Table III—Comparison of the HPLC Method with the USP XX or Current Methodology

Assay	Number of Batches Assayed	Percent Difference[a]
Epinephrine	115	−0.3 (USP–HPLC)
Methylparaben	87	+2.5 (UV–HPLC)
Lidocaine hydrochloride	87	−2.3 (USP–HPLC)

[a] Mean percent difference for batches assayed.

as Fe^{2+} (11) and did not interfere with the assay. However, addition of 0.1 m*M* edetate disodium to the mobile phase resulted in complete elimination of the Fe^{2+} peak with no other effect on the chromatogram.

Comparison of HPLC Method with USP or Current Methodology—Production samples[21] of lidocaine hydrochloride with epinephrine in injectable solutions, which included vials, ampuls, and dental cartridges from batches < 1 month old to batches at their expiration date, were assayed by HPLC, fluorometry (epinephrine), titrimetry (lidocaine hydrochloride), and spectrophotometry (methylparaben). All analyses were performed on individual dosage units (if enough solution was available) or on aliquots from a pool of several dosage units (if one unit was inadequate). The results (Table III) indicate good agreement between the methods.

DISCUSSION

The HPLC procedure provides a significant advance over existing methodology for assaying components in epinephrine-containing local anesthetic injectable solutions. Three of the major components (epinephrine, lidocaine hydrochloride, and methylparaben) of these types of dosage forms may be assayed simultaneously in one injection. The current methodology requires separate analytical procedures for each component.

The small sample size (2 μl) allows replicate analysis of the three components from individual dosage units. The current methodology in some cases requires units to be pooled to obtain a sufficient sample for analysis.

No preparation of the sample is necessary. The dosage unit is opened, a 2-μl aliquot is removed with a 10-μl syringe, and the sample is injected into the chromatograph. The current methodology requires extensive sample preparation for each component.

The HPLC method is selective and specific for all three components and is stability indicating.

Although both the HPLC and current methodology may be automated, the HPLC method offers a distinct advantage since only one setup of automated equipment is needed. The current methodology requires three separate and different automated setups since the procedure for each component is different. The HPLC methodology can be used to assay other local anesthetic injectable solutions such as prilocaine hydrochloride, etidocaine hydrochloride, mepivacaine hydrochloride, and bupivacaine hydrochloride with little or no change in the mobile phase.

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ACKNOWLEDGMENTS

The authors thank Mr. Allard Edhorn, Dr. Peter Kissinger, and Dr. Ronald Shoup for valuable discussions on electrochemical detectors. They also thank Mr. Gerard Boyce and Mr. Bruce Manning for encouragement and Ms. Debbie Soloski and Ms. Patricia Murray for assistance in the comparison of the methods.

GC and GC–Mass Spectrometric Determination of *p*-Hydroxyphenobarbital Extracted from Plasma, Urine, and Hepatic Microsomes

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Received September 10, 1980, from the *Epilepsy Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20205.* Accepted for publication March 19, 1981.

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Abstract □ Analytical methodology was developed for the quantitation of *p*-hydroxyphenobarbital extracted from plasma, urine, and hepatic microsomes. *p*-Hydroxyphenobarbital was derivatized with an appropriate *n*-alkyl iodide in the presence of a methanolic base in aprotic solvent medium. The peralkylated derivatives were stable indefinitely and were quantitated by the sensitive and selective method of GC nitrogen-selective detection and/or selected ion monitoring. The accuracy, precision, and cross verification of all methods were good. The analysis was subsequently used to study the effects of other drugs on phenobarbital biodisposition.

Keyphrases □ *p*-Hydroxyphenobarbital—determination by GC and GC–mass spectrometry in urine, plasma, and hepatic microsomes □ Phenobarbital—quantitation of *p*-hydroxyphenobarbital by GC and GC–mass spectrometry in urine, plasma, and hepatic microsomes □ GC—nitrogen-selective detection of *p*-hydroxyphenobarbital in urine, plasma, and hepatic microsomes

Phenobarbital (I) is commonly used in the treatment of epilepsy, and *p*-hydroxyphenobarbital (II) is its major metabolite. Even though *p*-hydroxyphenobarbital is considered to be devoid of antiepileptic activity, its quantitation is important when studying phenobarbital biodisposition. During a study on the mechanisms of val-

