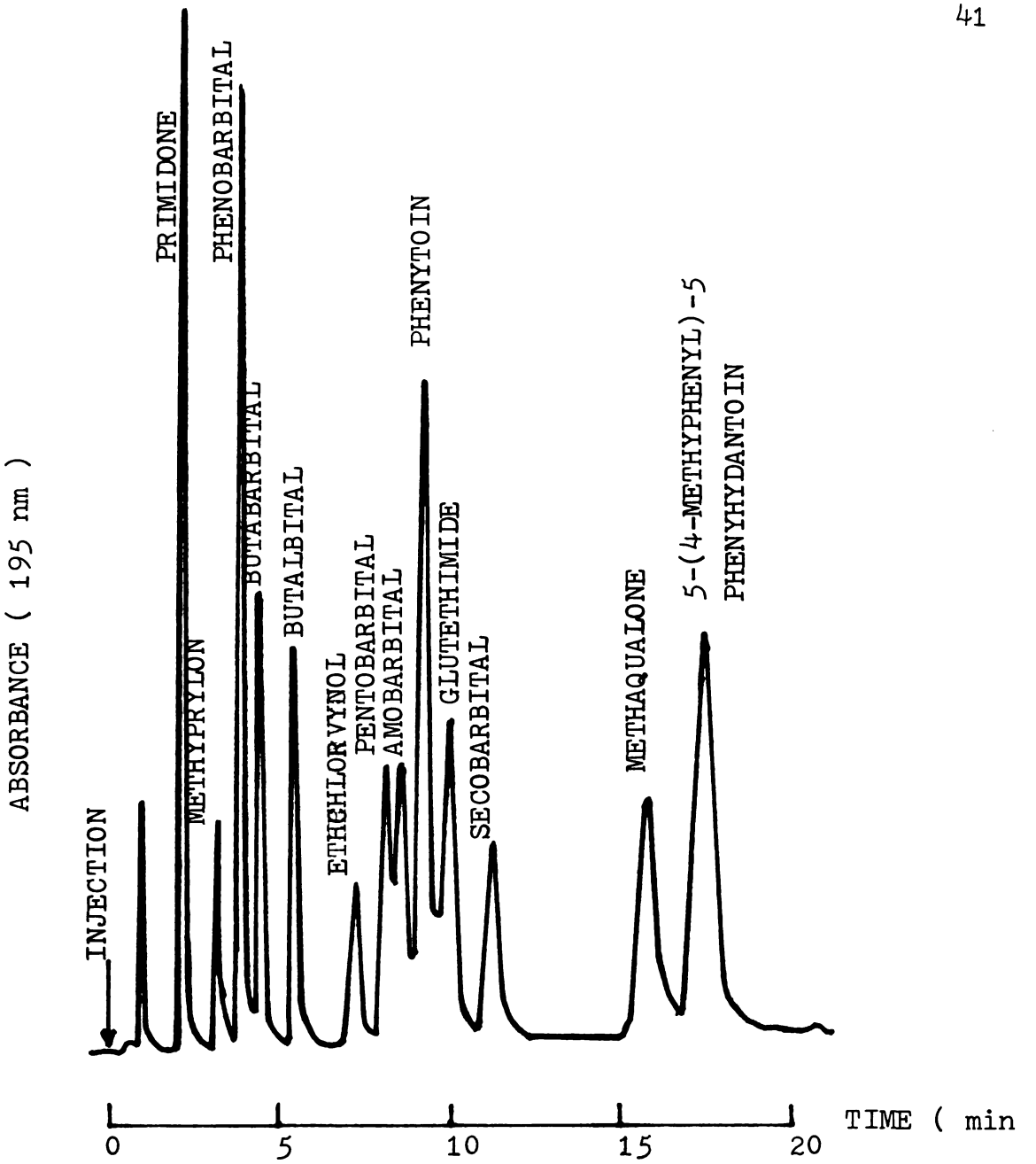


Figure 9. Chromatogram of a standard mixture of drug. 250 ng of each drug was chromatographed except for the internal standard, 5-(4-methyl-phenyl)-5 phenyhydantoin, which was 500 ng. Mobile phase = 21.5 % of acetonitrile. Flow rate = 3 ml/min.

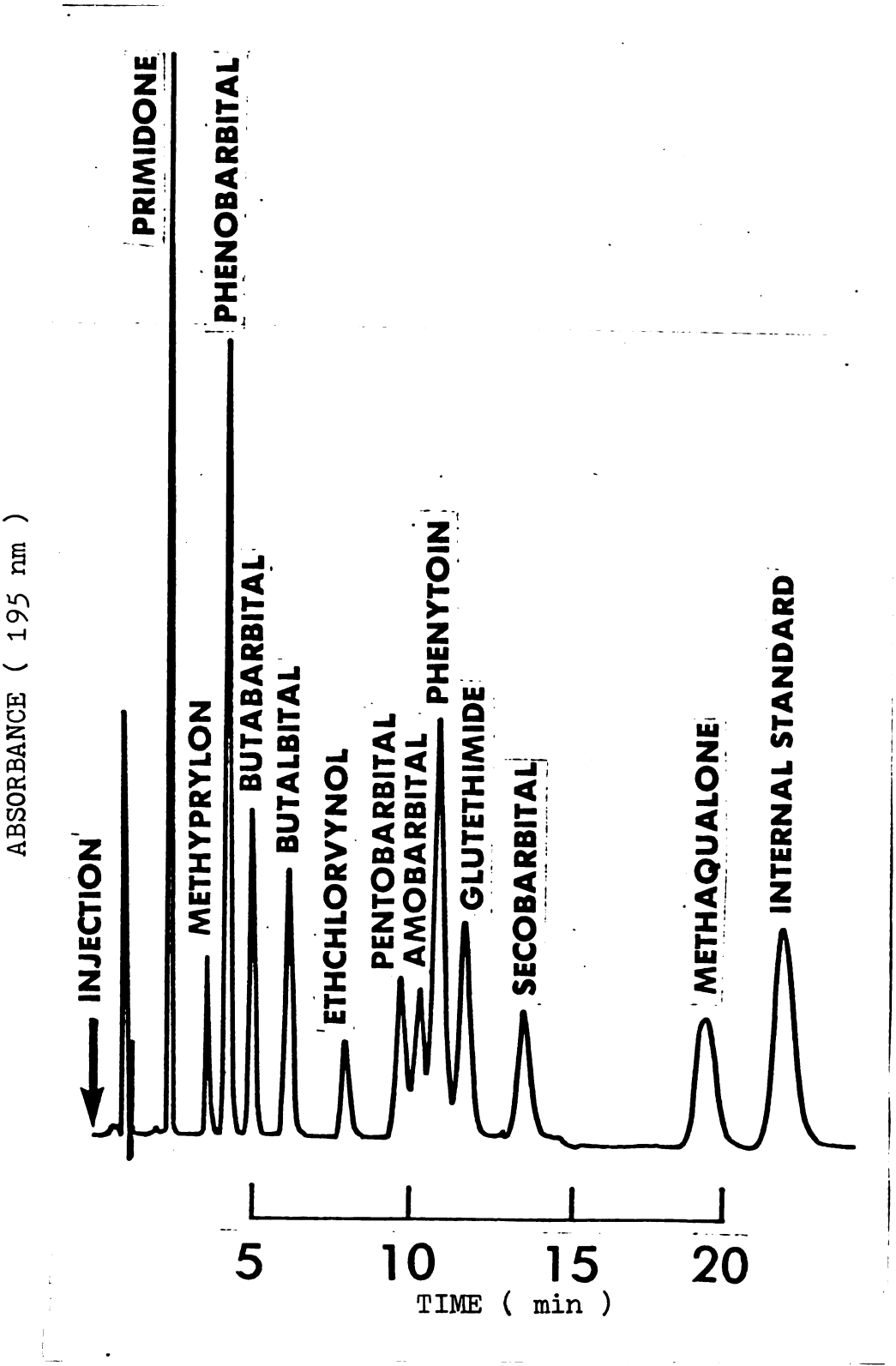


Figure 10. Chromatogram of a standard mixture of 250 ng of each drug and 500 ng of the internal standard. Mobile phase = 20.5 % of acetonitrile. Flow rate = 3 ml/min.

