Title

Simultaneous measurement of phenobarbital, diphenylhydantoin, primidone, ethosuximide, and carbamazepine in serum by high pressure liquid chromatography

Author

Stafford, Brian Edwin

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SIMULTANEOUS MEASUREMENT OF PHENOBARBITAL, DIPHENYLHYDANTOIN, PRIMIDONE, ETHOSUXIMIDE, AND CARBAMAZEPINE IN SERUM BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

by
Brian Stafford
B.A. Chemistry Colorado College 1975

THESIS

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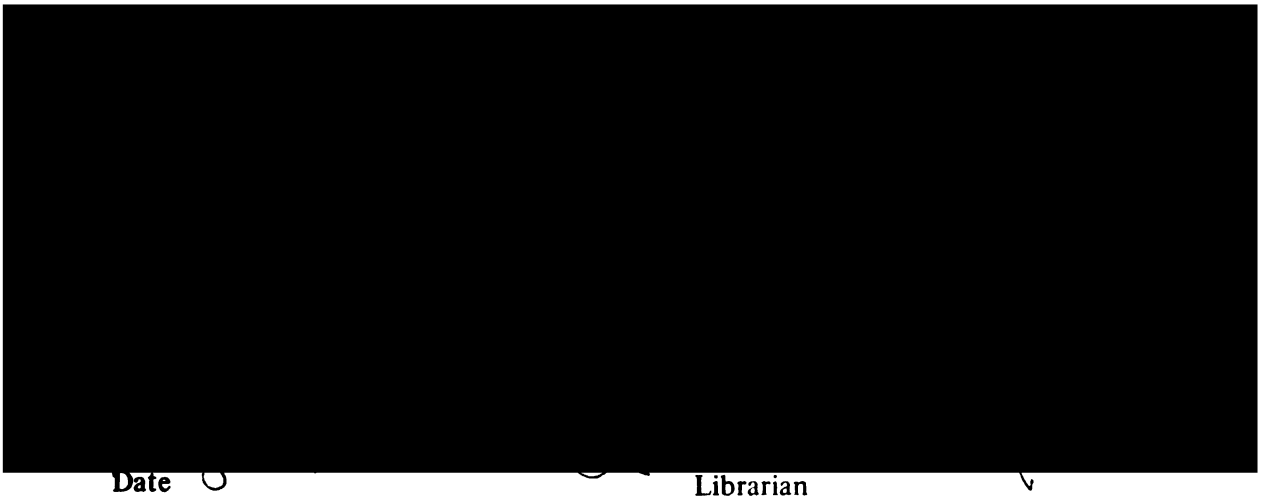

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ABSTRACT

A method for simultaneously determining five anticonvulsant drugs (phenobarbital, diphenylhydantoin, primidone, ethosuximide and carbazepine) in as little as 25 µl of serum is presented. The procedure involves precipitation of proteins with an acetonitrile solution containing hexobarbital as an internal standard. The anticonvulsants are eluted from a reversed-phase column with a mobile phase consisting of an acetonitrile/phosphate buffer (19/81 by volume) at a flow rate of 3.0 ml/minute. The eluted drugs are detected by their absorption at 195 nm, and quantities estimated from their peak heights. Each analysis requires about 14 minutes, at an optimum column temperature of 50° C. The lower limit of detection for all of these drugs is less than 10 ng. Sensitivities, for serum samples, of 1.0 mg/liter are attained routinely. Recoveries for all of these drugs varied from 97% to 107% with good day-to-day precision with coefficients of variation between 3.9% and 5.9%. Over 30 drugs have been tested for possible interference. Out of these drugs only ethotoin interferes with the analysis of phenobarbital. The method is linear up to at least 100 mg/liter for all of these drugs. The method compared well with GLC with r values of 0.995, 0.980 and 0.990 for ethosuximide, primidone, phenobarbital and diphenylhydantoin respectively. Background values for twenty drug-free serum samples was less than 0.1 mg/liter for all of these drugs. The column utilized for the assay showed no appreciable deterioration in the resolution of these drugs after 2,000 analyses. The method is now used for clinical monitoring of anticonvulsant drug therapy in the Clinical Laboratories, University of California, San Francisco, California.

INTRODUCTION

The first use of phenobarbital as an anticonvulsant was reported by Hauptmann in 1912. He investigated the dose-response relationship of this drug in human subjects (1). In 1938 Merritt and Putman (2) reported the use of diphenylhydantoin in the treatment of convulsant disorders. The impetus generated by the discovery of diphenylhydantoin resulted in the laboratory screening of thousands of other compounds and the introduction of a host of new antiepileptic drugs. All of these drugs were either the result of a deliberate laboratory search for antiepileptic action or a spin-off from other therapeutic programs. The discovery of diphenylhydantoin, for example, resulted from a systematic laboratory search, whereas diazepam was originally employed clinically as a sedative and was subsequently shown to have antiepileptic action (2). Conversely, the therapeutic management of other diseases has benefited from research on drugs first used as antiepileptics, e.g., diphenylhydantoin in trigeminal neuralgia and cardiac arrythmias, and carbamazepine in trigeminal neuralgia (3).

Primidone, a desoxybarbiturate, was synthesized by Bogue and Carrington in 1949 (4), however, its anticonvulsant activity was not reported until 1952 by Handley and Steward (5). Ethosuximide, although synthesized in 1951 in the Parke-Davis laboratories by Miller et al. (6-9), wasn't used as an anticonvulsant until Vossen (10) studied its efficacy in 1958. Carbamazepine was introduced in the early fifties when Domenjoz (11) examined the side effects of antihistaminic drugs derived from iminodibenzyl analogues. Carbamazepine, which possesses a urea moiety like many of the other anticonvulsants, demonstrated strong anticonvulsant activity.

Although the relationship between blood levels and clinical response is well studied for the anticonvulsants, there are several other parameters that the clinician should be familiar with. The expected blood level ranges in relation to the dose are given in Table 1.

Table 1.

Pharmacologic Properties of the Anticonvulsant Drugs

DRUG-	Diphenylhydantoin	Phenobarbital	Primidone	Ethosuximide	Carbamazepine
Therapeutic Range (mg/liter)	10-20	10-40	2-10	10-40	2-10
Dosage to achieve therapeutic range (mg/d)	300	180	750	500	1,200
Days to achieve steady-state blood level	5-10	14-21	4-7	14-21	2-4
Serum half-life (hr.)	24 (± 12)	96 (± 12)	12 (± 6)	30 (± 6)	12 (± 3)
Toxic level (mg/liter)	>20	>40	>12	>100	>12

Whether an individual patient will achieve mid, high, or low values with a certain dose cannot be predicted with any degree of certainty, therefore, the actual blood level should be determined. With an increase in dosage, a linear elevation of blood level occurs with a slope characteristic to the individual. However, a saturation point may eventually be reached beyond which a further increase in dosage produces a disproportionately high elevation of blood level. This may occur with relatively low doses in some patients (12, 13).

The most frequent cause of low blood levels of antiepileptic drugs

in relation to the dose is the failure of patients to take their medication as prescribed (12). There are many genetic factors that influence the rate of absorption, biotransformation, and elimination of drugs by man. Drug effect may further be influenced by exposure to other chemicals, normal and pathologic variations in the central nervous system, and psychologic factors affecting the patient. Different formulations of the marketed product may affect absorption by the gastrointestinal tract and is thus an important consideration facing the clinician (14). Waddel and Butler (15) showed that acid-base balance can have an influence on phenobarbital distribution. Phenobarbital has a pKa of about 7.2 thus making it possible to alter its degree of ionization. This may result in altered partition between the brain and plasma. Drug-drug interaction is another consideration. For example, Plaa et al. (16) demonstrated that the combined use of primidone and phenobarbital could result in toxicity due to the increased conversion of primidone to phenobarbital. Warfarin, methylphenidate, isoniazid, disulfiran and sulfaphenazole have all been shown to inhibit diphenylhydantoin metabolism (17).

Although phenobarbital and diphenylhydantoin remain the two most frequently prescribed anticonvulsant drugs, patients frequently receive other anticonvulsants. Thus, comprehensive therapeutic monitoring of anticonvulsant therapy demands the availability of suitable methods for separating and measuring various combinations of these drugs. The method should be reliable, precise, and accurate. Additionally it would seem valuable for it to be adaptable to pediatric sample sizes (50-200 µl) and at the same time be rapid and simple.