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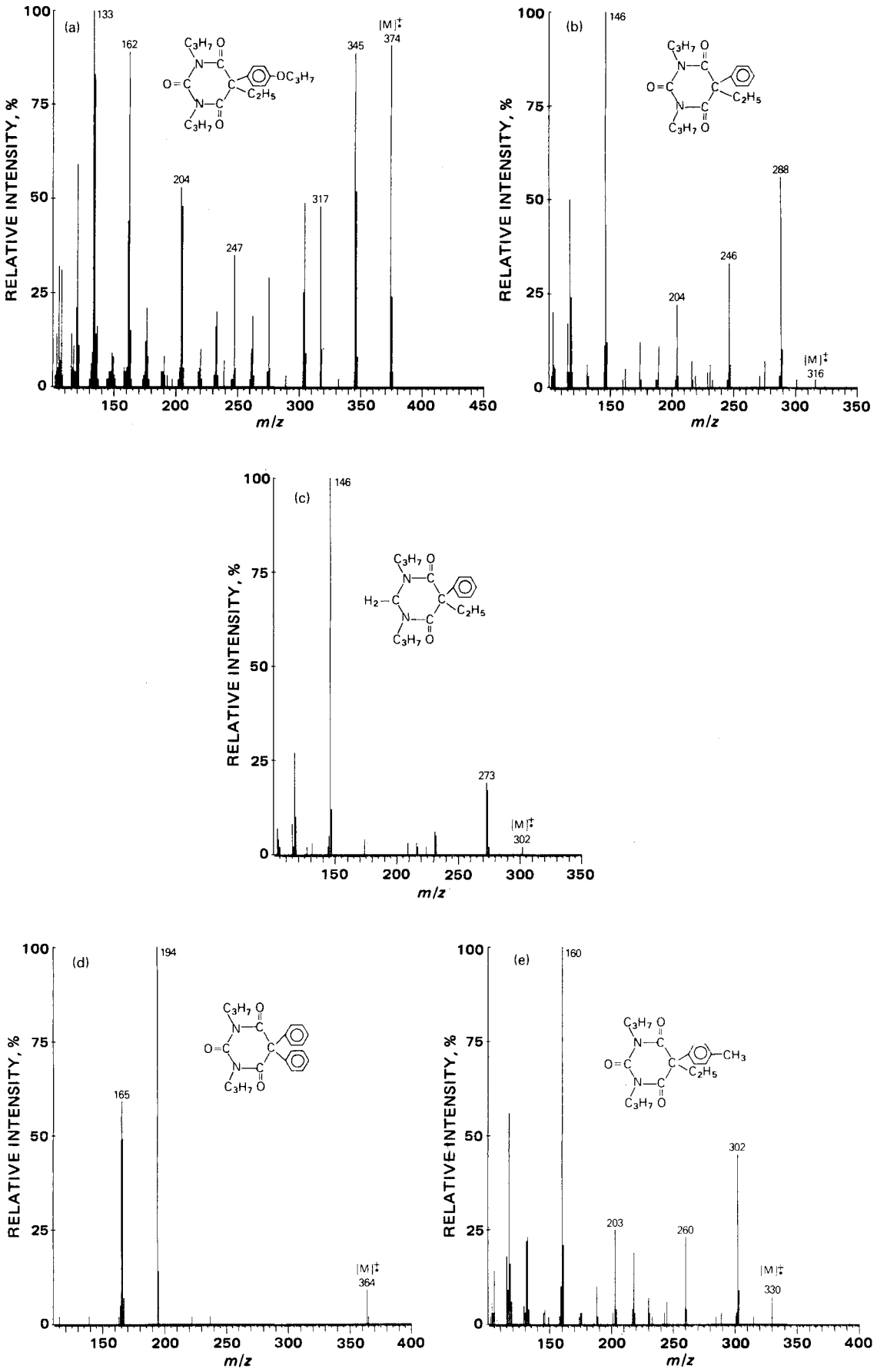

Figure 1—*Electron-impact mass spectra of propylated reference compounds and internal standards. Key:* a, p-*hydroxyphenobarbital;* b, *phenobarbital;* c, *primidone;* d, *diphenylbarbituric acid; and* e, p-*methylphenobarbital.*

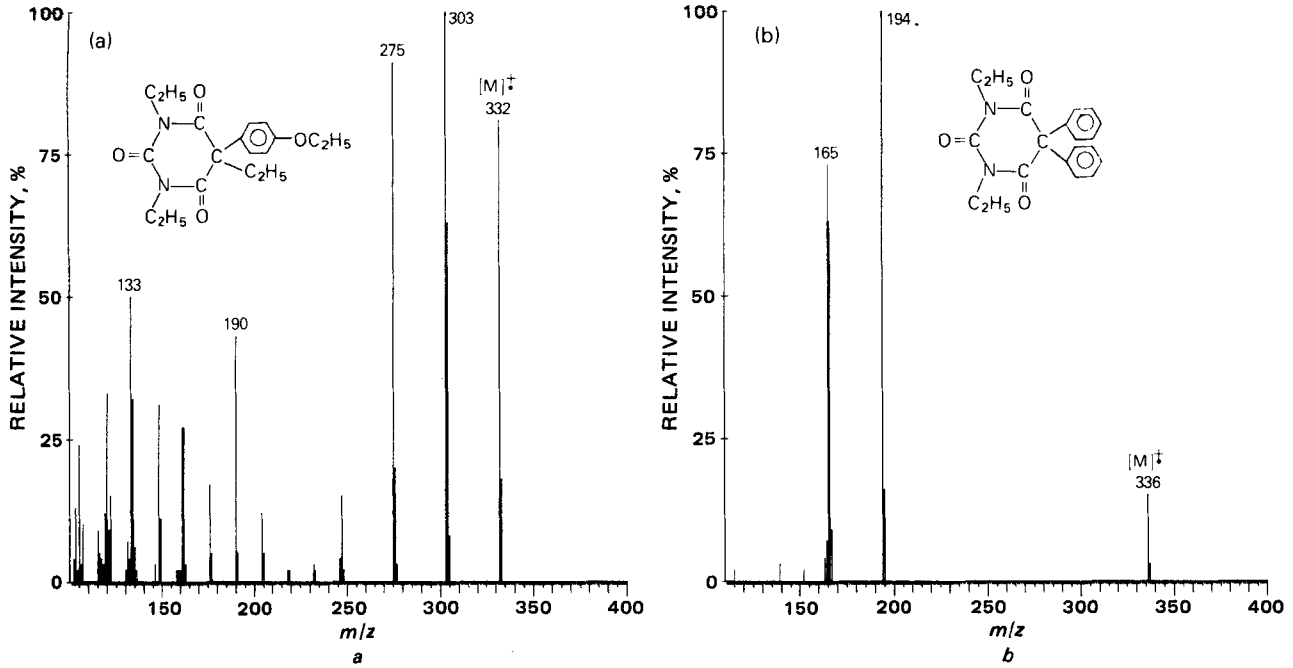

Figure 2—*Electron-impact spectra of ethylated* p-*hydroxyphenobarbital* (a) *and diphenylbarbituric acid* (b).

proate-caused elevation of plasma phenobarbital levels, it became necessary to quantitate *p*-hydroxyphenobarbital extracted from plasma, urine, and hepatic microsomes. The reported procedures for its quantitation lacked sensitivity or selectivity, and some methods had additional drawbacks. For example, countercurrent distribution and TLC in conjunction with liquid scintillation (1) are laborious and require the use of a radioactive tracer. TLC methods (2, 3) usually require a separate means of detection. GC methods using on-column methylation (4) can yield incomplete derivatization, and they also accelerate column degradation; furthermore, samples are not very stable once the alkylating base is added. The high-pressure liquid chromatographic method (5) has limited sensitivity due to a relatively poor extinction coefficient of unionized barbiturates.