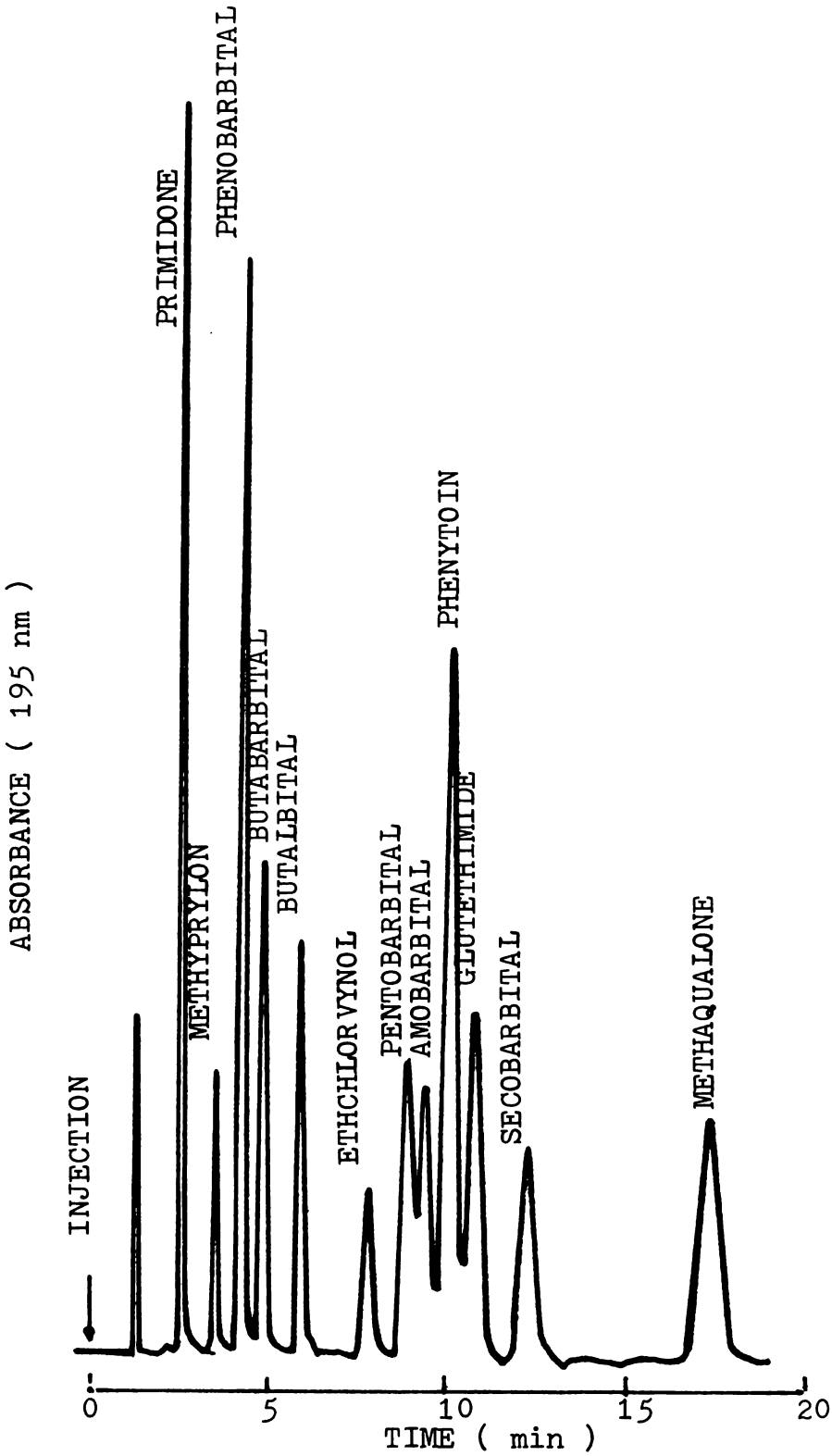


Figure 11. Chromatogram of a standard mixture of drugs without hexobarbital as the internal standard

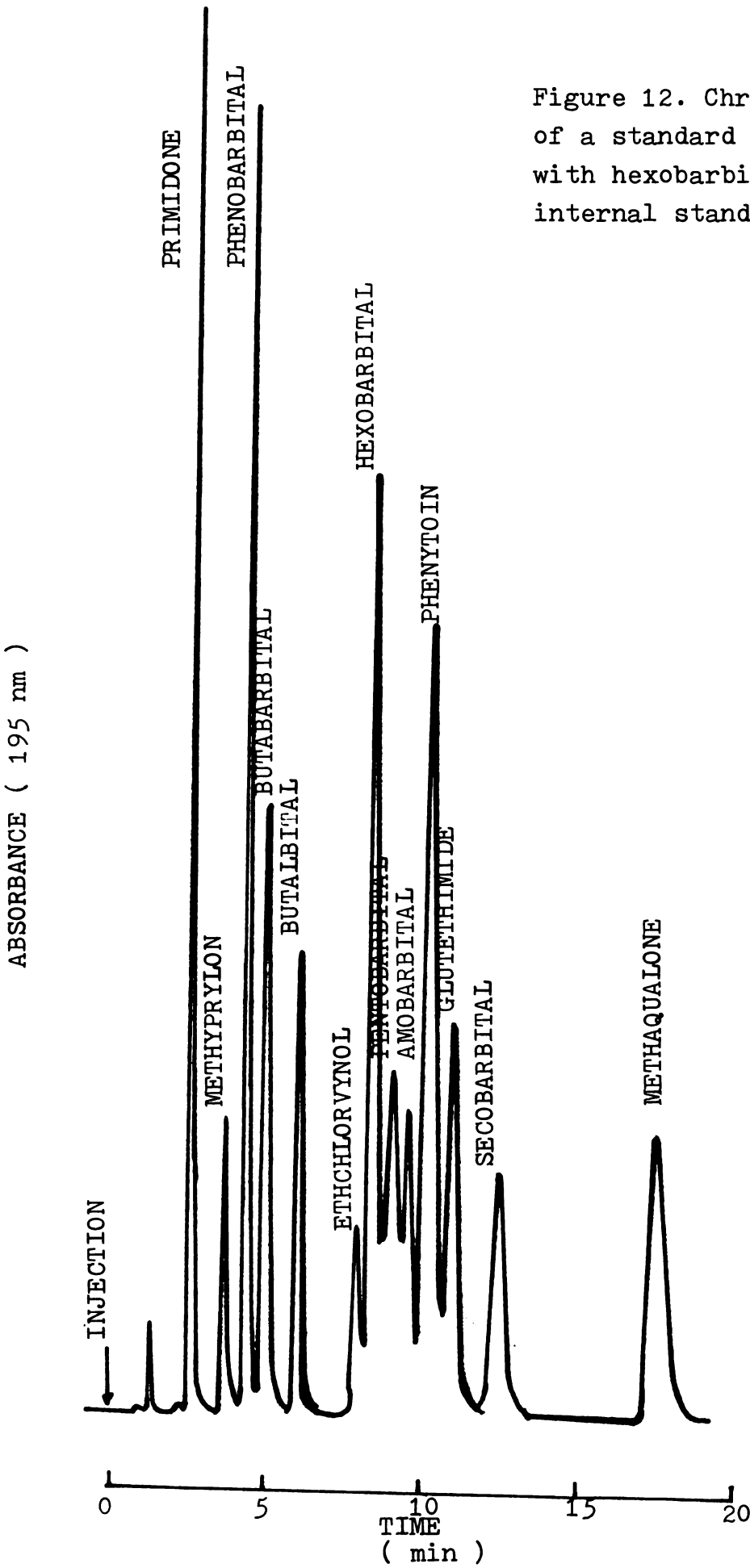


Figure 12. Chromatogram of a standard mixture of drugs with hexobarbital as the internal standard

observed when the acetonitrile concentration was $<$19 parts and $>$23 parts. In addition, the phenytoin and glutethimide peaks began to fuse when the acetonitrile concentration was $<$20 parts. Figures 13 to 18 illustrate the effect of acetonitrile concentration upon the resolution of pentobarbital/amobarbital and phenytoin/glutethimide peaks.

5-(4-methylphenyl)-5-phenyhydantoin was then used as the internal standard and it was found to be satisfactory. It eluted after all the other drugs and the analysis time was still only 20 min. when the acetonitrile concentration was 21.5%. The analysis time can be reduced to 18 minutes with only minimal loss in resolution by increasing the acetonitrile concentration to 22.5 parts (figure 19).

The effects of pH was most marked for a number of potentially interfering compounds such as salicylates, phenacitin, and caffeine. The retention time of these compounds could be adjusted at will by adjusting the pH of the mobile phase. pH 4.4 was selected for routine use because it allowed for the least amount of interference. During the course of the pH study, it was observed that elution of the drugs of interest was not affected until the pH was $>$ 6.5. At pH 6.5 phenytoin eluted after glutethimide.

The effect of column temperature was evaluated previously by Kabra et al. (91). The temperature selected

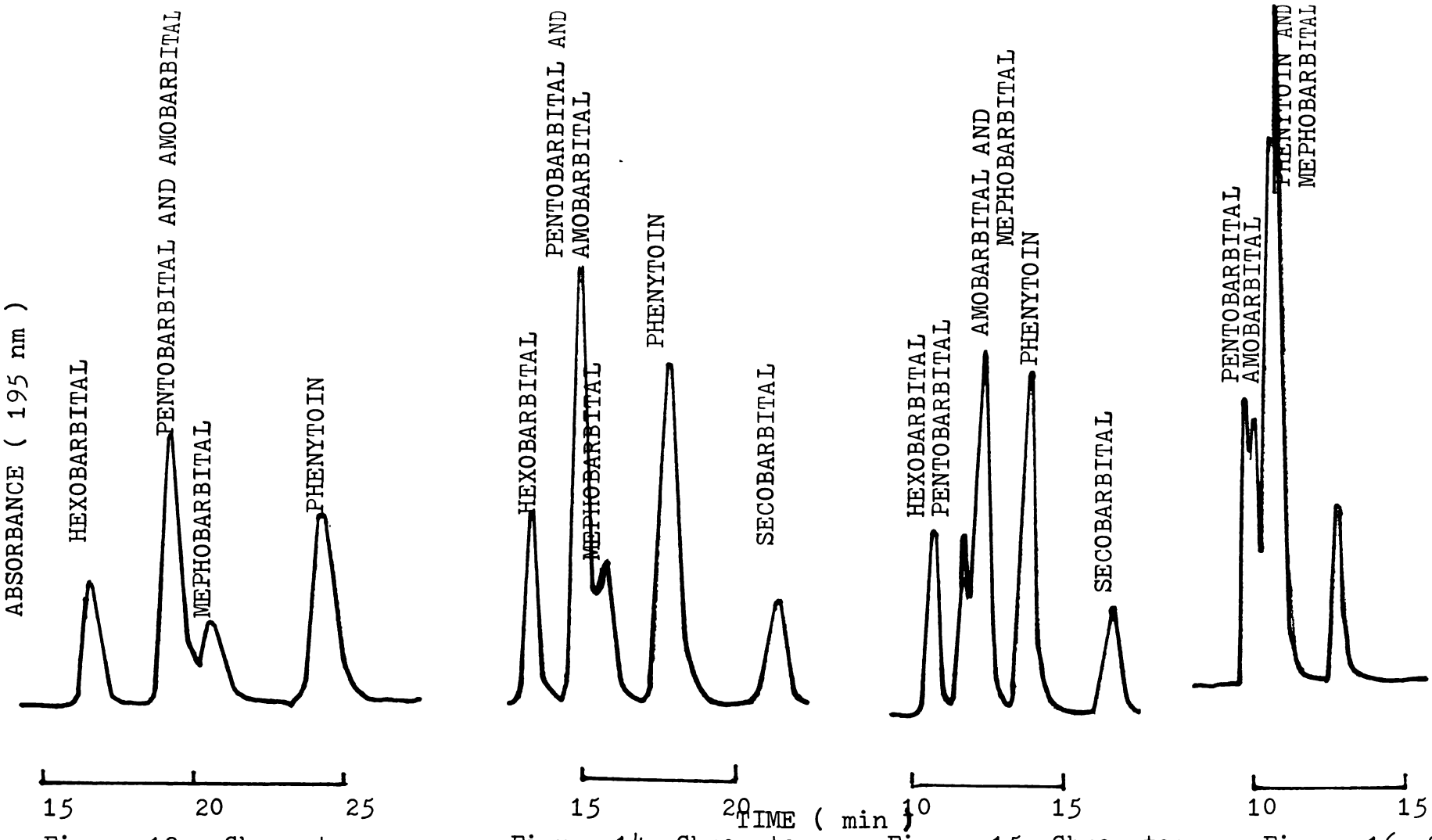


Figure 13. Chromatogram run at 15 parts acetontrile and 85 parts phosphate buffer