The first method attempted for the analysis of diphenylhydantoin

was a colorimetric method by Kozelka and Hine (18) in 1941. They chlorinated diphenylhydantoin quantitatively on the hydantoin ring by treatment with sodium hypochlorite, removed excess reagent, added potassium iodide and then titrated iodine with thiosulfate. Although the method was adequate for pharmaceutical preparations, it lacked the sensitivity for analyzing sub-milligram quantities in biological fluids. In fact, it soon became apparent that all chemical methods for the anticonvulsants lacked sufficient sensitivity. Additional methods were devised based on the separation of diphenylhydantoin from phenobarbital by ion-exchange chromatography followed by nonaqueous titration or spectrophotometry (19, 20). However, all of these methods lacked the necessary sensitivity for therapeutic monitoring. Then in 1956 a major analytical breakthrough was reported simultaneously by Plaa and Hine (21) and Dill *et al*. (22). Hine used a mixture of cyclohexane and n-butanol (20:1) to extract plasma at a pH of 8.5. The extract was then washed with 1M HCl to eliminate phenobarbital interference. The diphenylhydantoin was extracted into a carbonate buffer (pH 11) and analyzed spectrophotometrically. It was determined later, however, that the separation of phenobarbital from diphenylhydantoin was incomplete (23). Dill (22) extracted plasma with chloroform at a pH of 6.8. The extract was then subjected to countercurrent distribution, as described by Craig (24), which separates phenobarbital from diphenylhydantoin. The diphenylhydantoin was then transformed to a highly colored dye. The modified Dill Procedure (25) was highly specific for diphenylhydantoin but very time consuming. This method was used for many pharmacokinetic studies (14). Several modifications and/or combinations of these two methods have since been developed (26-28).

Then in 1965 Oleson described a thin-layer chromatographic method for the determination of diphenylhydantoin (29). Various other procedures included scraping the spots off the plate, derivitizing diphenylhydantoin and analyzing it either spectrophotometrically or colorimetrically. Simon et al. (30) developed probably the most sensitive and accurate thin-layer chromatographic analysis of diphenylhydantoin in 1971. The concentrated extract was subjected to thin-layer chromatography on silica gel plates. The diphenylhydantoin spot was then scraped off and analyzed by the colorimetric procedure of Dill (22). Reported recoveries were excellent and correlation with gas liquid chromatography (GLC) was 0.97.

Several other methods for the quantitation of diphenylhydantoin have been reported including the benzophenone-extraction method by Wallace et al. in 1965 (31). Dill and Glazko (32) improved Wallace's procedure in 1972 by using fluorometry instead of U.V. spectrophotometry, thus increasing sensitivity.

With the advent of GLC, analysis of drugs became quicker and more precise. Derivitization was employed in the first GLC methods for diphenylhydantoin. Sandberg et al. (33) prepared methylated derivitives with diazomethane and Chang and Glazko (34) prepared trimethylsilyl derivitives. Diazomethane was an explosion hazard while trimethylsilyl derivitization required anhydrous conditions. Kupferberg (35) used trimethylphenylammonium hydroxide (TMPAH) to form methyl derivitives. This agent was found to be stable and safe and did not require anhydrous conditions. Several methods without derivitization have been described, however, the underivitized GLC methods tend to produce asymmetric peaks which are difficult to quantitate by peak heights.

A relatively new method for anticonvulsant analysis, using GLC equipped with a nitrogen-specific detector, has recently been described by Brugmann et al. (36), Goudie and Burnett (37), Toseland et al. (38) and Lehane et al. (39). The nitrogen-specific detector, which can detect nitrogen-containing compounds in very low concentrations, is a modification of the standard flame-ionization detector.

Gas-chromatography coupled with mass-spectrometry has been demonstrated to be the most sensitive method available to monitor levels in the picogram range (40-43). However, the cost of such instrumentation seems to be prohibitive and unnecessary for routine determination. However, the value of determining metabolites on a research basis is recognized.

In 1975 Montgomery and co-workers (44-45), with the assistance from Syva Research Institute, applied electron-spin resonance (ESR) for the determination of diphenylhydantoin. They demonstrated that displacement of spin-labeled drugs from drug-specific antibodies in human serum can be measured by this method. The drugs are labeled with a stable nitroxide-free radical which is bound to the drug-specific antibody ("immobilized"). The nitroxide tumbles in solution at rates that are slow relative to their hyperfine frequencies (~40 MHz). The ESR spectrum then appears as a broad envelope of lines resulting from the summing of different signals from molecules in all possible orientations relative to the magnetic field. This broadened, weak ESR signal represents the background value for the assay. Antibody-bound, immobilized drug in the serum is competitively displaced from the free unlabeled drug. The displaced labeled drug now tumbles rapidly in solution causing averaging of the magnetic field positions, and yields three sharp, distinct peaks

in the ESR spectrum. The amplitude of these is directly proportional to the number of spin-labeled drug molecules displaced from the antibody. After the background ESR signal has been subtracted the original concentration of free unlabeled drug in the biological fluid can be estimated (46).

Homogeneous enzyme immunoassay uses antibodies that attach themselves to a specific drug molecule that has been covalently linked to an enzyme (47). The enzyme in the complex is thus deactivated until the antibody releases it upon coming into contact with the free drug molecules, allowing the enzyme to react with its substrate. The inhibition of enzyme activity by antibody is probably caused either by the conformational changes induced by antibody binding to an active group of the enzyme or by prevention of conformational changes necessary for catalytic activity (48). Because there is no need for a separation step, this type of assay is called homogeneous enzyme immunoassay or enzyme multiplied immunoassay (EMIT).

Booker and Darry (49) reported good correlation between EMIT and GLC for these drugs. This assay technique is now commercially available as a kit from Syva Corporation, Palo Alto, California. The technique has distinct advantages over radioimmunoassay (RIA) because, as previously mentioned, there is no need for separation of the free from bound drug. Additionally, no radioactive tracer material is required. The method has been adapted to measure phenobarbital, primidone, ethosuximide and carbamazepine using, of course, specific antibodies in each case.

RIA procedures are not that common for the anticonvulsants. Tigelaar and Kupferberg (50) described an RIA method for diphenylhydantoin in 1973. They prepared antisera by immunizing animals with a diphenylhydantoin-

chicken gamma globulin conjugate. The assay depends on the capacity of unlabeled diphenylhydantoin to compete with a known amount of ^{14}C-diphenylhydantoin for the antibody-binding sites. Correlation with GLC was good ($r = 1.07$) and few drugs interfere substantially. The aforementioned advantage of the EMIT system would seem to discourage further development of RIA methods for the determination of the anticonvulsants.

High-pressure liquid chromatography (HPLC), also referred to as high-performance liquid chromatography, offers particular advantages for the analysis of the anticonvulsant drugs in that compounds may be analyzed without derivitization. The uniform and balanced packing of particulate material in the column results in high resolution, constant flow and low back-pressure. On line UV detectors monitor the elution of drugs and measures them nondestructively so that they can be collected for further investigation. The inherent problem in GLC of achieving volatility of the drug in the gas phase without decomposition is eliminated.

The use of high-pressure liquid chromatography has been reported for the analysis of anticonvulsant drugs in a number of publications. However, most of these methods require extensive sample preparation before chromatography and some of these methods apply only to the analysis of one or more specific anticonvulsant drugs.

Evans (51) utilized a silica-gel column for separation and analysis of diphenylhydantoin and phenobarbital. The mobile phase used for the elution of these compounds was methylene chloride/methanol/28% ammonium hydroxide (92:7:1, v/v/v). The method correlated well with GLC and precision was good (CV = 6%). Although the method could be adapted to pediatric samples (capillary tubes), it required extensive sample clean-up to remove basic and neutral compounds and no internal standard was used.

Analytical recoveries for these drugs were about 80%.

Eichelbaum and Bertilsson (52) described a method in which carbamazepine and its epoxide metabolite were analyzed by normal phase HPLC. The sample preparation involved several steps. Correlation with mass fragmentography was excellent. The precision of the method was adequate (standard deviation 0.22, n = 21, concentration range 1.2-16.5 µg/ml).