Therefore, a GC nitrogen-selective detection method was developed for the determination of *p*-hydroxyphenobarbital. The nitrogen-selective detector is commercially available and is a reasonably priced accessory for the gas chromatograph. Its simplicity of operation is similar to that of the regular flame-ionization detector, but its sensitivity and selectivity are superior. The barbiturates were derivatized to optimize their chromatographic sep-

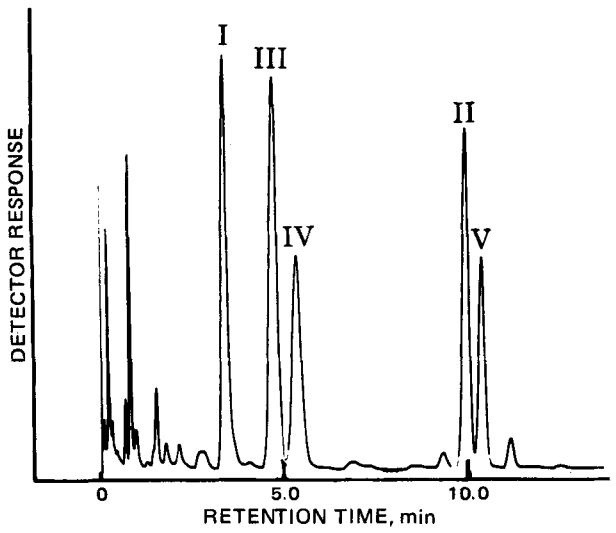

Figure 3—*Gas chromatogram of propylated human urine (hydrolyzed) extract using nitrogen–phosphorus flame-ionization detector. Levels of phenobarbital (I),* p-*hydroxyphenobarbital (II), and primidone (IV) were 34.6, 40.3, and 31.9 μg/ml, respectively. Diphenylbarbituric acid (V, 25 μg/ml) and* p-*methylphenobarbital (III, 50 μg/ml) were the internal standards.*

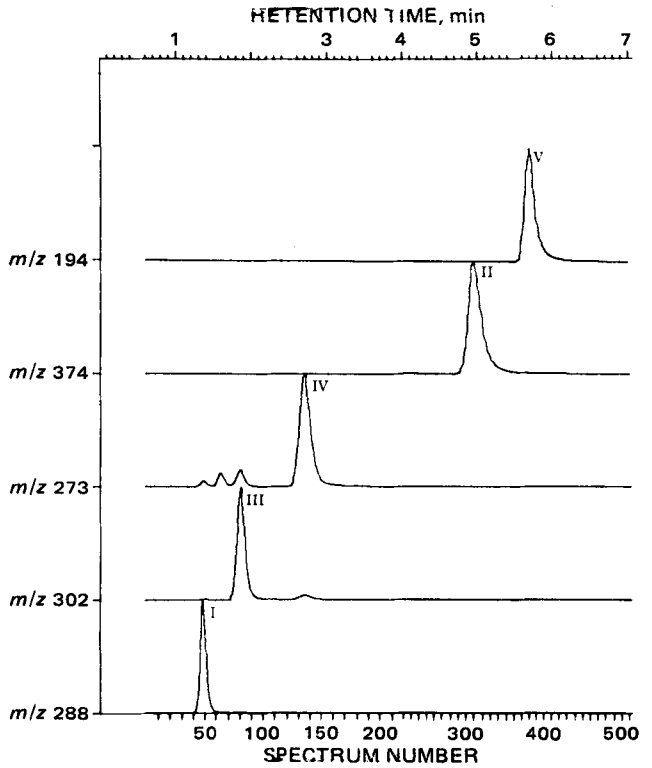

Figure 4—*Selected ion monitoring (electron-impact mode) tracing of propylated human urine (hydrolyzed) extract. For additional details, see Fig. 3.*

Table I—Accuracy and Precision of Determination of Urinary *p*-Hydroxyphenobarbital (II), Phenobarbital (I), and Primidone (IV)

Actual II, I and IV, μg/ml	Experimental[a], μg/ml		
	II	I	IV
10.00	10.08 ± 0.27	9.12 ± 0.28	10.32 ± 0.24
100.00	100.40 ± 1.23	101.10 ± 1.54	97.60 ± 1.72

[a] Values expressed as mean ± *SD* of four determinations.

aration and to stabilize them. Derivatization was performed by modification of a reported alkylation procedure for barbiturates (6).

These analytical methods were satisfactorily employed for *in vitro* animal experiments and *in vivo* human studies dealing with valproate-phenobarbital interaction (7–9).

EXPERIMENTAL

Instrumentation—The gas chromatograph[1] was equipped with a flame-ionization detector and a nitrogen–phosphorus flame-ionization detector. The injection port and detector temperatures were 260 and 300°, respectively. Helium was used as the carrier at a flow rate of 30 ml/min.