Figure 14. Chromatogram run at 17 parts acetontrile and 83 parts phosphate buffer

Figure 15. Chromatogram run at 19 parts of acetontrile and 81 parts phosphate buffer

Figure 16. Chromatogram run at 23 parts acetonitrile and 77 parts phosphate buffer

Figures 13 to 16. Loss of resolution between pentobarbital and amobarbital peaks when acetonitrile concentration was < 19 parts and > 23 parts. Mephobarbital was a source of interference when the acetonitrile concentration exceeded 17 parts

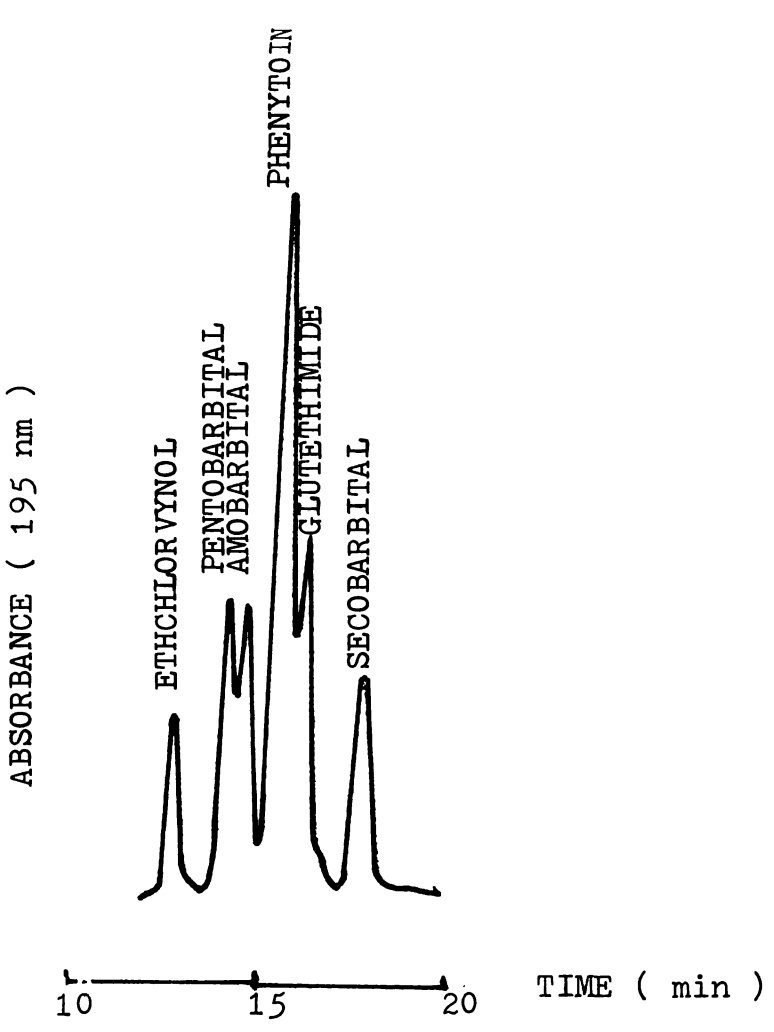


Figure 17. Chromatogram run at 20.5 parts acetontrile and 79.5 parts phosphate buffer. Resolution between phenytoin and glutethimide is poor

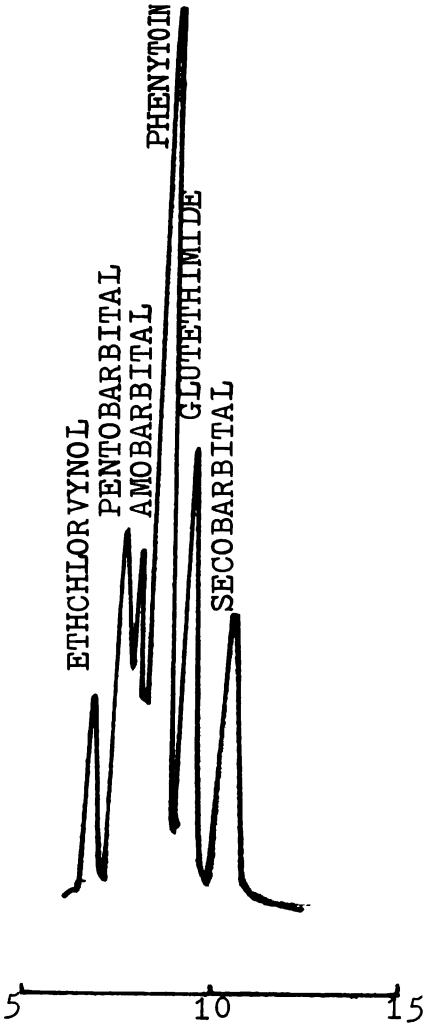


Figure 18. Chromatogram run at 22.5 parts acetontrile and 77.5 parts phosphate buffer. Excellent resolution between phenytoin and glutethimide was obtained

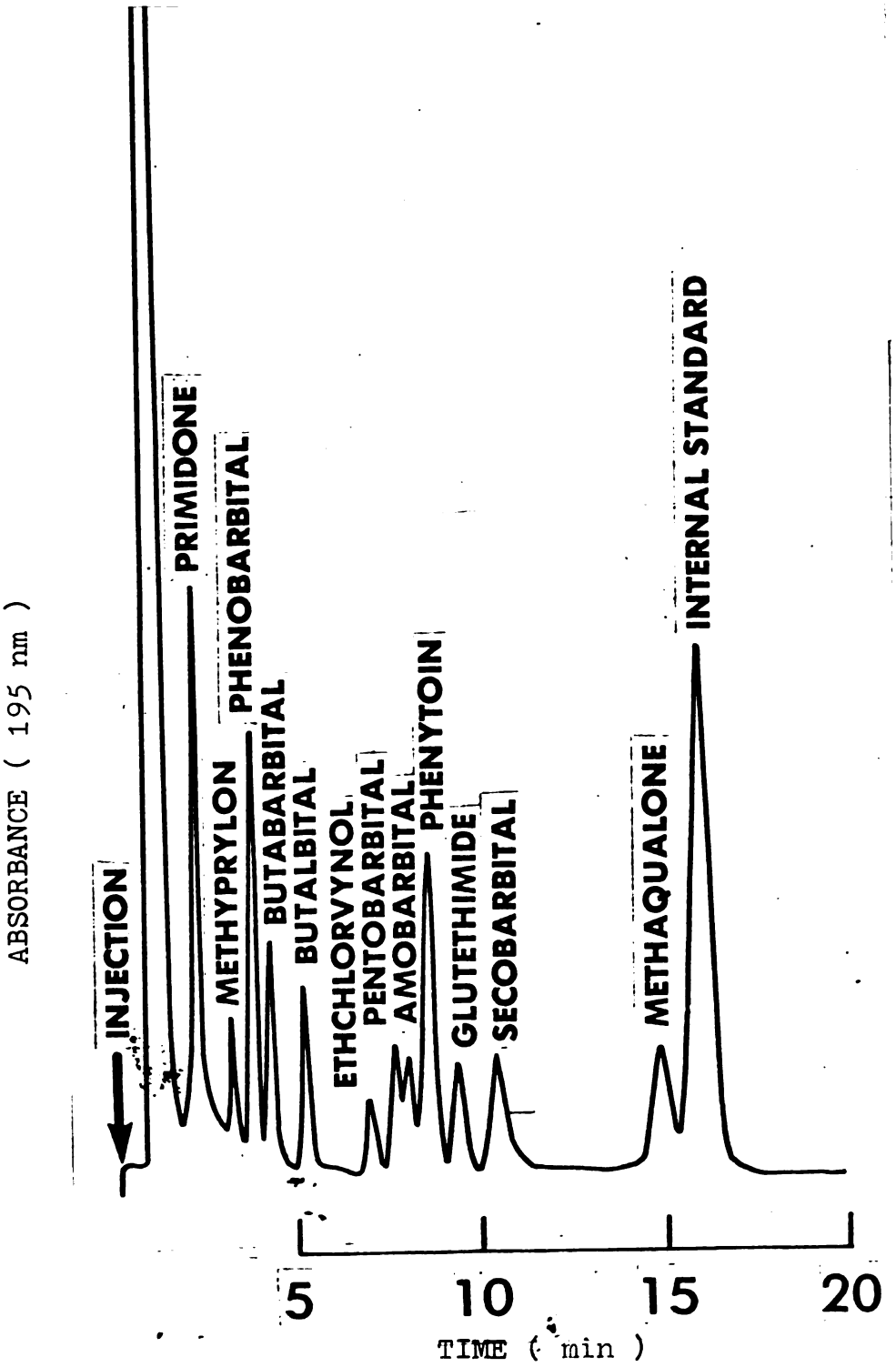


Figure 19. Chromatogram of a serum supplemented with 200 ng of each drug except for the internal standard, which was 500 ng. The chromatogram was run at 22.5 parts of acetonitrile and 77.5 parts phosphate buffer

for routine use was 50°C, a temperature high enough to avoid ambient variation and to increase column efficiency.