Atwell (53) determined phenobarbital and diphenylhydantoin simultaneously using normal-phase HPLC. They used a simple one-step solvent extraction of the serum. The recovery of diphenylhydantoin and phenobarbital was close to 100%. The method correlated well with GLC, however, no precision or interference data were reported.

Kabra et al. (54) reported a method for the simultaneous determination of phenobarbital, diphenylhydantoin and primidone by normal phase HPLC. Whole blood was extracted at acidic pH with chloroform after addition of an internal standard. Recovery was excellent (95-110%) and both within-day and day-to-day precision were good (CV's averaging from 5.7% to 7.4%). The method correlated well with GLC and few drugs were found to interfere. They also reported a method for the determination of carbamazepine by reversed-phase HPLC utilizing their previously described extraction procedure (55).

Kitazawa and Komuro (56) described the use of an anion-exchange HPLC method for the simultaneous determination of diphenylhydantoin, phenobarbital, and carbamazepine in plasma. Napthol was used as an internal standard. Although analytical recovery was good (88% to 107%), the extraction procedure was lengthy and difficult and the reported precision was poor (CV% ≃ 15%).

Adams et al. (57) described the use of reversed-phase HPLC for the analysis of primidone, diphenylhydantoin, carbamazepine, ethosuximide,

methsuximide and some less common anticonvulsant drugs. Reproducibility was adequate (CV's between 4.2 and 6.6%) and sensitivity was good enough to quantitate 0.5 mg/l of these drugs. Correlation with GLC was good (r values from 0.92 - 0.96). Few drugs were found to interfere in the analysis of these drugs. The compounds were adsorbed from the serum to charcoal which was subsequently washed with a mixed solvent (dichloromethane, isopropanol, diethyl ether, (6/10/25, v/v/v). The charcoal adsorption technique may have been responsible for their poor recovery (41 - 73%).

While our studies were in progress, Soldin and Hill (58) described the use of reversed-phase HPLC for the analysis of five anticonvulsant drugs from 25 µl serum samples. He used a C_{18} µBondapack (Waters Associates) column and eluted the drugs with an equivolume mixture of potassium phosphate buffer (10 mmoles/liter, pH 8.0) and acetonitrile at a flow rate of 0.8 ml/minute. He detected the drugs by their absorbance at 200 nm and quantitated by measuring peak areas with an electronic integrator. An acetonitrile solution containing cyheptamide (as an internal standard) was utilized to precipitate proteins before chromatography. With the exception of primidone, precision and correlation with GLC were adequate for clinical monitoring of these drugs (CV's from 2.8 to 12.8%). Analytical recovery ranged from 80 to 94%. However, their chromatograms did not show baseline resolution for most of these drugs. It may not be possible to detect low therapeutic levels of these drugs at 200 nm. They did not report the lower limit of sensitivity for these drugs. No comprehensive interference study was undertaken. Recently they reported that they could not separate phenylethylamalonamide (PEMA, a major metabolite of primidone) from primidone (59). These two compounds coelute under their chromatographic conditions. Since PEMA is consistently present along with primidone in clinical samples, primidone values were falsely elevated by their method. Although PEMA is known

to have anticonvulsant activity (60), the pharmacokinetic properties of PEMA have not been studied. The ratio of these two compounds cannot be predicted in a given serum sample. Because of this limitation their method will not give true primidone values in clinical samples. In addition, the use of any buffer over a pH of 7.5 can result in serious deterioration of packing material.

In contrast, the HPLC method we describe can also be used for as little as 25 µl of plasma or serum, requires minimal sample preparation and provides almost complete analytical recovery for all of these anticonvulsants. The method provides adequate sensitivity to analyze 1.0 mg/liter concentrations of these drugs. We use phosphate buffer (pH 4.4) to elute these drugs which prolongs column life. We have injected over 2,000 samples on the column without appreciable loss in the resolution of these drugs. Additionally, we can separate and quantitate PEMA and primidone simultaneously by our method. Of the 35 drugs tested so far, only ethotoin interfered with the analysis of phenobarbital.

MATERIALS AND METHODS

Apparatus: We used a Model 601 (Perkin-Elmer Corp., Norwalk, Conn. 06856) high-pressure liquid chromatograph equipped with a variable wavelength detector (Perkin-Elmer LC55) and a temperature controlled 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, CA 94710) mounted on the chromatograph. We used a C_{18} μBondapack (Waters Associates, Milford, MA 01757) reverse phase column. Reverse phase columns are generally preferred over normal phase columns in biomedical application because polar compounds elute first. This allows relatively non-polar compounds (i.e. drugs) to be separated from most of the biological matrix. Packing materials for reverse phase columns are prepared by reacting the appropriate organosilane with silica as shown (61):

$$SiOH + ClSiC_{(n)} \rightarrow SiOSiC + HCl$$

$$RP\text{-}2 = (CH_2)_2 \qquad RP\text{-}8 = (CH_2)_8 \qquad RP\text{-}18 = (CH_2)_{18}$$

where the number following RP (reverse phase) indicates the length of the carbon chain. The choice of the RP sorbent can influence the retention time and phase ratio for specific separations. Because of the SiOSiC bonding, these RP sorbents possess superior hydrolytic and thermal stability as compared to esterified supports with SiOC bonding. They are stable over a pH range of 1-7.5 without appreciable deterioration or change in selectivity. The C_{18} μBondapack column has an average particle size of 10 microns. The smaller the particle size the more the theoretical plates in the column. However, the denser the column packing the more pressure required for elution.

Reagents: All reagents were reagent grade with the exception of the acetonitrile (ultraviolet grade), which was distilled in glass by Burdick and Jackson Laboratories, Inc., Muskegan, MI 49442.

Drug Standards: We obtained 5,5-diphenylhydantoin from Eastman Kodak Co., Rochester, NY 14650. Phenobarbital was obtained from the hospital pharmacy. Primidone was supplied as a gift from Ayerst Laboratories Inc., New York, NY 10017. Ethosuximide was obtained from Parke, Davis & Co., Detroit, MI 48232. Carbamazepine was supplied by Ciba-Geigy Corp., Summit, NJ 07901. Hexobarbital was purchased from Sigma Chemical Co., St. Louis, MO 63178. A chromatography mixture for standardization was prepared as follows: 25 mg each of primidone, ethosuximide, carbamazepine, diphenylhydantoin, phenobarbital and 50 mg of hexobarbital were dissolved in 100 ml of methanol. The solution is stable at 4° C for at least three months. Drug free serum spiked with known amounts of these drugs was used as a control.

Internal Standard: The internal standard was made up by dissolving 5 mg of hexobarbital in 100 ml of acetonitrile. Because of the wide fluctuation in the pH of the distilled water in our laboratory, we elected to buffer the mobile phase to maintain a constant pH.

The following chromatographic conditions were tried initially: 1) mobile phase- 67% acetic acid-water (100 µl of acetic acid in 500 ml of distilled water) pH-3.3; 33% acetonitrile. 2) Ambient temperature. 3) Flow-rate- 1.5 ml/minute. This system was selected based on a previous study by Kabra et al. (55). With this system we could not separate diphenylhydantoin and carbamazepine (retention times of 8.0 and 8.2 minutes respectively) nor could we separate primidone and ethosuximide (retention times of both were 3.0 minutes). The internal standard (5-(p-methylphenyl)-5-phenylhydantoin had a retention time of 11.7 minutes. Phenobarbital was well separated with a retention time of 5.0 minutes. When the acetonitrile concentration was reduced to 30% we began to see resolution between carbamazepine and diphenylhydantoin but primidone and

ethosuximide still coeluted. Subsequently, the acetonitrile concentration was reduced to 25%. At this concentration of acetonitrile primidone and ethosuximide began to resolve from each other, however, the retention times of diphenylhydantoin and carbamazepine increased substantially. Also the internal standard was retained on the column for a long period of time (retention time >16 minutes). Still we didn't see complete separation of diphenylhydantoin and carbamazepine. Reducing the acetonitrile concentration would not separate these two drugs but it could separate primidone and ethosuximide. We found that by increasing the oven temperature we could separate diphenylhydantoin and carbamazepine. We also discovered that hexobarbital (a rarely prescribed drug) could be used as an appropriate internal standard. It eluted between phenobarbital and diphenylhydantoin with good resolution. By reducing the acetonitrile concentration and increasing the temperature we arrive at the following optimum parameters for analysis: 1) mobile phase- 81% acetic acid buffer, 19% acetonitrile; 2) oven temperature- 50° C; 3) flow-rate- 3 ml/minute.