The dodecapole mass spectrometer[2] was equipped with electron-impact and chemical-ionization sources and was coupled to the gas chromatograph[3] by a glass-lined single-stage jet separator. Data acquisition and reduction were computer automated[4]. The temperatures of the injection port, transfer line, and ion source were 300, 300, and 180°, respectively. In the electron-impact mode, helium was used as the carrier at a flow rate of 25 ml/min; the operating pressure of the ion source was 3.7×10^{-6} torr, and the mass spectrometer was operated with an electron energy of 70 ev. In the chemical-ionization mode, methane was used as the carrier at a flow rate of 15 ml/min and as a reagent gas. The operating pressure of the ion source was 0.4 torr, and an ionizing energy of 250 ev was used to produce the reactant ions.

Materials—All organic solvents[5] were used as obtained after their purity was checked. Reference compounds (phenobarbital[6], primidone[6], and *p*-hydroxyphenobarbital[7]) and internal standards (*p*-methylphenobarbital[7] and 5,5-diphenylbarbituric acid[8]) were also checked for purity before use. Methyl, ethyl, *n*-propyl, and *n*-butyl iodides[9] and tetraethylammonium hydroxide (25% methanolic solution[9]) were used as obtained.

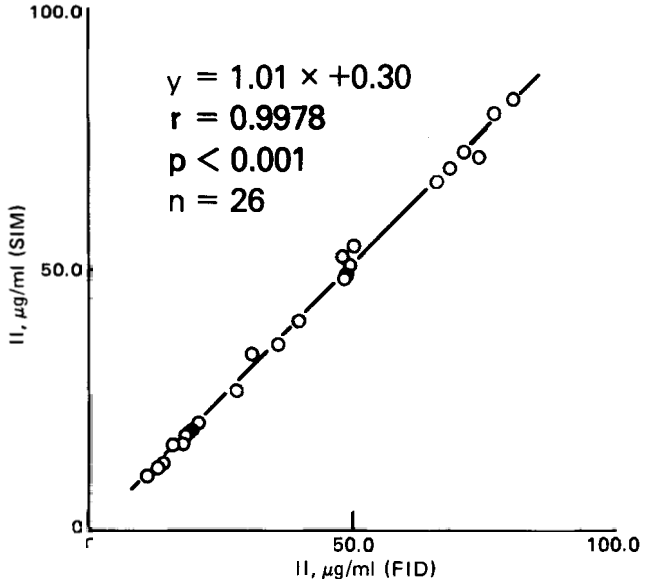

Figure 5—*Relationship between selected ion monitoring (SIM) at electron-impact mode and GC nitrogen–phosphorus flame-ionization detector (FID) methods for quantitation of urinary* p-*hydroxyphenobarbital (II).*

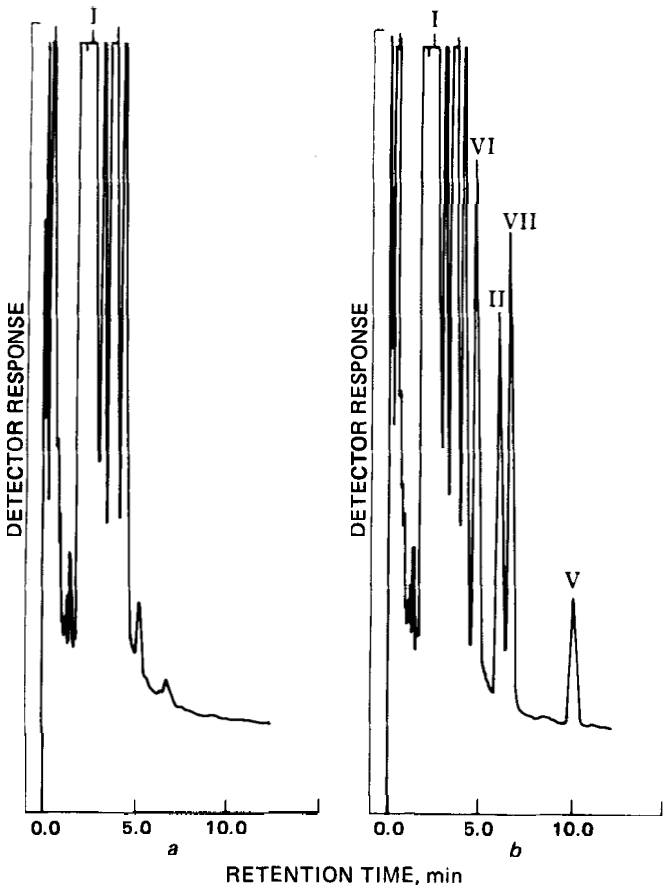

Figure 6—*Gas chromatogram of extract from microsomal blank (a) and microsomal phenobarbital reaction mixture containing phenobarbital (b) using nitrogen–phosphorus flame-ionization detector. The reaction occurred in the presence of 4 mM phenobarbital, and 0.875 μg of* p-*hydroxyphenobarbital was formed. Diphenylbarbituric acid (0.50 μg) was the internal standard. Peaks VI and VII represent other metabolites of phenobarbital. The microsomal blank was run parallel to the microsomal phenobarbital reaction, but NADPH was added after termination of the reaction and no internal standard was added. The chromatogram of the blank shows a lack of interferences for* p-*hydroxyphenobarbital and diphenylbarbituric acid.*

Silanized GC glass columns[10] for heated on-column injection were packed with commercially available packings[10].

Sodium salt of phenobarbital[11] was used for animal treatment.

A commercial source of β-glucuronidase–arylsulfatase and NADPH was used[12].

All other chemicals were reagent grade.

Human Subjects—The subjects were epileptic patients[13] who consented to participate in a clinical study of drug–drug interactions in antiepileptic therapy. Blood samples were obtained at 7 am, just prior to administration of the patients' morning medication (including phenobarbital and/or primidone); 24-hr urine samples were also collected. For the clinical study, blood and urine samples were obtained while the plasma barbiturate levels were at steady state.