Quantitation

Quantitation was done by peak height measurement, a good method when peaks are symmetrical and sharp. Relative retention time (RRT) and response factors (RF) were calculated as follows:

$$\text{RRT of the drug} = \frac{\text{Retention time of the drug from the point of injection}}{\text{Retention time of the I.S. from the point of injection}}$$

$$\text{RF} = \frac{\text{Peak height of internal standard}}{\text{Peak height of drug}} \times \frac{1}{2}$$

The unknow drugs were identified by their RRT's. The RF's were used to calculate the concentration of the drug in the unknown serum as follows:

$$\text{mg/liter of drug in unknown serun sample} = \frac{\text{Peak height of drug X RF X Conc. of I.S.}}{\text{Peak height of I.S.}}$$

Analytical Variables

Standards: 250 ng of each drug and 500 ng of 5-(4-methylphenyl)-4-phenylhydantoin (internal standard) were injected to ascertain the adequacy of the chromatographic conditions. This chromatogram was used to calculate the relative retention times and response factors for these

drugs. Figure 9 shows a chromatogram of the drug reference standard.

Sensitivity

Method A:

Primidone and phenobarbital are detected and reproducibly quantitated at a concentration of 0.25 mg/liter of serum. Butabarbital, butalbital, phenytoin and glutethimide can be quantitated at a concentration of 0.5 mg/liter of serum. Methyprylon, ethchlorvynol, pentobarbital, amobarbital, secobarbital and methaqualone can be quantitated at a concentration of 1.0 mg/liter concentration of serum.

Method B:

Sensitivity is increased 3-4 fold for all of the above drugs if a suitable aliquot of extracted sample is injected into the chromatograph. All of these drugs are detected and reproducibly quantitated at a concentration of less than 0.5 mg/liter in serum samples.

Linearity

Each drug was added to a drug-free serum in amounts equivalent to 5 mg to 100 mg/liter concentration. A constant amount of internal standard was added to each sample and processed as described. Concentrations and peak heights were linearly related over the stated ranges (Method A) (figures 20 and 21).

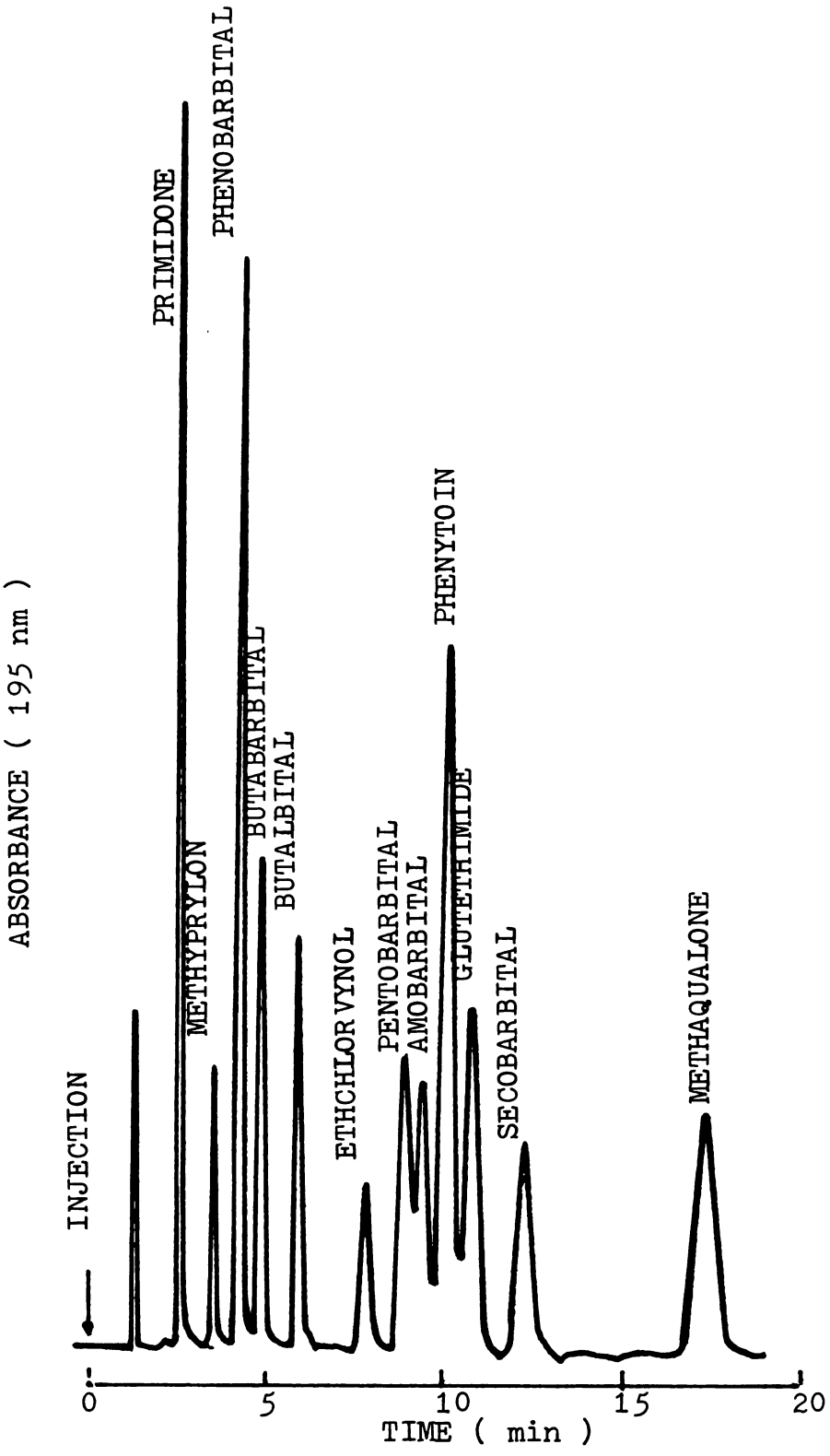


Figure 11. Chromatogram of a standard mixture of drugs without hexobarbital as the internal standard

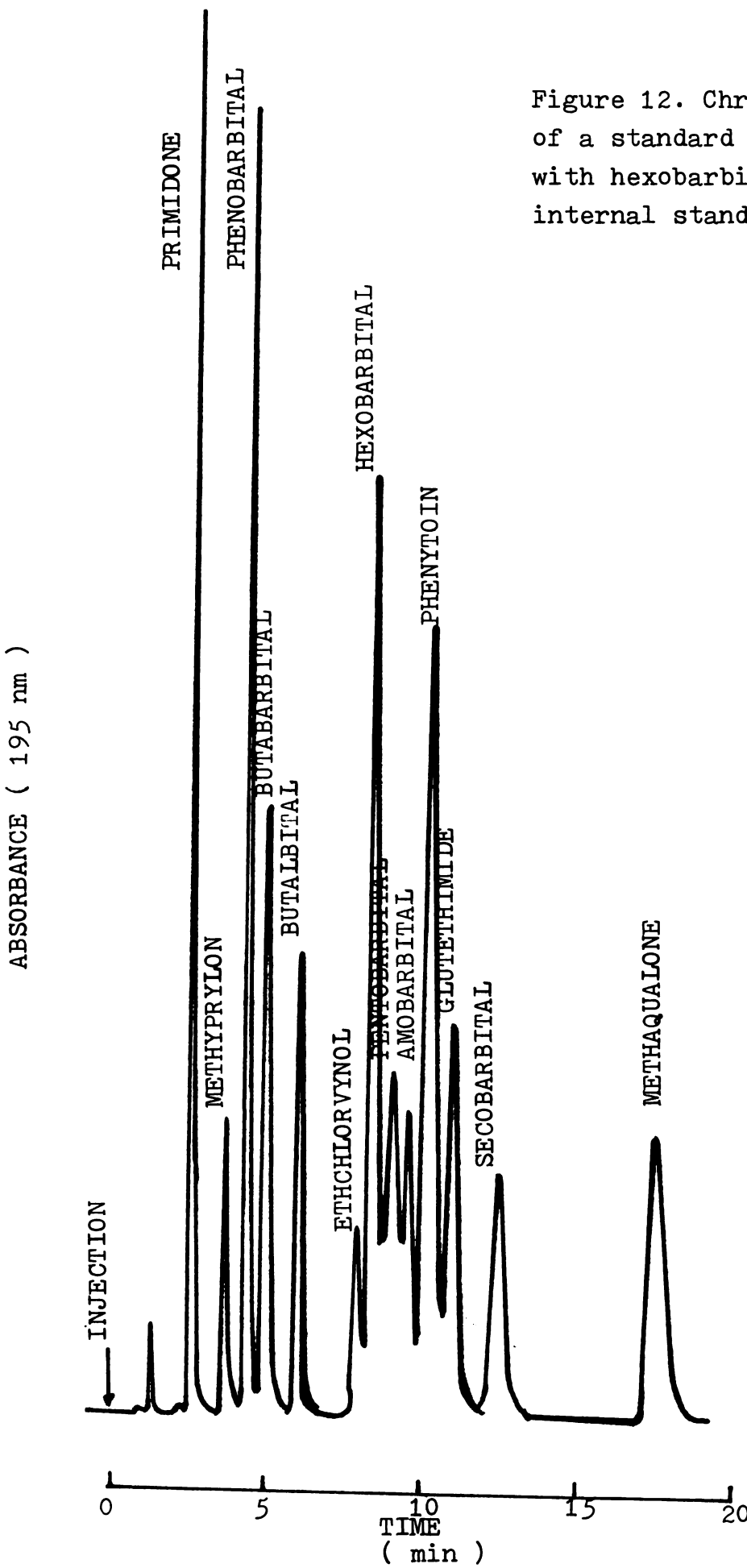


Figure 12. Chromatogram of a standard mixture of drugs with hexobarbital as the internal standard

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The effects of pH was most marked for a number of potentially interfering compounds such as salicylates, phenacitin, and caffeine. The retention time of these compounds could be adjusted at will by adjusting the pH of the mobile phase. pH 4.4 was selected for routine use because it allowed for the least amount of interference. During the course of the pH study, it was observed that elution of the drugs of interest was not affected until the pH was $>$ 6.5. At pH 6.5 phenytoin eluted after glutethimide.