With these parameters we were able to separate the drugs under study with a total chromatographic time of less than 14 minutes. The oven temperature of 50° C eliminated day-to-day ambient temperature variations. At higher temperatures greater column efficiency was achieved resulting in better resolution and shorter retention times. Higher temperatures also substantially reduced the viscosity of the mobile phase resulting in a pressure of only 1,600 psi at a flow rate of 3.0 ml/minute.

Sensitivity and Wavelength Selection: In order to achieve adequate sensitivity we required a minimum detection limit of 10 nanograms for phenobarbital, ethosuximide, diphenylhydantoin, carbamazepine and primidone. This was based on the therapeutic ranges of these drugs (see Table 1) and

assuming that we could inject 10 µl of pure serum (or 20 µl of diluted specimen containing an internal standard).

It is well known that many chemical compounds having little or no ultraviolet absorption in the near U.V. region may have fairly strong absorption in the region below 200 nm (63). This phenomenom is commonly referred to as "end absorption" and is due to n-σ^* and π-π^* electronic energy transitions (63). Thus, compounds containing an isolated carbon-carbon double bond as a chromophore group are strongly detected in the region near 200 nm, but are transparent to radiation of higher wavelengths. In addition, the presence of auxochromes such as -OH, -NH_2, -S, or halogens in a molecule may lead to n-σ^* transitions that permit detection in the region at or below 200 nm (62). Most aromatic compounds are detectable at 254 nm with a fixed wavelength detector. However, the sensitivity is greatly enhanced when the detector wavelength is set at 220 nm or lower.

It was found that all of these compounds exhibited stronger absorbance at the lower wavelengths. Even carbamazepine, which exhibited marked absorbance at 254 nm, was shown to have much higher absorbance at the lower wavelengths. Primidone, which has almost no absorbance at 254 nm in acidic media, exhibits great absorbance at 200 nm or lower.

During our investigation we found that acetic acid has a substantial absorbance at around 200 nm. This resulted in greater chromatographic noise. Therefore, we decided to substitute acetic acid with phosphoric acid (100 µl in 500 ml of H_2O, pH-3.2) due to its U.V. cutoff at 190 nm. This substitution did not effect the separation of the drugs while at the same time we eliminated the baseline noise caused by acetic acid. We selected 195 nm as our U.V. wavelength to give us adequate sensitivity for these drugs. This wavelength is above the U.V. cutoff of our mobile

phase and gives us a sensitivity of less than 10 ng for each drug.

During the course of our interference study we discovered that salicylate coeluted with phenobarbital and caffeine interfered with ethosuximide analysis. Because these two drugs are commonly encountered in serum we elected to modify our mobile phase slightly to eliminate these interferences. Salicylate is a stronger acid and caffeine is a stronger base than any of these anticonvulsant drugs. Therefore, it was possible to adjust the retention times of these drugs at will without changing the retention times of the anticonvulsant drugs appreciably by changing the pH of the mobile phase. We changed the buffer pH to 4.4 where salicylates and caffeine did not interfere with the anticonvulsant drugs.

Procedure: To an aliquot of serum (± 25 µl) in a 12x75 mm glass test tube add an equal amount of acetonitrile containing 50 mg/liter of hexobarbital (as an internal standard). Vortex-mix the mixture for 10 seconds, then centrifuge for 2 minutes. Inject about 20 µl of the supernate into the chromatograph and elute with the mobile phase at a flow rate of 3.0 ml/minute.

Detector sensitivity was set at 0.04 Abs. full scale. The µBondapack column was washed with acetonitrile and dimethylsulfoxide weekly to remove proteins and lipids from the column.

RESULTS AND DISCUSSION

We injected 750 ng of each drug and 1.5 µg of hexobarbital (internal standard) to ascertain the adequacy of the chromatographic conditions. The peak height measurement was sufficient for quantitation because the peaks were symmetrical and sharp. This chromatogram (Figure 1A) was used to calculate relative retention times (RRT) and response factors (RF) as follows:

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

$$\text{RF} = \frac{\text{Peak height of Internal Standard}}{\text{Peak height of drug (2)}}$$

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

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

A mixture of drug-free serum containing known quantities of ethosuximide, primidone, phenobarbital, diphenylhydantoin, and carbamazepine, each at a concentration of 20 mg/liter, was taken through the procedure. Figure 1B shows the resulting chromatogram.

Linearity: To assess the linearity of the chromatographic system, we injected various concentrations of the drugs containing a constant amount of internal standard. The peak height ratios of the anticonvulsant drugs and the internal standard were calculated. The peak height ratios and concentrations were linearly related from 2 to 100 mg/liter (Figure 2).