Animals—Adult male Holtzman rats[14], ~200 g, were injected intra-

[1] Hewlett-Packard model 5840A.
[2] Hewlett-Packard model 5982A.
[3] Hewlett-Packard model 5700 series.
[4] Hewlett-Packard model 5934A data system.
[5] Burdick & Jackson Laboratories, Muskegon, Mich.
[6] USPC, Rockville, Md.
[7] Aldrich Chemical Co., Milwaukee, Wis.
[8] Generous gift from Dr. A. Raines, Department of Pharmacology, School of Medicine and Dentistry, Georgetown University, Washington, DC 20007.
[9] Eastman Chemical Co., Rochester, NY.
[10] Supelco, Bellefonte, Pa.
[11] Merck & Co., Rahway, N.J.
[12] Boehringer-Mannheim Biochemicals, Indianapolis, Ind.
[13] Admitted to the Clinical Center, National Institutes of Health.
[14] Charles River, Wilmington, Mass.

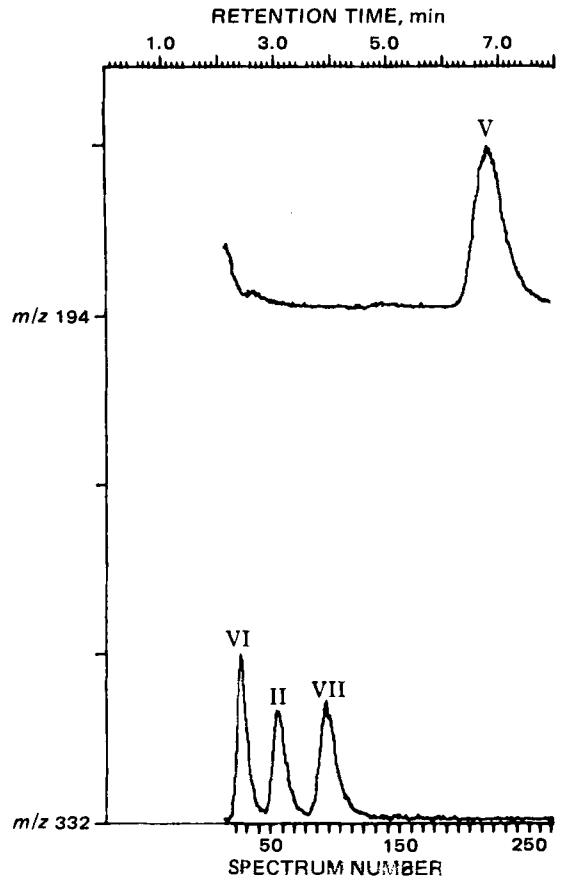

Figure 7—*Selected ion monitoring (electron-impact mode) tracing of ethylated extract from microsomal phenobarbital metabolic reaction. For additional details, see Fig. 6.*

peritoneally with aqueous phenobarbital sodium solution (75 mg/kg) on 3 consecutive days. The rats were then sacrificed, and hepatic microsomes were prepared as previously described (10).

Microsomal Reactions—Microsomal reactions were carried out in 0.1 *M* potassium phosphate buffer (pH 7.50) in the presence of 1.83 nmoles of microsomal cytochrome P-450 and various concentrations of the substrate, phenobarbital. Following preequilibration of the samples at 37° for 1 min, reaction was initiated with NADPH (final NADPH concentration in the reaction mixture was 0.25 m*M*). The samples were incubated at 37° for 8 min in 5 ml of reaction mixture.

Plasma Extraction—Stock methanolic solutions of reference compounds and internal standards were kept at 4° and were stable on storage. Methanolic solutions of reference compounds (10–200 μl) were added to the tubes to be used for standards, and methanolic solutions of internal standards were added to all of the tubes. Methanol was evaporated, using a rotary evaporator with aspirator vacuum[15]. Blank plasma or plasma from phenobarbital-treated subjects (1 ml) was added to the appropriate tubes. Spiked plasma standards were extracted along with the patients' samples.

For the determination of total (free plus conjugated) *p*-hydroxyphenobarbital, the samples were hydrolyzed. Free *p*-hydroxyphenobarbital was determined by omitting the hydrolysis. For determination of total *p*-hydroxyphenobarbital, 0.2 ml of 0.5 *M* sodium acetate buffer (pH 5.00) was added, followed by 50 μl of β-glucuronidase–arylsulfatase, and samples were incubated at 37° for 20–24 hr. To hydrolyzed and nonhydrolyzed samples, 1 ml of 1 *M* monobasic potassium phosphate was added; samples were then extracted with 7 ml of ethyl acetate, using a reciprocating shaker. Following centrifugation (~2000 rpm), the ethyl acetate phase was collected and washed with 2 ml of 0.1 *N* HCl and evaporated to dryness. Hexane was added to the residue, and the samples were extracted with 5 ml of acetonitrile. Hexane was used to remove endogenous lipoidal substances. The acetonitrile phase was collected and evaporated to dryness, and the residue was ready for derivatization.

Urine Extraction—The hydrolysis of urine samples was analogous to that of the plasma samples. Hydrolyzed and nonhydrolyzed urine samples were extracted in the same manner as the plasma samples.

[15] Buchler Instruments, Fort Lee, N.J.