The effect of column temperature was evaluated previously by Kabra et al. (91). The temperature selected

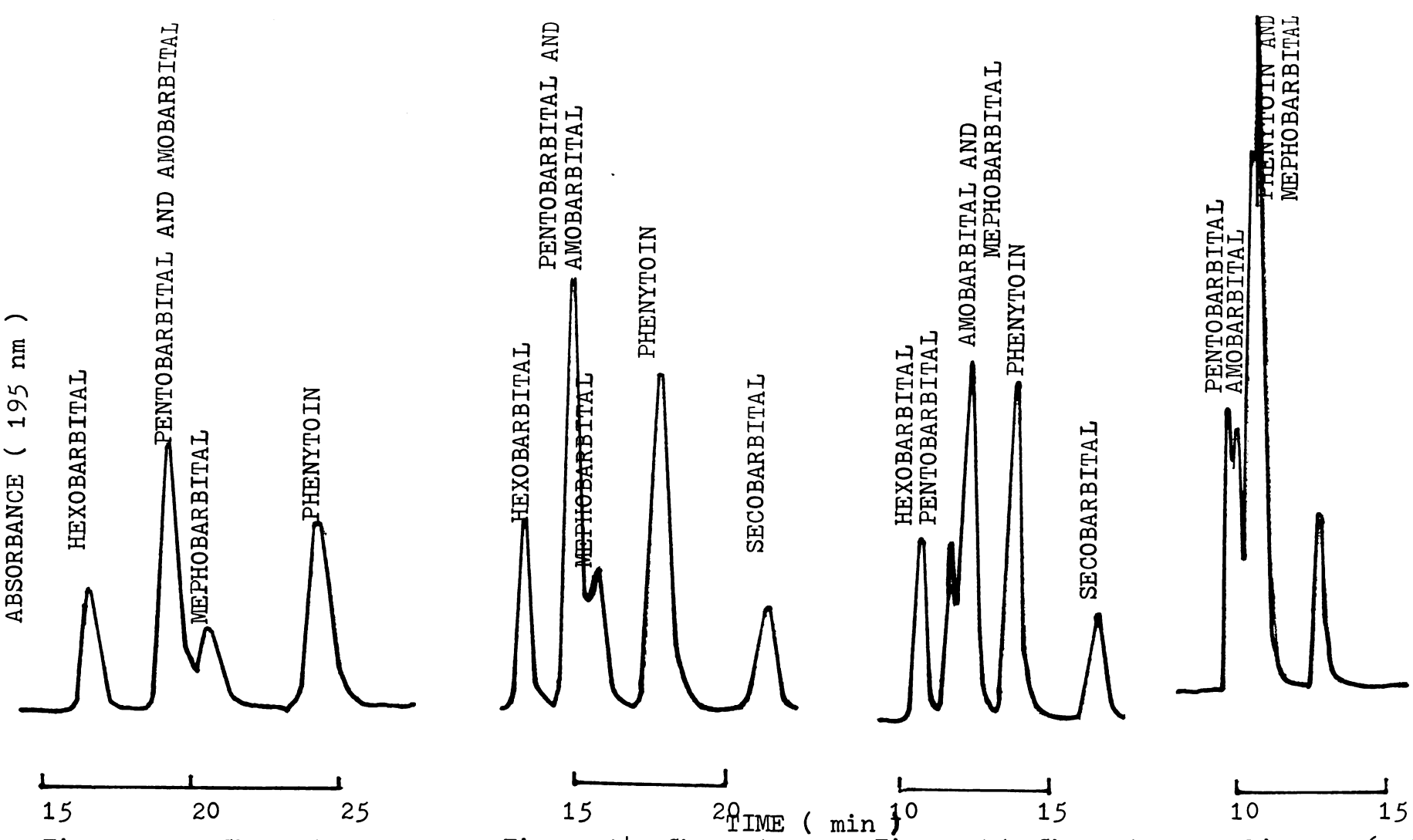

Figure 13. Chromatogram run at 15 parts acetontrile and 85 parts phosphate buffer

Figure 14. Chromatogram run at 17 parts acetontrile and 83 parts phosphate buffer

Figure 15. Chromatogram run at 19 parts of acetontrile and 81 parts phosphate buffer

Figure 16. Chromatogram run at 23 parts acetonitrile and 77 parts phosphate buffer

Figures 13 to 16. Loss of resolution between pentobarbital and amobarbital peaks when acetonitrile concentration was < 19 parts and > 23 parts. Mephobarbital was a source of interference when the acetonitrile concentration exceeded 17 parts

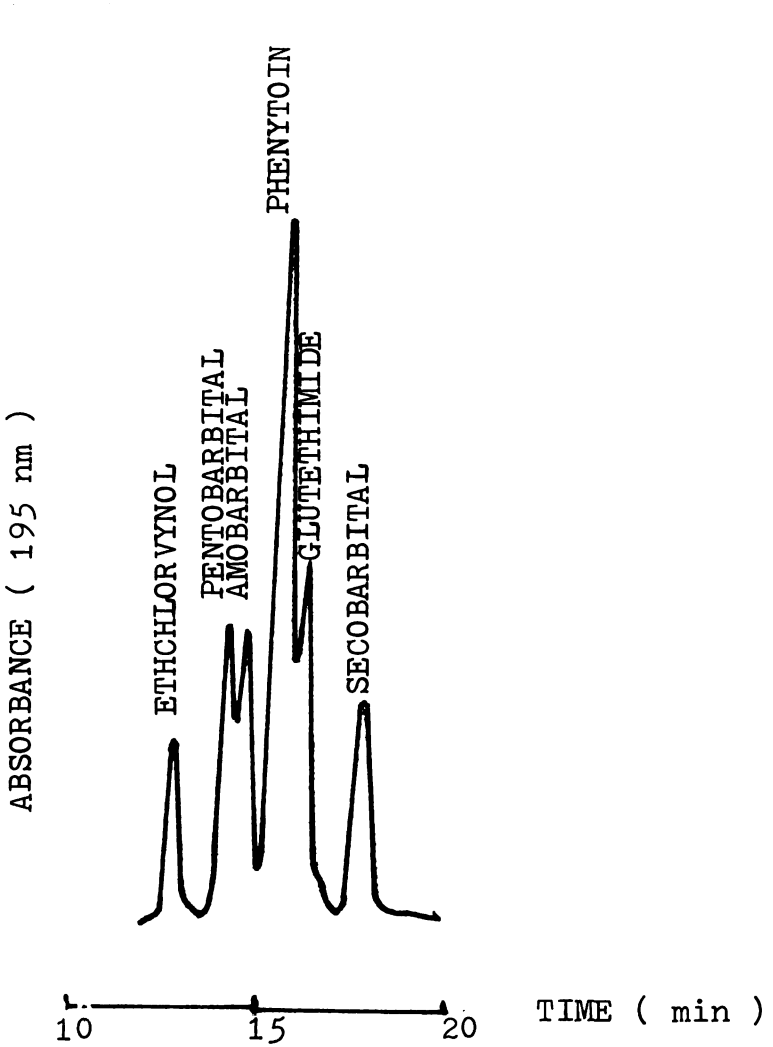

Figure 17. Chromatogram run at 20.5 parts acetontrile and 79.5 parts phosphate buffer. Resolution between phenytoin and glutethimide is poor

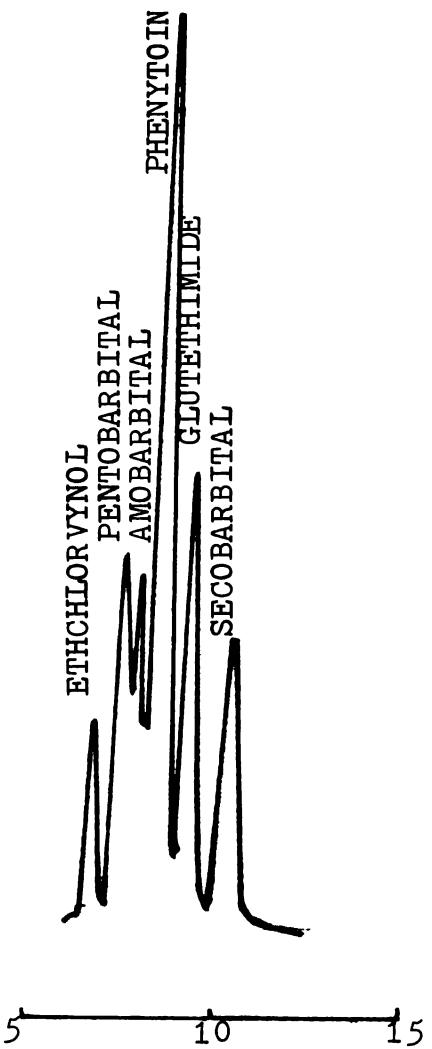

Figure 18. Chromatogram run at 22.5 parts acetontrile and 77.5 parts phosphate buffer. Excellent resolution between phenytoin and glutethimide was obtained