FIGURE 1A

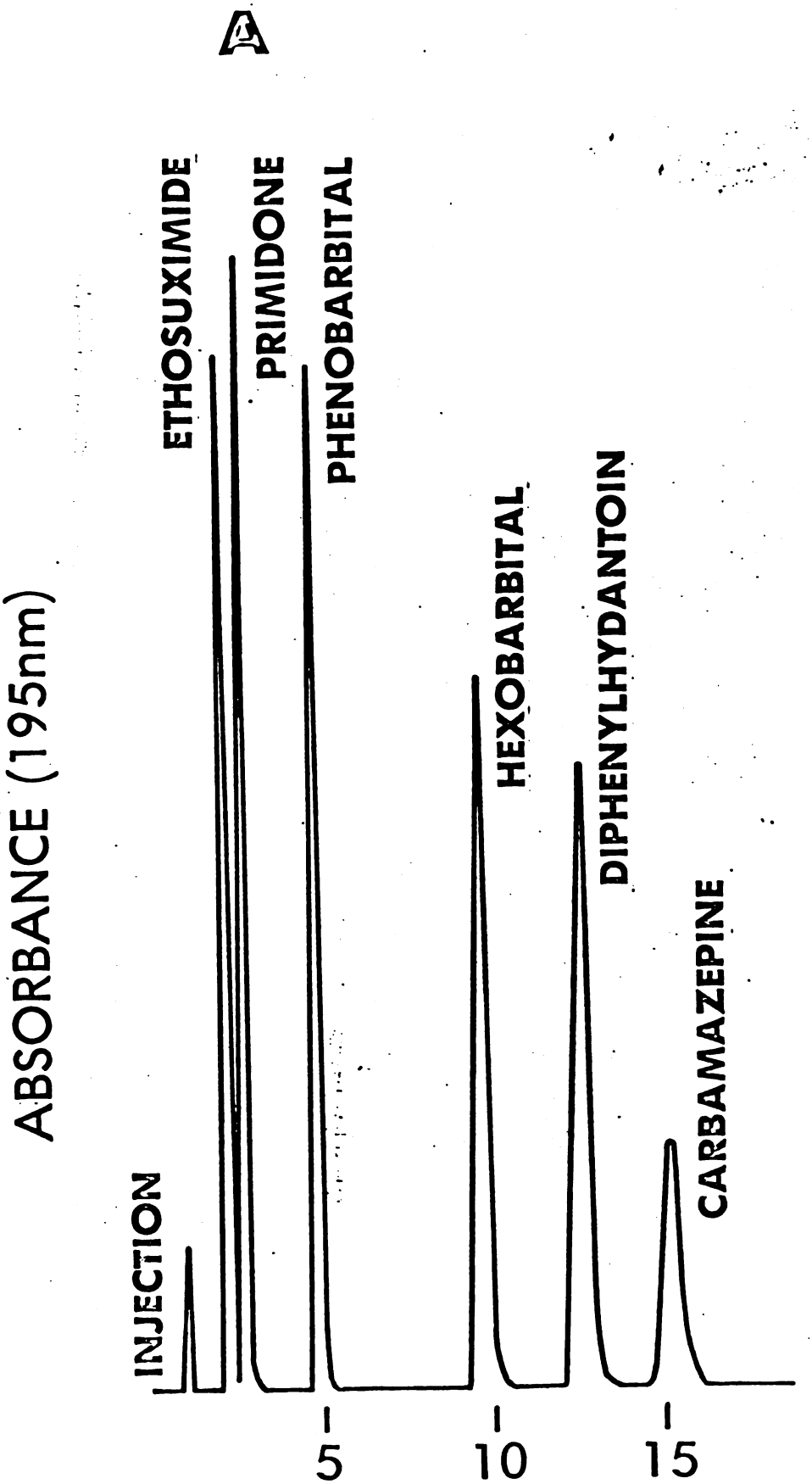

A
ABSORBANCE (195nm)
INJECTION
ETHOSUXIMIDE
PRIMIDONE
PHENOBARBITAL
HEXOBARBITAL
DIPHENYLHYDANTOIN
CARBAMAZEPINE
5
10
15

FIGURE 1B

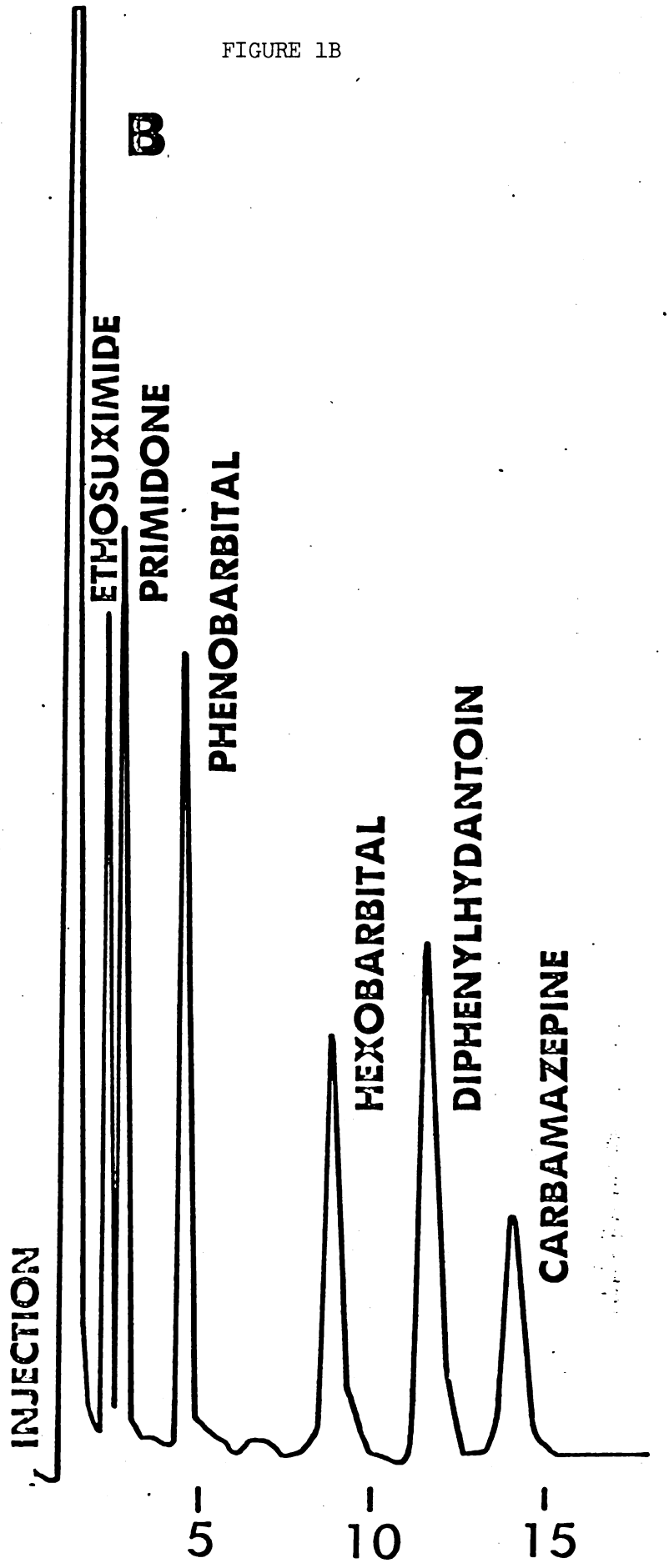
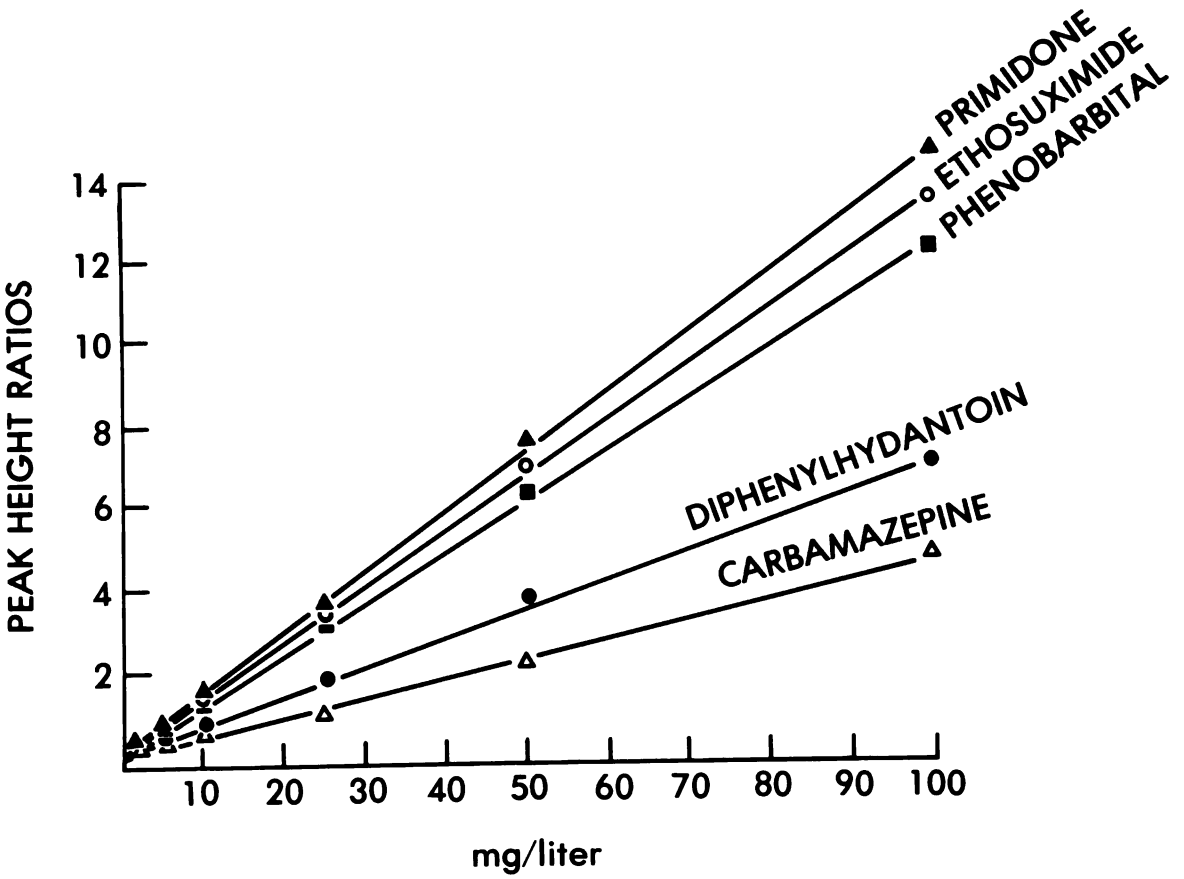

B
INJECTION
ETHOSUXIMIDE
PRIMIDONE
PHENOBARBITAL
HEXOBARBITAL
DIPHENYLHYDANTOIN
CARBAMAZEPINE
5
10
15
TIME (min)

FIGURE 2

Linearity of Methanol Standards



Precision: We assessed the precision of the method by repeated analysis of serum specimens containing known amounts of the drugs being investigated. The standard deviation, S.D., was calculated by the following equation:

$$S.D. = \left[\frac{\Sigma x^2}{N} - \left(\frac{\Sigma x}{N}\right)^2\right]^{\frac{1}{2}}$$

Where x = values in mg/liter
N = # of specimens

Comparisons of different methods cannot be made by directly comparing their means and standard deviations, because the magnitude of the statistics and units in which the results are expressed may vary from one to another. Such comparisons can be made if the standard deviation is expressed as a percentage of the mean. The coefficient of variation provides a measure of relative variability and is calculated as:

$$CV\% = \frac{S.D.}{\overline{X}} \qquad \text{Where } \overline{X} = \text{mean}$$

As shown in Table 2, within day precision varied between 4.2% and 5.5% and day-to-day precision varied between 3.9% and 5.9% for all of these drugs.