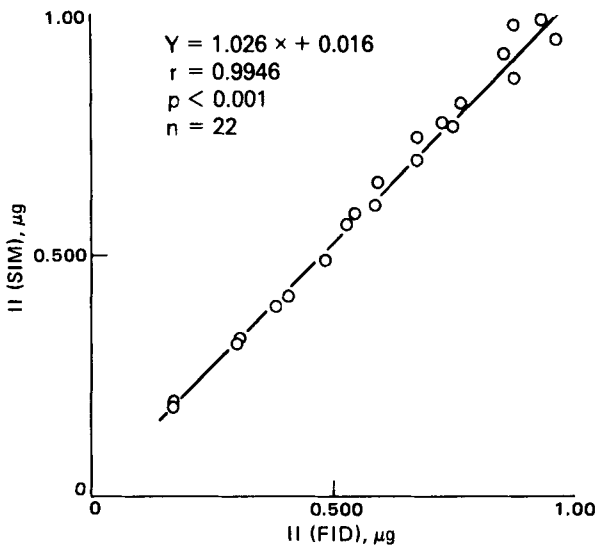

Figure 8—*Relationship between selected ion monitoring (SIM) (electron-impact mode) and GC nitrogen–phosphorus flame-ionization detector (FID) methods for quantitation of* p*-hydroxyphenobarbital (II) formed in microsomal reaction.*

Microsome Extraction—Microsomal reactions were terminated by the addition of 7 ml of cold benzene (or toluene). For consistency, phenobarbital was added such that its final concentration prior to the addition of benzene was always the same. Aqueous and organic phases were mixed by hand inversion, samples were centrifuged, and the benzene layer was discarded. The samples were then washed again with 7 ml of benzene. A double benzene wash was employed to eliminate some of the large excess of phenobarbital, which would otherwise swamp the peak corresponding to the relatively small amounts of generated *p*-hydroxyphenobarbital. *p*-Hydroxyphenobarbital was not extractable into benzene under these conditions. Following the benzene washes, diphenylbarbituric acid (the internal standard) was added, and the samples were extracted with 7 ml of ethyl acetate; the ethyl acetate phase was collected and evaporated to dryness.

Derivatization Reactions—The derivatization reaction was essentially the same for the extracts from plasma, urine, and microsomes. The main difference was in the choice of the *n*-alkyl iodide; the selection of alkyl iodide was made to optimize chromatographic separations. Ethyl iodide was selected for derivatization of plasma and microsomal extracts, whereas *n*-propyl iodide was used for urinary extracts. To the residue from the extract were added 200 μl of acetonitrile and 50 μl of methanolic (25%) tetraethylammonium hydroxide, and the sample was mixed well. The appropriate *n*-alkyl iodide (25 μl) was added, and the sample was mixed again and placed in a water bath at 60° for 60 min, the optimum conditions for all derivatizations.

At the end of this time, 7 ml of chloroform was added, and the sample was washed sequentially with 2 ml of 0.1 *N* HCl, 0.1 *N* NaOH, and deionized water. The washed chloroform phase was evaporated to dryness, and the residue was dissolved in methanol or isoamyl acetate for analysis. Derivatized samples were stable at 4° or at room temperature for at least 2 months.

The major modification of the reported derivatization procedure for barbiturates (6) was the use of a different aprotic solvent. Acetonitrile was selected instead of *N*,*N*-dimethylacetamide because the former is more easily removed by evaporation. It has also been shown (11) that acetone serves equally as well as *N*,*N*-dimethylacetamide in alkylation of the phenolic group; but because higher temperatures were required for propylation of *p*-hydroxyphenobarbital, the low boiling point of acetone precluded its use. It was confirmed that acetonitrile or 2-butanone serves equally as well as *N*,*N*-dimethylacetamide in the derivatization of *p*-hydroxyphenobarbital or other barbiturates, and all three were superior to a protic solvent such as methanol.

Analysis—The samples were analyzed on a gas chromatograph equipped with a nitrogen–phosphorus flame-ionization detector (urine and microsomes) or a gas chromatograph–mass spectrometer–computer system (plasma). Quantitation was done using peak heights (urine and microsomes) or selected ion monitoring using peak areas (plasma).

Cross-verification of the analytical methods was carried out on the gas

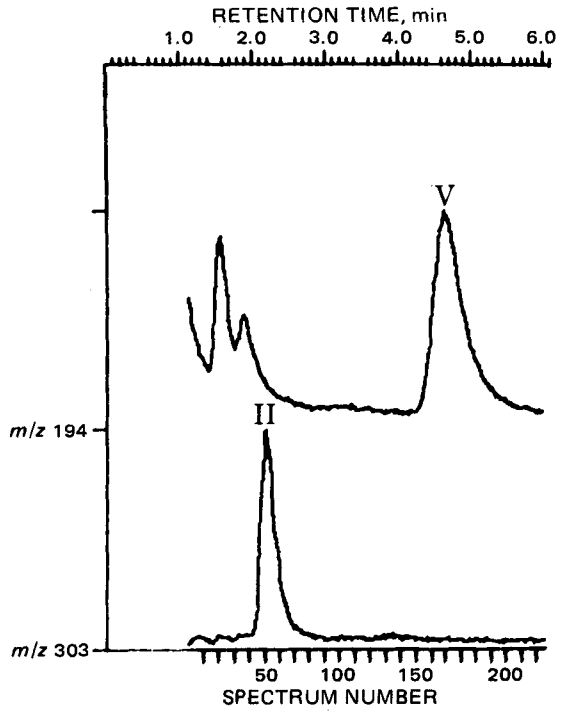

Figure 9—*Selected ion monitoring (electron-impact mode) tracing of ethylated human plasma (nonhydrolyzed) extract. The* p-*hydroxyphenobarbital level was 0.29 μg/ml. Diphenylbarbituric acid (0.50 μg/ml) was the internal standard.*