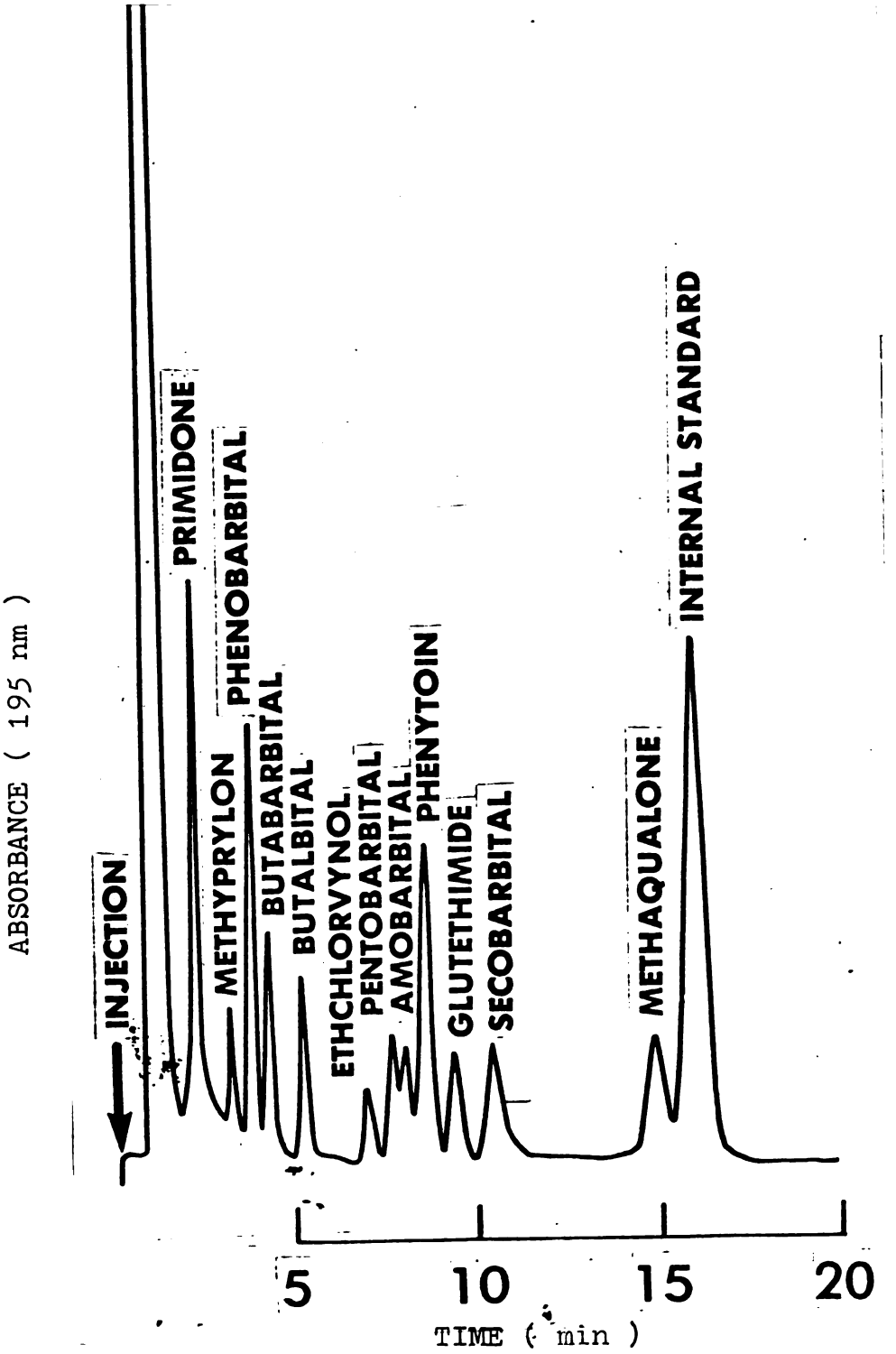

Figure 19. Chromatogram of a serum supplemented with 200 ng of each drug except for the internal standard, which was 500 ng. The chromatogram was run at 22.5 parts of acetonitrile and 77.5 parts phosphate buffer

for routine use was 50°C, a temperature high enough to avoid ambient variation and to increase column efficiency.

Quantitation

Quantitation was done by peak height measurement, a good method when peaks are symmetrical and sharp. Relative retention time (RRT) and response factors (RF) were calculated as follows:

$$\text{RRT of the drug} = \frac{\text{Retention time of the drug from the point of injection}}{\text{Retention time of the I.S. from the point of injection}}$$

$$\text{RF} = \frac{\text{Peak height of internal standard}}{\text{Peak height of drug}} \times \frac{1}{2}$$

The unknow drugs were identified by their RRT's. The RF's were used to calculate the concentration of the drug in the unknown serum as follows:

$$\text{mg/liter of drug in unknown serun sample} = \frac{\text{Peak height of drug X RF X Conc. of I.S.}}{\text{Peak height of I.S.}}$$

Analytical Variables

Standards: 250 ng of each drug and 500 ng of 5-(4-methylphenyl)-4-phenylhydantoin (internal standard) were injected to ascertain the adequacy of the chromatographic conditions. This chromatogram was used to calculate the relative retention times and response factors for these

drugs. Figure 9 shows a chromatogram of the drug reference standard.

Sensitivity

Method A:

Primidone and phenobarbital are detected and reproducibly quantitated at a concentration of 0.25 mg/liter of serum. Butabarbital, butalbital, phenytoin and glutethimide can be quantitated at a concentration of 0.5 mg/liter of serum. Methyprylon, ethchlorvynol, pentobarbital, amobarbital, secobarbital and methaqualone can be quantitated at a concentration of 1.0 mg/liter concentration of serum.

Method B:

Sensitivity is increased 3-4 fold for all of the above drugs if a suitable aliquot of extracted sample is injected into the chromatograph. All of these drugs are detected and reproducibly quantitated at a concentration of less than 0.5 mg/liter in serum samples.

Linearity

Each drug was added to a drug-free serum in amounts equivalent to 5 mg to 100 mg/liter concentration. A constant amount of internal standard was added to each sample and processed as described. Concentrations and peak heights were linearly related over the stated ranges (Method A) (figures 20 and 21).

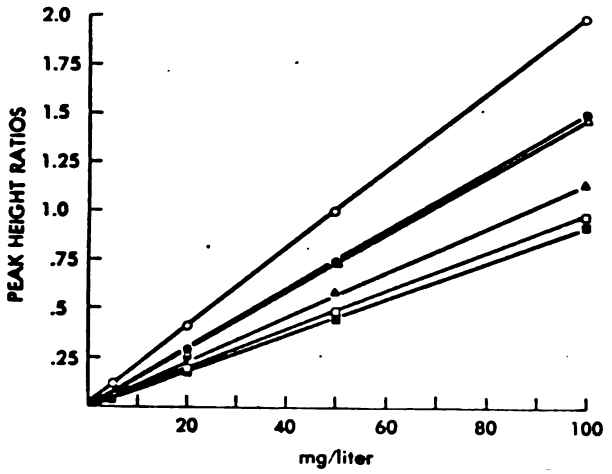

Figure 20. Peak-height ratios (drug / internal std.) for (top to bottom) butalbital, methyprylon, pentobarbital, methaqualone, secobarbital, and ethchlorvynol plotted vs. conc. of each drug
Each point is the average of triplicate determination (method A)

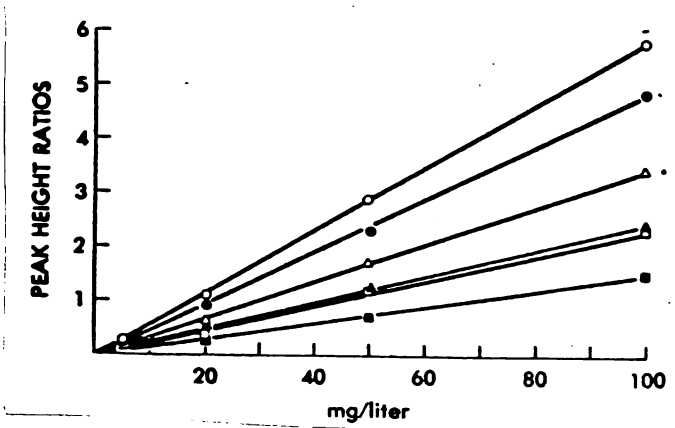

Figure 21. Peak-height ratios (drug/internal std.) for (top to bottom) primidone, phenobarbital, phenytoin, butabarbital, glutathimide, and amobarbital plotted vs. conc. of each drug
Each points is the average of triplicate determination (method A)

Analytical Recovery

Known amounts of each drug in methanol were added to drug-free serum to achieve the concentration shown in Table 3. A constant amount of internal standard was added to each sample and processed as described in Method A. At least 5 samples were processed at each concentration over the stated range. Analytical recoveries are tabulated in Table 3.

Precision

Within-run precision was evaluated by processing aliquots of a pooled plasma containing each of these drugs at concentrations shown in Table 4. Day-to-day precision was similarly evaluated on consecutive days. Precision data are tabulated in Table 4.

Background

Over 30 drug-free serum and plasma samples were processed to obtain data on the amount of background appearing at elution times corresponding to those drugs of interest. The background calculated from these samples ranged from 0 to 0.1 mg/liter. Figure 23 illustrates a drug-free serum sample.