TABLE 2

Precision Assays for Anticonvulsants in Serum

n	Drug	Range mg/liter (±SD)	CV%
		Within Day	
13	Ethosuximide	10.3 ± 0.50	4.9
17	Primidone	11.4 ± 0.63	5.5
17	Phenobarbital	10.5 ± 0.47	4.5
14	Diphenylhydantoin	10.2 ± 0.52	5.1
13	Carbamazepine	11.0 ± 0.46	4.2
		Day-to-Day	
10	Ethosuximide	19.1 ± 0.75	3.9
13	Primidone	19.9 ± 1.07	5.8
13	Phenobarbital	19.9 ± 1.04	5.2
13	Diphenylhydantoin	20.1 ± 1.18	5.9
10	Carbamazepine	20.1 ± 0.93	4.6

Absolute Recovery: The absolute recovery of these drugs and internal standard was measured in the following way: The drugs and internal standard were added to drug-free serum to give concentrations ranging from sub-therapeutic to toxic levels as shown in Table 3. This serum was then analyzed by our method, except that the acetonitrile used to precipitate proteins did not contain any internal standard. Carefully measured

aliquots of the supernates were then injected and their peak heights measured. Absolute recovery was calculated by comparing these peak heights obtained by the direct injection of pure drug standards. As shown in Table 3, absolute recoveries of the drugs ranged from 95% to 106%.

TABLE 3

Absolute Recovery of Drugs Added to Serum

Drug	Conc, mg/liter	Recovery, %
Ethosuximide	50	99
	25	103
	10	106
	5	95
Primidone	25	100
	10	101
	5	104
Phenobarbital	50	97
	25	107
	10	101
	5	101
Diphenylhydantoin	50	105
	25	102
	10	106
	5	106
Carbamazepine	25	101
	10	95
	5	105
Hexobarbital	50	102

The relative recovery of these drugs were also calculated. In this study, serum pools containing different concentrations of these anticonvulsant drugs were prepared. To each sample a constant amount of internal standard was added and the sample was processed as above. The concentration of each drug was calculated by internal standardization. The relative recovery of these drugs are included in Table 4.

TABLE 4

Relative Recovery of Anticonvulsants from Serum (N=5)

Drug	Drug Added mg/liter	Drug Recovered mg/liter	Recovery %
Ethosuximide	100	99.5	100
	40	39.2	98
	20	20.1	100
	10	10.1	101
	5	99.0	99
	2	2.0	100
Primidone	40	39.8	99
	20	19.9	100
	10	9.8	98
	5	5.1	102
	2	1.9	93
Phenobarbital	100	92.0	92
	40	39.3	98
	20	19.2	96
	10	10.0	100
	5	5.1	101
	2	2.0	100
Diphenylhydantoin	60	61.8	103
	40	39.4	99
	20	19.4	97
	10	9.9	99
	5	5.0	100
	2	2.0	100

Correlation with GLC: The method was further evaluated by comparing our method with gas-liquid chromatography for ethosuximide, primidone, phenobarbital and diphenylhydantoin. The GLC procedure was developed in our laboratory by Gotelli and Stanfill (46). The gas chromatograph was a Varian Aerograph Model 1200 (Varian Aerograph, Walnut Creek, CA 94598) equipped with a flame ionization detector and a 6 foot, 2 mm I.D. glass coil packed with 1.5% OV-17 on chromosorb W H/P. The following GLC conditions were used: Hydrogen flow rate: 20 ml/minute; Air flow rate: 360 ml/minute; Nitrogen flow rate: 20 ml/minute; Injector and detector temperature: 290° C. We used temperature programming from 200 to 260° C

at 6°/minute to minimize peak tailing. Sensitivity setting was 10^{-11} A.F.S., 32X. The samples for GLC analysis were prepared as follows:

Plasma or Serum (2 ml) plus:
2 ml of 25 mg/liter internal standard
↓ plus 75 µl of glacial acetic acid

Vortex-Mix 10 seconds
1) extract with 10 ml of $CHCl_3$
↓ 2) centrifuge 5 minutes

Evaporate $CHCl_3$
1) reconstitute with 50 µl of CH_3OH
2) inject 2 µl onto GLC column

If ethosuximide was to be analyzed, the extract was injected isothermally at 130° C.

Additional aliquots of the same sera were analyzed by the proposed HPLC method. To investigate the degree of association between the two sets of data, scatter plots were constructed with values for GLC on the x-axis and HPLC values on the y-axis. The regression coefficient (r) was then calculated by the equation:

$$r = \frac{N(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[N\Sigma x^2 - (\Sigma x)\ ]\ [N\Sigma x^2 - (\Sigma y)^2]}}$$

Where:
x is the independent variable (GLC)
and y is the dependent variable (HLPC).

Next, a value for the slope (a) was calculated using the formula:

$$a = \frac{N\Sigma xy - \Sigma x\Sigma y}{N\Sigma x^2 - (\Sigma x)^2}$$

the intercept (b) was calculated by the equation:

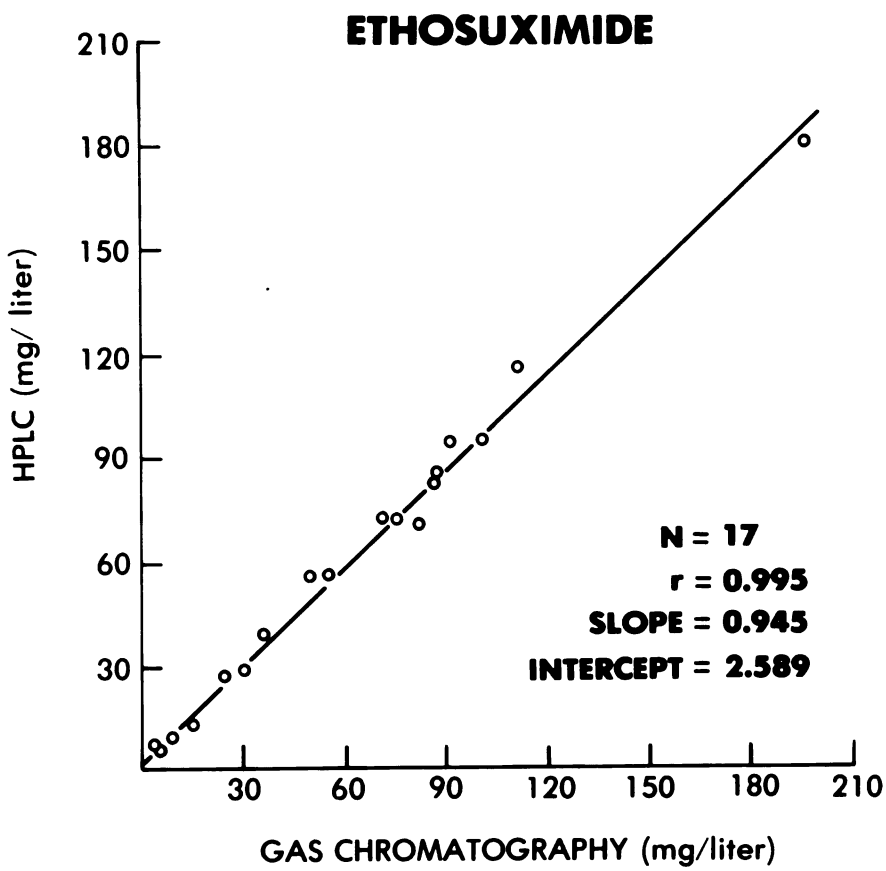
$$b = \frac{\Sigma y\Sigma x^2 - \Sigma x\Sigma xy}{N\Sigma x^2 - (\Sigma x)^2}$$

Although linear regression and correlation are not true measures of accuracy when comparing two methods, it is necessary to evaluate the new

method with an already accepted method. We analyzed 17 samples containing ethosuximide by the two methods (HPLC and GLC), and calculated a correlation coefficient (r) of 0.995; slope, 0.945 and an intercept of 2.589 (Figure 3).