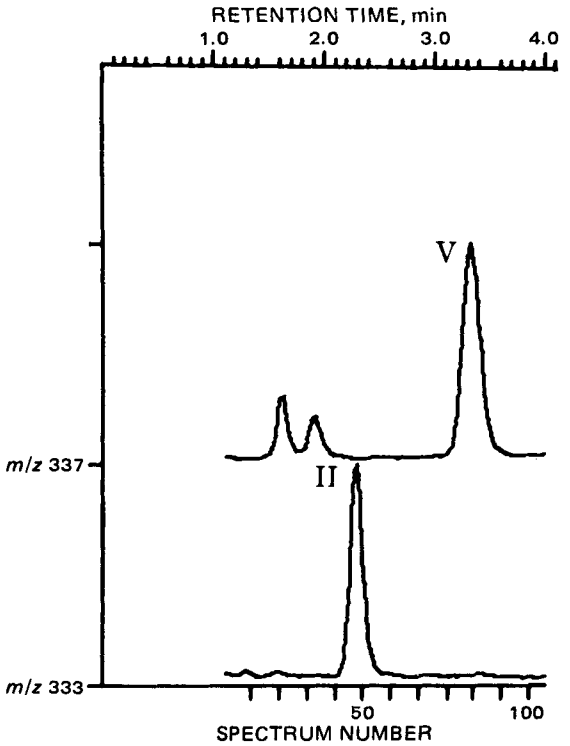

Figure 10—*Selected ion monitoring (chemical-ionization mode) tracing of ethylated human plasma (nonhydrolyzed) extract. For additional details, see Fig. 9.*

chromatograph–mass spectrometer–computer system. Quantitation was done by selected ion monitoring, using peak areas expressed as percent relative to internal standard. The selected ion monitoring technique was used to optimize sensitivity and specificity.

All samples, including the standards, were always injected in random order; some samples were injected in duplicate. Reproducibility between duplicate injections was always >95%. Random samples were analyzed in duplicate on the same day and on different days, and reproducibility was also >95%.

Urine Analysis—The gas chromatograph was equipped with a glass column (1.8 m × 2 mm i.d.) packed with 3% SE-30 on 100–120-mesh Gas Chrom Q. Oven temperature was maintained at 205° for 6 min, followed by temperature programming at 10°/min to 250°. Analysis of urinary *p*-hydroxyphenobarbital also allowed simultaneous quantitation of phenobarbital and primidone. *p*-Methylphenobarbital (III) was the internal standard for phenobarbital and primidone (IV); diphenylbarbituric acid (V) was the internal standard for *p*-hydroxyphenobarbital.

For verification of the analytical method, the gas chromatograph–mass spectrometer–computer system, operated in the electron-impact mode, was equipped with a glass column (0.9 m × 2 mm i.d.) packed with 3% SP 2250 DA on 100–120-mesh Supelcoport. Oven temperature was maintained at 200° for 4 min and then was programmed at 32°/min to 250°. The ions of *m/z* 374, 288, 273, 194, and 302 were monitored for propylated *p*-hydroxyphenobarbital (Fig. 1*a*), phenobarbital (Fig. 1*b*), primidone (Fig. 1*c*), diphenylbarbituric acid (Fig. 1*d*), and *p*-methylphenobarbital (Fig. 1*e*), respectively.

Microsome Analysis—The gas chromatograph was equipped with a glass column (1.8 m × 2 mm i.d.) packed with 2% OV3 on 100–120-mesh Supelcoport, and the analysis was done isothermally at 210°. Of the several packings tested, this one gave the most satisfying resolution for the peaks of interest. Diphenylbarbituric acid was the internal standard.

The gas chromatograph–mass spectrometer–computer system used for verification of the analytical method was operated in the electron-impact mode and was fitted with a glass column (0.9 m × 2 mm i.d.) packed with 3% SP 2250 DA on 100–120-mesh Supelcoport. The analysis was done isothermally at 190°. The ions of *m/z* 332 and 194 were monitored for *p*-hydroxyphenobarbital (Fig. 2*a*) and diphenylbarbituric acid (Fig. 2*b*), respectively.

Plasma Analysis—The gas chromatograph–mass spectrometer–computer system, operated in the electron-impact mode, was equipped with a glass column (0.9 m × 2 mm i.d.) packed with 3% SP 2250 DA on 100–120-mesh Supelcoport, and the analysis was done isothermally at 200°. Diphenylbarbituric acid was the internal standard. The ions of *m/z* 303 and 194 were monitored for *p*-hydroxyphenobarbital and diphenylbarbituric acid, respectively.

The analytical method was verified by a gas chromatograph–mass spectrometer–computer system operated in the chemical-ionization mode, using a glass column (1.2 m × 2 mm i.d.) packed with 3% OV-1 on Gas Chrom Q. The analysis was done isothermally at 220°. The ions of *m/z* 333 and 337 were monitored for *p*-hydroxyphenobarbital and diphenylbarbituric acid, respectively. The monitored ions corresponded to the quasimolecular ions for the two compounds, and they both were the base peak as well.

RESULTS AND DISCUSSION

Urine—The capability of monitoring urinary phenobarbital and primidone along with *p*-hydroxyphenobarbital by this method is convenient because phenobarbital is a major metabolite of primidone, a closely related antiepileptic agent. Thus, if primidone is administered, its urinary output can be quantitated at the same time as its primary and secondary metabolites, phenobarbital and *p*-hydroxyphenobarbital, respectively. A gas chromatogram (Fig. 3) and selected ion monitoring tracing (Fig. 4) of urinary *p*-hydroxyphenobarbital, phenobarbital, and primidone are illustrated. Standard curves were set up for all three barbiturates, and the concentrations selected for all three ranged from 10 to 100 μg/ml. This range bracketed the range of experimental values for all three barbiturates.