Interference

Potential interference caused by other drugs was

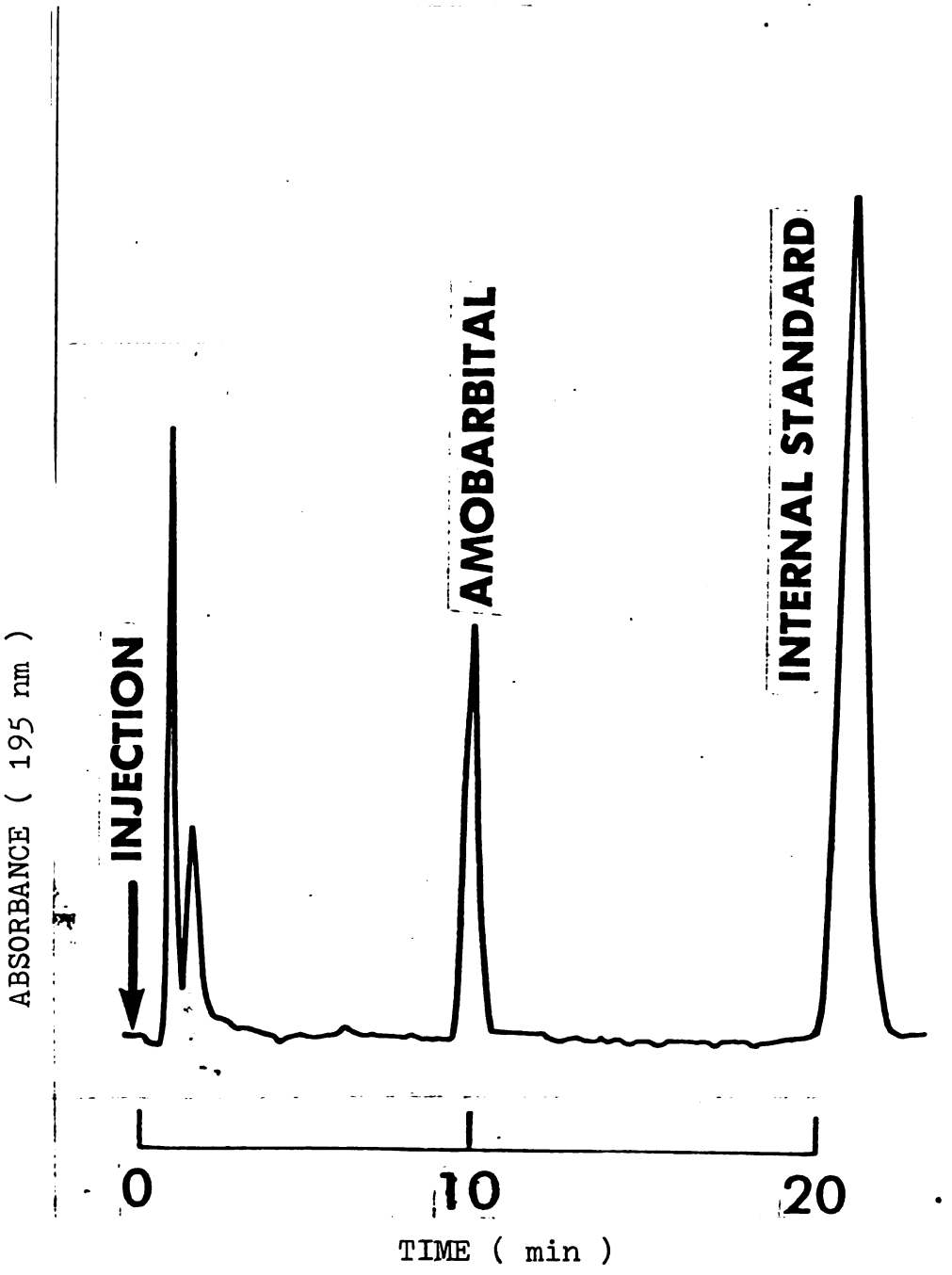

Figure 22. Chromatogram of serum containing 38.0 mg/liter of amobarbital. The sample was prepared by the ethylacetate extraction (method B)

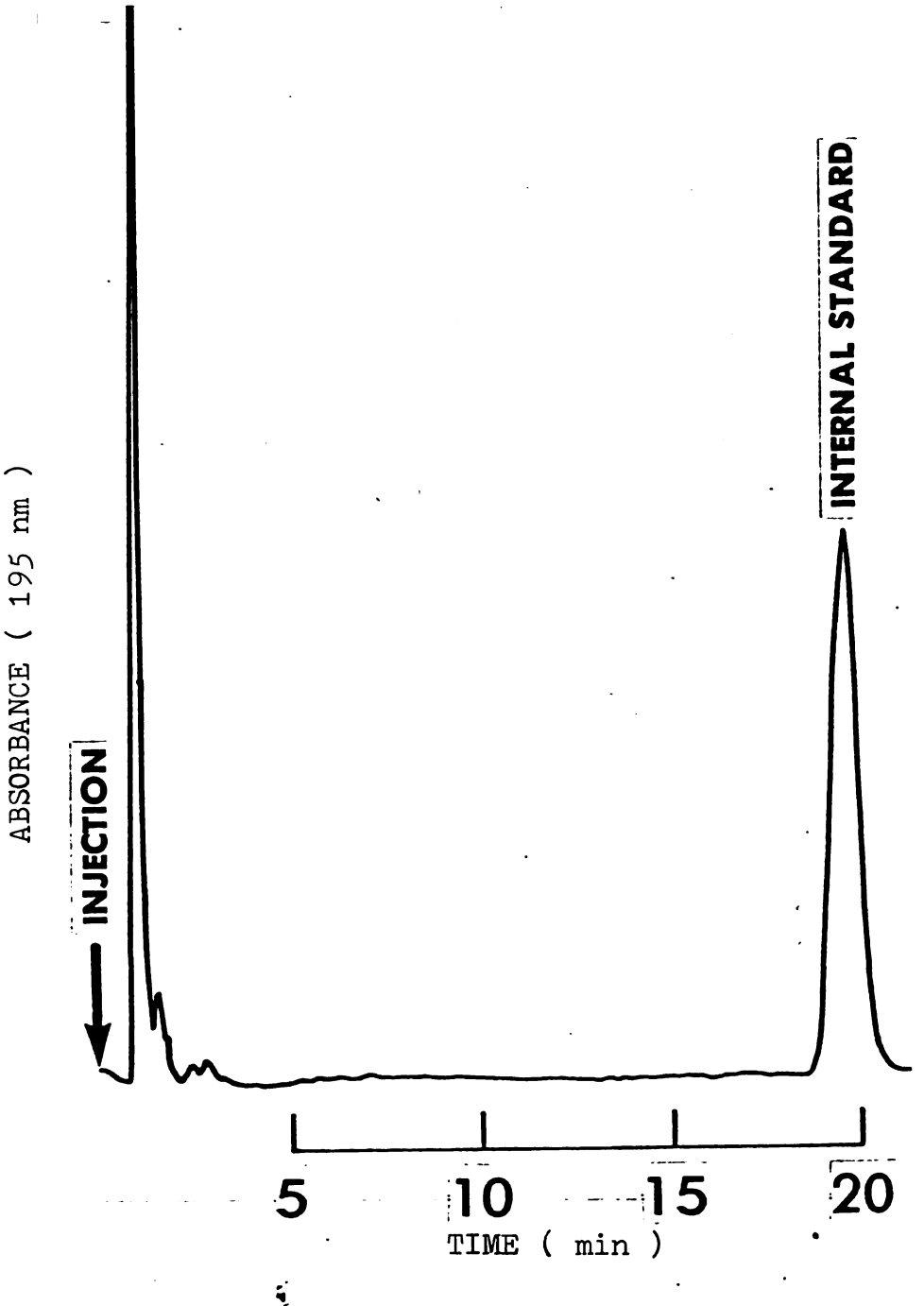

Figure 23. Chromatogram of a drug free serum containing 50 mg/liter of added internal standard. The sample was prepared by acetonitrile precipitation (method A)

Table 3. Analytical Recovery of Drugs from Serum (n = 5)

Drug	Added	Recovered	Recovery
	mg/liter		%
Primidone			
	40.0	39.8	99
	20.0	20.0	100
	5.0	5.1	102
Methyprylon			
	100.0	101.5	101
	50.0	48.8	98
	20.0	20.2	101
	5.0	5.4	108
Phenobarbital			
	100.0	94.0	94
	40.0	39.3	98
	20.0	19.2	96
	5.0	5.1	102
Butabarbital			
	100.0	104.2	104
	50.0	51.6	103
	20.0	20.2	101
	5.0	4.8	97
Butalbital			
	100.0	99.8	100
	50.0	46.4	93
	20.0	19.0	95
	5.0	5.6	112
Ethchlorvynol			
	100.0	109.1	109
	50.0	52.7	105
	20.0	21.6	108
	5.0	5.1	102

Table 3 continued

Drug	Added	Recovered	Recovery
	mg/liter		%
Pentobarbital			
	100.0	99.8	100
	50.0	48.7	97
	20.0	20.8	104
	5.0	5.3	106
Amobarbital			
	100.0	101.2	101
	50.0	50.9	102
	20.0	21.0	105
	5.0	5.5	110
Phenytoin			
	100.0	97.2	97
	50.0	50.0	101
	20.0	19.4	97
	5.0	5.0	100
Glutethimide			
	100.0	112.3	112
	50.0	53.2	106
	20.0	20.0	100
	5.0	4.8	96
Secobarbital			
	100.0	93.5	94
	50.0	49.9	100
	20.0	19.0	95
	5.0	4.9	98
Methaqualone			
	100.0	103.5	103
	50.0	51.9	104
	20.0	21.8	109
	5.0	5.3	106

Table 4. Precision of Assays for Hypnotics in Serum[a]

Drug	Within day Range, ± SD mg/liter	CV, %	Day to day Range, ± SD mg/liter	CV, %
Primidone	18.70 ± 0.8	4.0	18.75 ± 0.70	3.8
Methyprylone	19.40 ± 1.2	6.1	18.90 ± 1.00	5.3
Phenobarbital	15.40 ± 0.8	4.9	16.7 ± 0.87	5.2
Butabarbital	17.10 ± 0.8	4.7	18.2 ± 0.95	5.3
Butalbital	17.90 ± 0.88	4.9	18.35 ± 1.25	6.8
Ethchlorvynol	20.0 ± 1.8	9.2	19.7 ± 2.00	10.4
Pentobarbital	18.30 ± 1.1	6.0	18.9 ± 1.5	7.8
Amobarbital	18.20 ± 1.4	7.5	18.4 ± 1.4	7.7
Phenytoin	17.90 ± 0.9	5.0	20.1 ± 1.1	5.9
Glutethimide	14.40 ± 0.9	6.3	16.4 ± 1.0	6.2
Secobarbital	21.40 ± 1.2	5.4	20.5 ± 1.5	7.3
Methaqualone	21.30 ± 1.3	6.0	19.9 ± 1.1	5.4

[a] n = 10 in each case for within-day, 11 for day-to-day.

studied by chromatographing each drug in methanol. Any drug that eluted sufficiently close to the drug of interest was further studied by adding known amounts of the interfering drug to serum and evaluating the quantitative effect. Table 5 lists retention times for the drugs studied. Of the more than 35 drugs studied thus far, only ethotoin coeluted with phenobarbital. Ethotoin is a rarely prescribed anticonvulsant. At high concentrations and at certain acetonitrile concentrations (figures 12 to 15) mephobarbital is a source of potential interference, however, mephobarbital is rapidly metabolized into phenobarbital in the body and is seldom seen in detectable amounts in the serum as parent drug. During the course of the patients' comparison study, a possible glutethimide metabolite was often seen and can interfere with the analysis of butalbital (figure 7). Hemolyzed, lipemic, icteric samples do not interfere with the analysis. Figure 9 illustrates a chromatogram from a grossly hemolyzed sample.