FIGURE 3

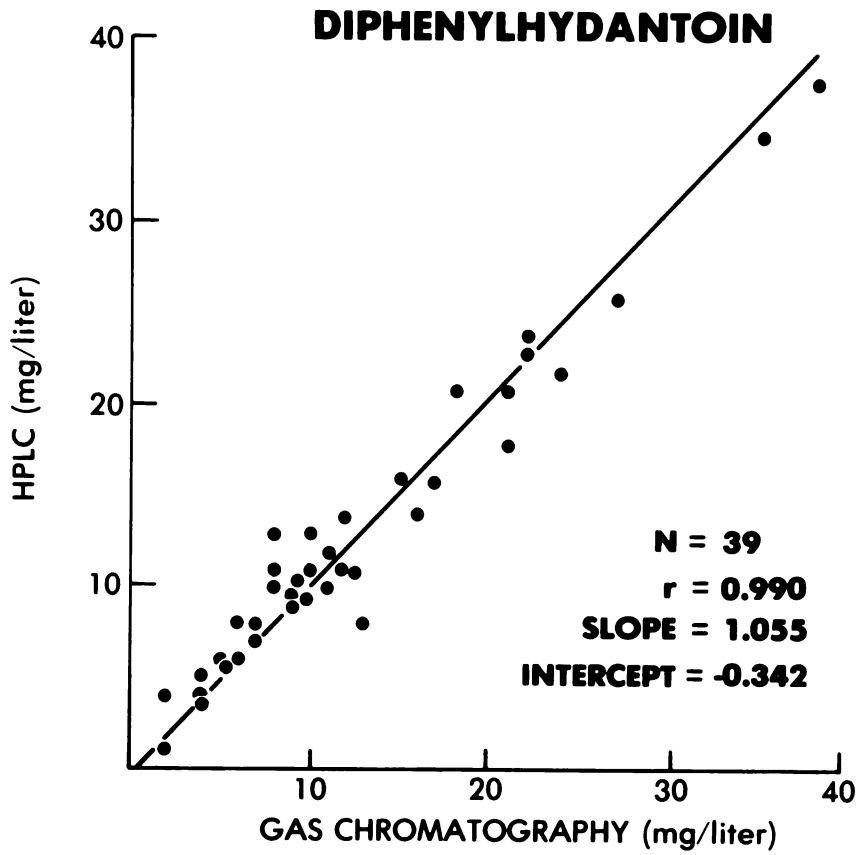
Ethosuximide Analysis, GLC vs. HPLC



Of 39 specimens containing diphenylhydantoin we calculated the r of 0.990, the slope of 1.055 and the intercept of 0.342 (Figure 4).

FIGURE 4

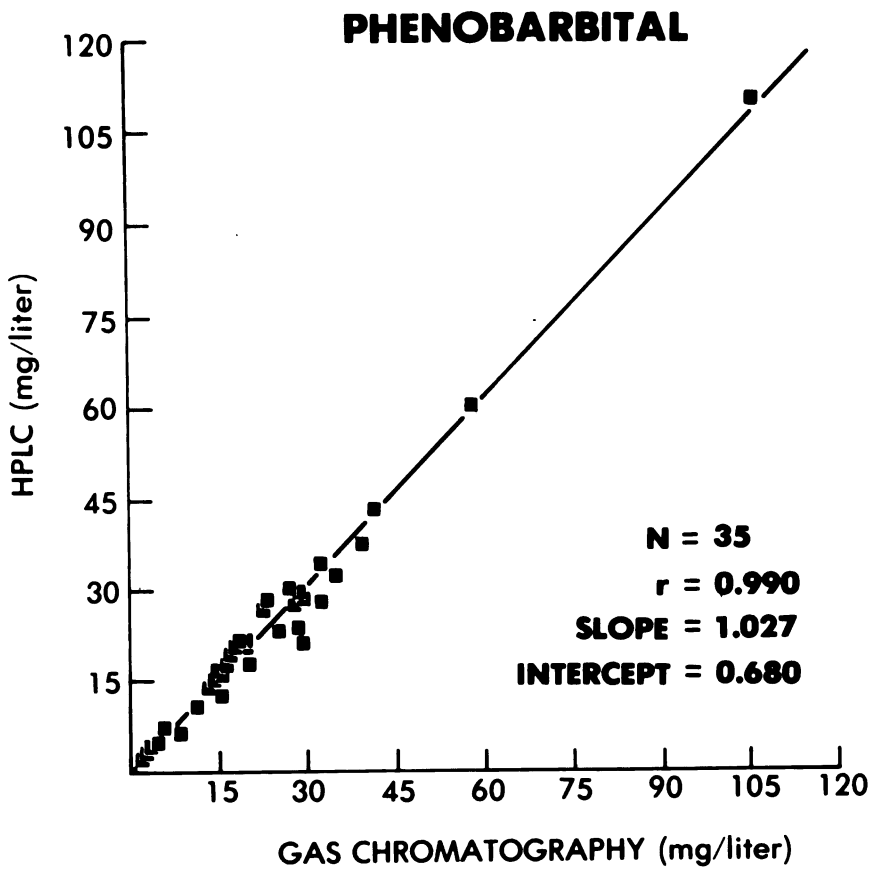
Diphenylhydantoin Analysis, GLC vs. HPLC



Phenobarbital (n=35) had an r value of 0.990, slope of 1.027 and an intercept of 0.680 (Figure 5).

FIGURE 5

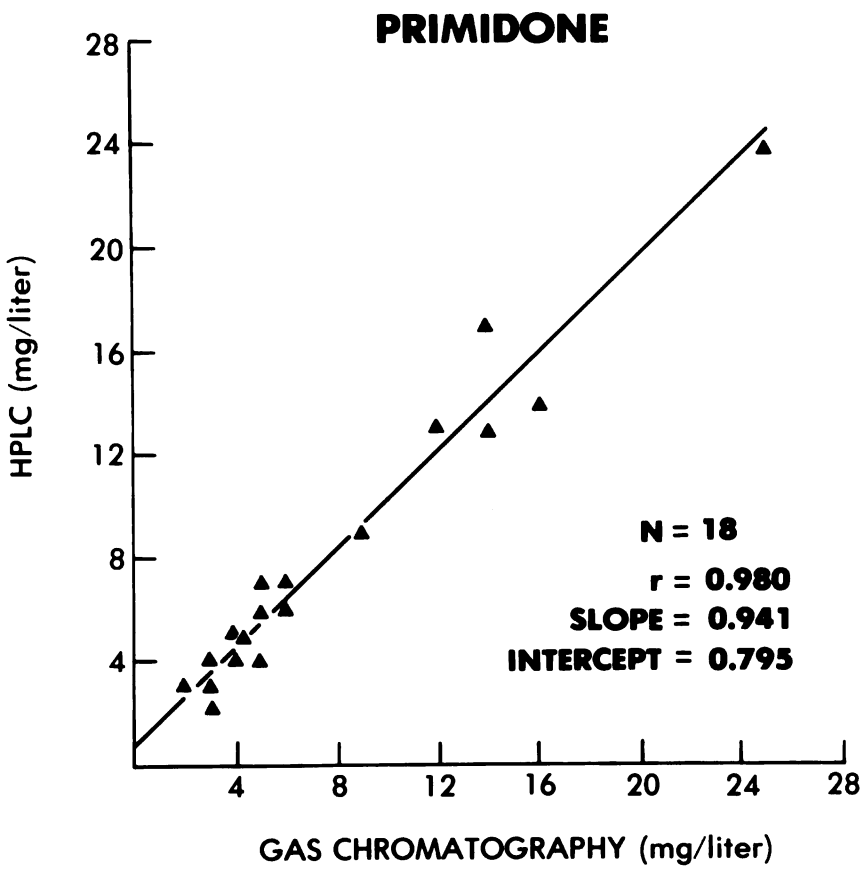
Phenobarbital Analysis, GLC vs. HPLC



Of 18 specimens containing primidone we calculated an r = 0.980, slope of 0.941, and an intercept of 0.795 (Figure 6).