Representative standard curves obtained from the GC nitrogen–phosphorus flame-ionization detector data exhibited the following linear least-squares regression equations and correlation coefficients (r^2): $y = 0.0204x + 0.006$ and $r^2 = 0.9996$ for *p*-hydroxyphenobarbital; $y = 0.0313x + 0.072$ and $r^2 = 0.9995$ for phenobarbital; and $y = 0.0167x + 0.004$ and $r^2 = 0.9965$ for primidone. Furthermore, the accuracy and precision of the method were evaluated by quadruplicate analyses of spiked samples; the results (Table I) demonstrated good accuracy and precision.

There was a good correlation between the GC nitrogen–phosphorus flame-ionization detector and selected ion monitoring methods for quantitation of urinary *p*-hydroxyphenobarbital (Fig. 5). A similar correlation was also obtained for the quantitation of phenobarbital and primidone in the urine. The 24-hr urinary output of phenobarbital, free *p*-hydroxyphenobarbital, and total (free plus conjugated) *p*-hydroxyphenobarbital from a patient on a therapeutic regimen of phenobarbital alone (180 mg/day) was 33.0 ± 4.9 μg/ml, 27.3 ± 2.7 mg, and 60.9 ± 4.1

mg, respectively (mean ± *SD* of five determinations). The urinary output of phenobarbital is reported in concentration units rather than in absolute amount of excreted phenobarbital because the amount of urinary phenobarbital is dependent on urinary volume, which is not the case for free or conjugated *p*-hydroxyphenobarbital.

Microsomes—A gas chromatogram (Fig. 6*b*) and selected ion monitoring tracing (Fig. 7) of *p*-hydroxyphenobarbital generated in the microsomal reaction are illustrated. A representative standard curve obtained from the GC nitrogen–phosphorus flame-ionization detector data was described by the linear least-squares regression equation as $y = 3.34x + 0.005$, $r^2 = 0.9993$. The standards ranged from 0.10 to 1.00 μg of *p*-hydroxyphenobarbital and bracketed the range of experimental values. The accuracy and precision of the method were measured by quadruplicate analyses of spiked samples. The results were 0.098 ± 0.001 and 1.04 ± 0.03 μg of *p*-hydroxyphenobarbital for samples spiked with 0.10 and 1.00 μg of *p*-hydroxyphenobarbital, respectively (mean ± *SD* of four determinations).

There was good correlation between the GC nitrogen–phosphorus flame-ionization detector and selected ion monitoring methods for quantitation of *p*-hydroxyphenobarbital in the microsomal phenobarbital metabolic reaction (Fig. 8). In the microsomal oxidative metabolism of phenobarbital, two metabolites in addition to *p*-hydroxyphenobarbital were detected (peaks VI and VII in Figs. 6*b* and 7). Compound VI was subsequently shown to correspond to *m*-hydroxyphenobarbital on the basis of the same retention times as those of the synthetic reference compound[16] in several chromatographic systems. Furthermore, ratios for the relative abundance of several ions for this metabolite were virtually identical to those of the reference compound, as determined by the gas chromatograph–mass spectrometer–computer system. Compound VII, however, could possibly be the *ortho* analog on the basis of some limited electron-impact mass spectrometric data. However, a complete mass spectrometric analysis was not performed, and the reference standard was not available. Thus, the identity of VII remains speculative. The values for K_m and V_{max}, the kinetic parameters for *p*-hydroxylation of phenobarbital, were determined to be 1.16 m*M* and 0.325 nmole of *p*-hydroxyphenobarbital formed min^{-1} $nmole^{-1}$ of P-450, respectively.

Plasma—Occasional interferences were encountered in the analysis of plasma *p*-hydroxyphenobarbital by the GC nitrogen–phosphorus flame-ionization detector method. Since it proved difficult to eliminate them satisfactorily with column packing phases of different polarities, a more specific selected ion monitoring method was developed. Selected ion monitoring tracings in the electron-impact mode (Fig. 9) and chemical ionization mode (Fig. 10) for the analysis of plasma *p*-hydroxyphenobarbital are illustrated. A representative standard curve obtained from the selected ion monitoring (electron-impact) data was described by the linear least-squares equation as $y = 49.68x + 0.017$, $r^2 = 0.9978$. The standards ranged from 0.25 to 1.50 μg/ml of *p*-hydroxyphenobarbital and bracketed the range of experimental values. The accuracy and precision of the method were evaluated by quadruplicate analyses of spiked samples. The results were 0.268 ± 0.014 and 1.05 ± 0.03 μg/ml of *p*-hydroxyphenobarbital for samples spiked with 0.250 and 1.00 μg/ml of *p*-hydroxyphenobarbital, respectively.

There was good correlation between the electron-impact and chemical-ionization modes of the selected ion monitoring method. For example, quadruplicate analyses of a plasma sample yielded mean (±*SD*) levels of 0.344 ± 0.008 and 0.338 ± 0.014 μg/ml of *p*-hydroxyphenobarbital in the electron-impact and chemical-ionization modes, respectively. Plasma levels of free *p*-hydroxyphenobarbital and total (free plus conjugated) *p*-hydroxyphenobarbital in a patient maintained on a therapeutic regimen of phenobarbital alone (180 mg/day) were 0.516 ± 0.022 and 0.763 ± 0.057 μg/ml, respectively (mean ± *SD* of four determinations).

[16] Generous gift from Dr. K. H. Dudley, Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, N.C.

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ACKNOWLEDGMENTS

The authors thank Dr. R. J. Porter, Dr. E. Schulman, Dr. W. Theodore, and Dr. J. K. Penry for selecting and clinically managing the patients from the Clinical Epilepsy Section, Experimental Therapeutics Branch, National Institute of Neurological and Communicative Disorders and Stroke, and Ms. B. J. Hessie for editorial assistance.