Comparison with Other Methods

Over 40 samples from patients with therapeutic or toxic concentrations of the drugs of interest were analyzed. Aliquots of the same samples were analyzed by the other analytical methods described below. A portion of these results are illustrated in table 6. Some of the HPLC chromatograms from this comparison study are given in

Table 5. Retention Times for Some Drugs

Drugs	Retention time, min	Drugs	Retention time, min
Salicylate	1.5	Secobarbital	13.7
Acetaminophen	1.7	Cocaine	16.0
Theophylline	1.7	Methaqualone	19.5
Caffeine	2.0	5-(4-Methylphenyl)-5-phenylhydantoin	22.0
Ethosuximide	2.2	Methapyrilene	N.D.[a]
Primidone	2.9	Phenylpropanolamine	N.D.
Methyprylon	4.0	Quinidine	N.D.
Ethotoin	4.4	Gentamicine	N.D.
Phenobarbital	4.4	Propoxyphene	N.D.
Codeine	4.8	Diazepam	N.D.
Phenacetin	5.0	N-Desmethyl-diazepam	N.D.
Butabarbital	5.4	Chlordiazepoxide	N.D.
Butalbital	6.5	Flourazepam	N.D.
Mesentoin	7.1	Amitriptyline	N.D.
Ethchlorvynol	8.5	Nor-amitriptyline	N.D.
Methsuximide	9.0	Imipramine	N.D.
Pentobarbital	9.7		
Mephobarbital	10.2		
Amobarbital	10.5		
Phenytoin	11.2		
Glutethimide	12.0		
Carbamazepine	12.9		

[a]N.D. = not detctable

Table 6. Some Patients Comparison Study

	HPLC method		Other analytical methods					
No.	Drugs found	Conc. mg/l	Drugs found	Conc. mg/l	Method of screening	Method of quantitation	Method of confirmation	References
1	Pentobarbital	9.9	Pentobarbital	10.0	a	a	d	55, 56, *
	Ethchlorvynol	11.8	Ethchlorvynol	12.0	b	c		101
2	Secobarbital	5.2	Secobarbital	6.0	a	a	d	55, 56, *
3	Butabarbital	10.5	Butabarbital	12.0	a	a	d	55, 56, *
4	Amobarbital	38.0	Amobarbital	42.0	a	a	d	55, 56, *
5	Butabarbital	2.0	Butabarbital	2.0	a	a	d	55, 56, *
	Ethclorvynol	34.0	Ethchlorvynol	30.0	b	c		101
6	Methyprylon	13.5	Methyprylon	13.0	e	d	d	*
	Glutethimide	44.0	Glutethimide	40.0	e	d	f	*
7	Pentobarbital	4.0	Pentobarbital	9.5	a	a	d	55, 56, *
	Butabarbital	2.5	Butabarbital					
	Secobarbital	2.5	Secobarbital					
	Phenytoin	8.7	Phenytoin	9.0		g		91
8	Pentobarbital	5.6	Pentobarbital	6.0	a	a	d	55, 56, *
	Methaqualone	3.6	Methaqualone	4.0	h	h	i	##
9	Amobarbital	3.2	Amobarbital	8.0	a	a	d	55, 56, *
	Secobarbital	3.3	Secobarbital					

Table 6 continued

	HPLC method		Other analytical methods					
No.	Drugs found	Conc. mg/l	Drugs found	Conc. mg/l	Method of screening	Method of Quantitation	Method of confirmation	References
10	Phenobarbital	32.0	Phenobarbital	30.0	a	a	d	55, 56, *
	Phenytoin	30.0	Phenytoin	28.0		g		91
	Primidone	6.0	Primidone	5.0		g		91

Notes:

a = differential spectrophotometric analysis (UV)
b = qualitative color reaction test
c = colorimetric analysis
d = GLC - OV 1 and OV 17
Individual barbiturate is not quantitated separately but each barbiturate is identified and confirmed by GLC method using OV 1 and OV 17 columns
e = GLC - OV 1 Or OV 17
f = GLC - OV 1 and OV 17 with TMAH derivatization
g = HPLC
h = GLC -OV 17
i = UV and TLC

*Reynolds, P. C., Institute of Forensic Sciences, Oakland, Ca. Personal Communication
##Wall, J., Clinical Laboratories, University of California, San Francisco, Ca. Personal communication

figures 6 to 9 and 22 and 23. In addition to the analytical methods described below, chemical ionization mass spectroscopy was occasionally employed for confirmation.

Barbiturate Determinations (U.V., GLC)

5 ml of blood was extracted with 50 ml of chloroform. 40 ml of the chloroform extract was shaken with 5 ml of 0.45 N NaOH. 4 ml of the upper alkaline layer was removed for barbiturate determination. The remaining chloroform extract was saved for neutral drug and methaqualone analyses. For barbiturate determination, 4 ml of alkaline aliquots were divided into two 2 ml aliquots. 1 ml of 0.45 N NaOH was added to one of the aliquots and the mixture was placed into the sample cell of a recording spectrophotometer. 1 ml of boric acid was added to the other aliquot and the mixture was placed in the reference cell of the recording spectrophotometer. The pH's of these two mixtures were 13 and 10.5 respectively. A differential spectrum, with a negative absorption at 240 nm and a positive absorption at 260 nm, results if a barbiturate(s) is present in the sample. Quantitation utilized absorption at 260 nm. Identification of individual barbiturates and confirmation were carried out by GLC analysis in which OV-17 and OV-1 columns were used simultaneously.

Methyprylon and Glutethimide Determination (GLC).

5 ml of 0.5 N HCl was added to the 40 ml of the chloroform extract remaining from the barbiturate determination. 5 ml of the chloroform layer was transferred after the chloroform-HCl mixture was shaken for 5 minutes. 0.5 ml of meperidine (internal standard) was then incorporated into the 5 ml chloroform aliquot. The aliquot was evaporated to dryness and the extract was injected onto an OV-1 column. A positive methyprylon peak would be quantitated by its peak height and confirmation was carried out by injecting the same extract onto an OV-17 column. A positive glutethimide peak would also be quantitated by its peak height, however, confirmation is carried out by derivatizing the original extract with trimethylanaline hydroxide (TMAH). The derivatized extract was then injected onto OV-1 and OV-17 columns.

Ethchlorvynol Determination (Colorimetry)

1 ml of the chloroform extract prepared for the methyprylon and glutethimide determination was added to 3 ml diphenylamine color reagent. A positive etchlorvynol sample would yield a pink color reaction after the mixture was incubated for 10 minutes at 50°C.

Confirmation and quantitation of etchlorvynol was then carried out by the following procedure. (1) 0.5 ml plasma or serum was deproteinized with 4.5 ml of 10%

trichloracetic acid (TCA). (2) An aliquot of the supernatant was incubated with 3 ml of diphenylamine color reagent at 37°C for 30 minutes. Quantitation was accomplished by measuring the absorbance of ethchlorvynol at 510 nm against an ethchlorvynol standard.

Methaqualone Determination (U.V.)

30 ml of the chloroform extract prepared for methyprylon and glutethimide determination was evaporated to dryness, dissolved in 3.5 ml ethyl alcohol, and then scanned on a recording spectrophotometer from 190 nm to 350 nm using ethyl alcohol as a blank. One drop of 6 N HCl was then added to the sample and it was re-scanned. Methaqualone shows a characteristic absorption spectrum at 318 nm. Quantitation was carried out by measuring the difference in absorption at 318 nm between the acidified and non-acidified sample.

CONCLUSIONS

Most clinical laboratories use spectrophotometric methods for barbiturate screening. These methods lack both sensitivity and specificity. In phenobarbital intoxication, where plasma levels are relatively high, these methods may be adequate. On the other hand, with short and intermediate acting barbiturate intoxication, where plasma levels are much lower, these methods are not suitable and levels reported often are inaccurate. Besides the lack of sensitivity, spectrophotometric methods cannot differentiate accurately between long and short-acting barbiturates. This differentiation is important for the interpretation of blood levels and the institution of rational therapy. Additionally, with the increasing tendency for alcohol to be ingested along with barbiturates and other hypnotics, with resultant potentiation of the barbiturates, it has become necessary to detect and assay these drugs in lower concentrations.

These problems of specificity and sensitivity have been overcome by the proposed HPLC method. 12 of the most frequently abused barbiturates and hypnotics can be simultaneously identified and quantitated. The method is sensitive enough to detect most of these drugs at a

concentration of 0.5 mg/liter, a level more than adequate for short acting barbiturates. Because of the higher sensitivity, the method can easily be used for the therapeutic monitoring of many of these drugs. Method B (ethyl acetate extraction) was specifically developed to increase the sensitivity of the assay to detect the short acting barbiturates in low therapeutic concentrations.

As HPLC is a non-destructive method of analysis, the eluate from the column can be collected and further analyzed by suitable alternate methods to confirm the presence of any drug or metabolite.

Additionally the presence of a specific drug can be confirmed by the technique of absorption ratioing or U.V. scanning (97,98). This can be easily accomplished using stop-flow techniques. The eluted peak is retained in the detector cell by stopping the flow of the mobile phase while the peak is scanned.

Moreover, the method could be easily adapted for pediatric samples (as little as 25 µl of serum). This eliminates the need for the collection of several milliliters of blood often required for the analysis of these drugs by other screening methods.

Since the method is simple and rapid (total analysis time $<$30 min). It could be easily adapted for rapid screening of these drugs in the emergency room. This method eliminates the variety of techniques presently

employed for the isolation, derivatization and analysis of these drugs. This is the first example of a single set of analytical conditions to analyze for all of these drugs.

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