FIGURE 6

Primidone Analysis, GLC vs. HPLC

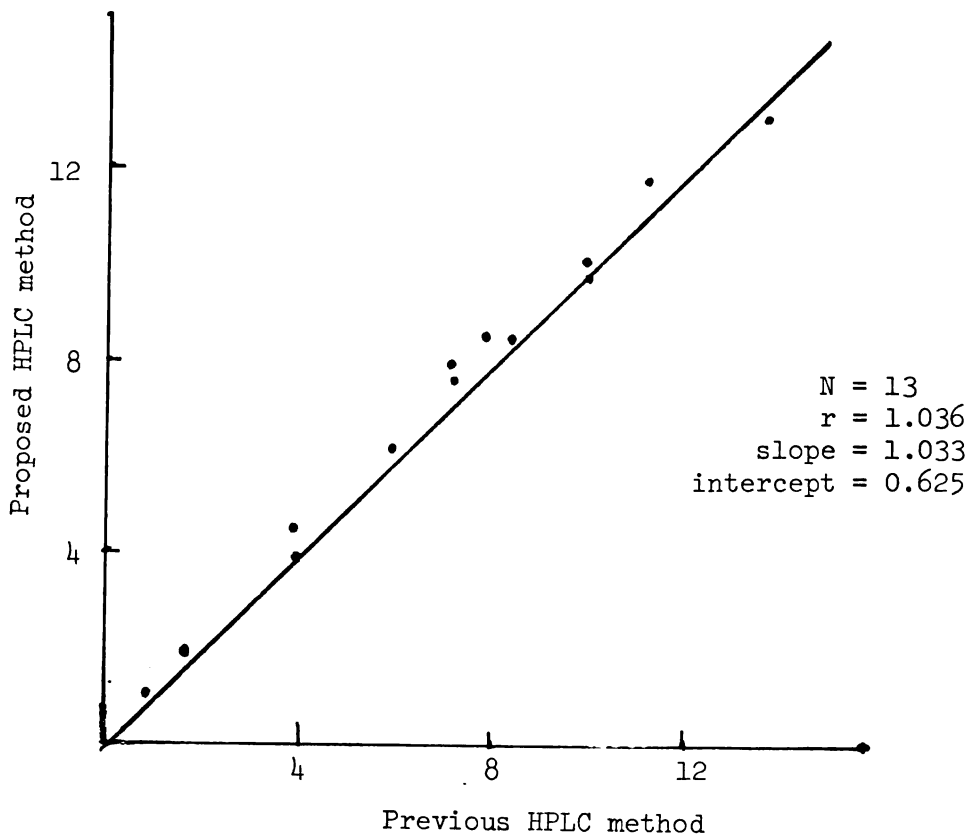


Our method shows better correlation for primidone in contrast to poor correlation reported by Soldin et al.(63). Strangely, Soldin shows low HPLC primidone concentrations compared to GLC even though he is measuring both primidone and PEMA in his primidone analysis.

Carbamazepine results were compared with a previously reported HPLC method (59). In 13 analyses we calculated a correlation coefficient of 1.036, slope of 1.033 and an intercept of 0.625 (Figure 7).

FIGURE 7

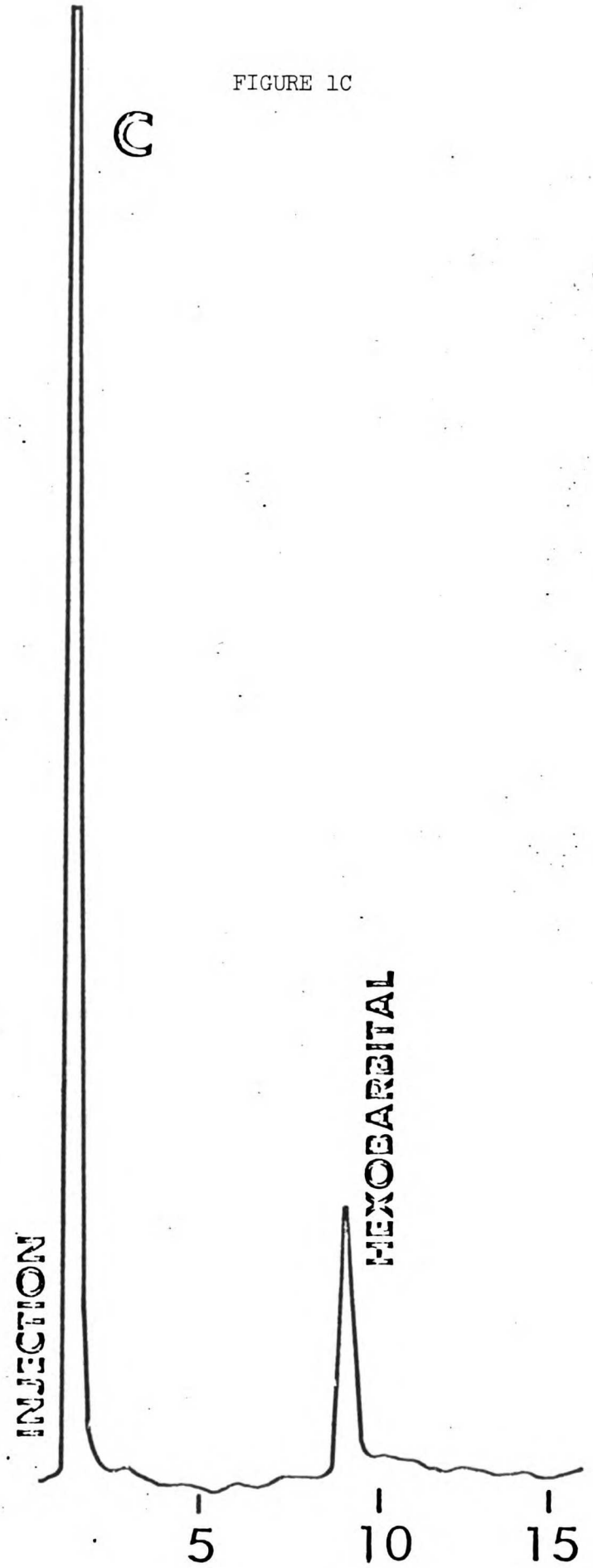
Carbamazepine Analysis, HPLC vs. Previous HPLC



Background: We processed twenty drug-free serum and plasma samples to determine the background interference. The serum or plasma samples were processed as above. We did not see any normal plasma or serum constituents eluting in the vicinity of the anticonvulsant drugs or internal standard. The backgrounds calculated from these samples ranged from 0 to 0.1 mg/liter (Figure 1C).

Interference: To determine the potential usefulness of the proposed HPLC methods, we undertook a systematic study to investigate the possible interference by other drugs. We injected pure drug standards of other less commonly used anticonvulsants in toxic concentrations to determine their potential interference in our method. Of the various other anticonvulsant drugs and barbiturates tested for interference, only ethotoin coeluted with phenobarbital. If ethotoin is present in the sample to be analyzed, a falsely high phenobarbital result will be calculated. Ethotoin is a rarely used anticonvulsant. Most of the benzodiazepines did not elute under our chromatographic conditions. Commonly used analgesics like salicylates, acetominophen, phenacetin, etc. did not interfere with any of the anticonvulsants. Caffeine, theophylline and other neutral and basic drugs listed in Table 5 did not interfere with the analysis. We have performed analysis on over 2,000 clinical samples without any noticeable interference by endogenous or exogenous substances. In addition, the method can be used for grossly hemolyzed samples without appreciable interference.

FIGURE 1C



CONCLUSION

Routine monitoring of anticonvulsant therapy requires specificity, speed and accuracy. Our method meets these requirements. None of the commonly used drugs tested interfered with the method. The method requires approximately ten minutes for sample preparation and approximately thirty minutes until the completion of the assay. Because the method can assay any combination of these drugs, serum from patients on multiple drug therapy must be analyzed only once. All of these time-saving factors reduce technologist costs.

The method can be adapted for pediatric samples (samples as little as 25 µl). Both day-to-day and within-day precision show that the method is suitable to be used routinely in a clinical laboratory.

The method has the potential for use in monitoring other anticonvulsant drugs in serum. Drugs such as mesantoin, phensuximide, methsuximide, and mephobarbital all separate from the five drugs studied in this paper. The same chromatographic conditions have been used to separate and quantitate acetaminophen and phenacetin in our laboratory (65). Many of the common barbiturates also separate which suggests that these conditions could be used for their analysis.

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