GLC Determination of Ethinamate and Its Hydroxy Derivative in Biological Fluids

J. W. KLEBER [x], L. D. BECHTOL, M. K. BRUNSON, and S. M. CHERNISH

Abstract □ A sensitive, specific GLC assay was developed for the determination of ethinamate in plasma and its major metabolite, *trans*-4-hydroxyethinamate, in urine. The assay uses a mass internal standard of dimethylethinamate. Ethinamate is extracted from alkalinized plasma with dichloromethane. Urine samples require β-glucuronidase hydrolysis prior to extraction of hydroxyethinamate. The dichloromethane is removed by evaporation, and the compounds are measured by GLC using a flame-ionization detector. By using GLC–chemical-ionization mass spectrometry, the compounds measured were identified as the intact ethinamates. Plasma and urine data are presented from a bioavailability study to demonstrate the utility of this method. From these data, the ethinamate plasma half-life was calculated as 1.9 ± 0.3 hr.

Keyphrases □ Ethinamate and hydroxy derivative—GLC analysis in biological fluids, bioavailability from tablets and capsules, humans □ GLC—analysis, ethinamate and hydroxy derivative in biological fluids □ Sedatives—ethinamate and hydroxy derivative, GLC analysis in biological fluids, bioavailability from tablets and capsules, humans

GLC of acetylenic hypnotic agents was first described in 1960 (1) and later applied to biological samples (2). A method also was developed[1] for the determination of ethinamate[2] in human plasma by GLC. This method was improved (3) by using benzene instead of carbon disulfide as the extracting solvent. None of the foregoing methods utilized a mass internal standard.

This paper describes a method for the assay of ethinamate (I) in plasma and of *trans*-4-hydroxyethinamate (III) (4) in urine; it employs a mass internal standard.

EXPERIMENTAL

Reagents—Ethinamate and dimethylethinamate (II) were synthesized[3]. Compound II was used as the internal standard for GLC analysis. Compound III was obtained from the urine of human subjects given ethinamate. All solvents were spectroquality. The GLC packing material was 2% OV-17 on 80–100-mesh Chromosorb G[4].

Apparatus—The gas–liquid chromatograph[5] was equipped with a flame-ionization detector and 1-mv recorder[6]. Disposable 15-ml centrifuge tubes[7] were used for the extractions. Tapered 15-ml glass centrifuge tubes[8], previously silanized, were used for evaporating dichloromethane (IV).

Chromatographic Conditions—The recorder was operated at a chart speed of 0.63 cm/min. Helium carrier gas (flow rate of 60 ml/min) was filtered through a sieve, which had been preconditioned overnight at 200° with dry nitrogen. A glass column (0.61 m × 3 mm i.d.) containing the packing material was heated isothermally at 140° for 4 min followed by 16°/min programming to 240° during the analysis to clear the column of late emerging peaks. The detector temperature was maintained at 250°. The oxygen and hydrogen flow rates were 240 and 60 ml/min, respectively.

Under these conditions, the following retention times were observed: I, 170 sec; II, 240 sec; and III, 420 sec (Fig. 1). The chromatogram obtained when a urinary extract was evaluated by GLC looked similar but was devoid of the ethinamate peak.

Table I—Precision and Accuracy in Measurement of Ethinamate Added to Plasma and *trans*-4-Hydroxyethinamate Added to Urine

Added, μg/ml	Measured μg/ml	*RSD*, %	*RE*, %
	Plasma		
1.0	0.91 (0.76–1.09)[a]	14.3	−9.0
4.0	3.76 (3.60–3.98)	3.5	−6.0
8.0	7.97 (7.38–8.51)	5.1	−0.4
	Urine		
300	310 (290–322)[b]	4.7	+3.3
600	616 (605–645)	3.1	+2.7

[a] Mean of eight replicate samples with range. [b] Mean of four replicate samples with range.

Procedure—The 15-ml tapered centrifuge tubes were washed with chromic acid cleaning solution, rinsed with water, dried, and then silanized using a 1% solution of dimethyldichlorosilane in toluene[9]. A standard solution of ethinamate in chloroform was added to 2 ml of blank plasma to give calibration concentrations of 0, 0.5, 2, 4, and 8 μg of drug/ml. To each tube was added 30 μl (6 μg) of the internal standard solution and 1 ml of 0.1 *N* NaOH. The mixture was then vortexed to ensure uniform distribution of the compounds in the plasma.

With a pipet[10], 10 ml of IV was added to each tube. The samples were shaken for 2 min and then centrifuged at 2000 rpm for 4 min. The upper aqueous phase was aspirated and discarded. The IV phase was transferred to a 15-ml silanized, tapered centrifuge tube and was evaporated to dryness at room temperature under a gentle stream of dry nitrogen. The residue was dissolved in 20 μl of carbon disulfide–chloroform (1:1) with the aid of a vortex mixer, and 2 μl of each sample solution was injected onto the GLC column.

Urine was assayed for III by the following procedure. A standard solution of III in chloroform was added to 0.1 ml of blank urine to give calibration concentrations of 100, 200, 400, and 800 μg of drug/ml. This solution was buffered with 1 ml of 0.2 *N* sodium acetate buffer (pH 5.5)

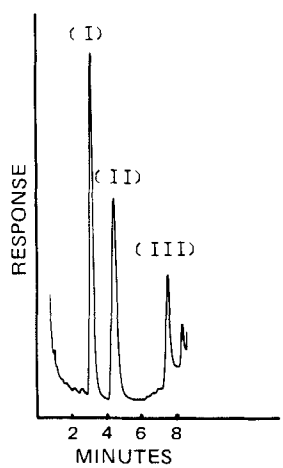

Figure 1—*Gas–liquid chromatogram of extract of plasma spiked with 4 μg/ml of I, 6 μg/ml of II, and 4 μg/ml of III.*

[1] D. M. Fuller, Lilly Research Center, Erl Wood, England, personal communication.
[2] Valmid, Eli Lilly and Co., Indianapolis, Ind.
[3] At the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.
[4] Ohio Valley Specialty Chemical, Marietta, Ohio.
[5] Model 5700A, Hewlett-Packard, Avondale, Pa.
[6] Model 7127A, Hewlett-Packard, Avondale, Pa.
[7] Corning Glass Co., Corning, N.Y.
[8] Matheson Scientific Co., Chicago, Ill.
[9] This treatment appears to prevent adsorption of the ethinamates to the glassware.
[10] Repipet, Labindustries, Berkeley, Calif.

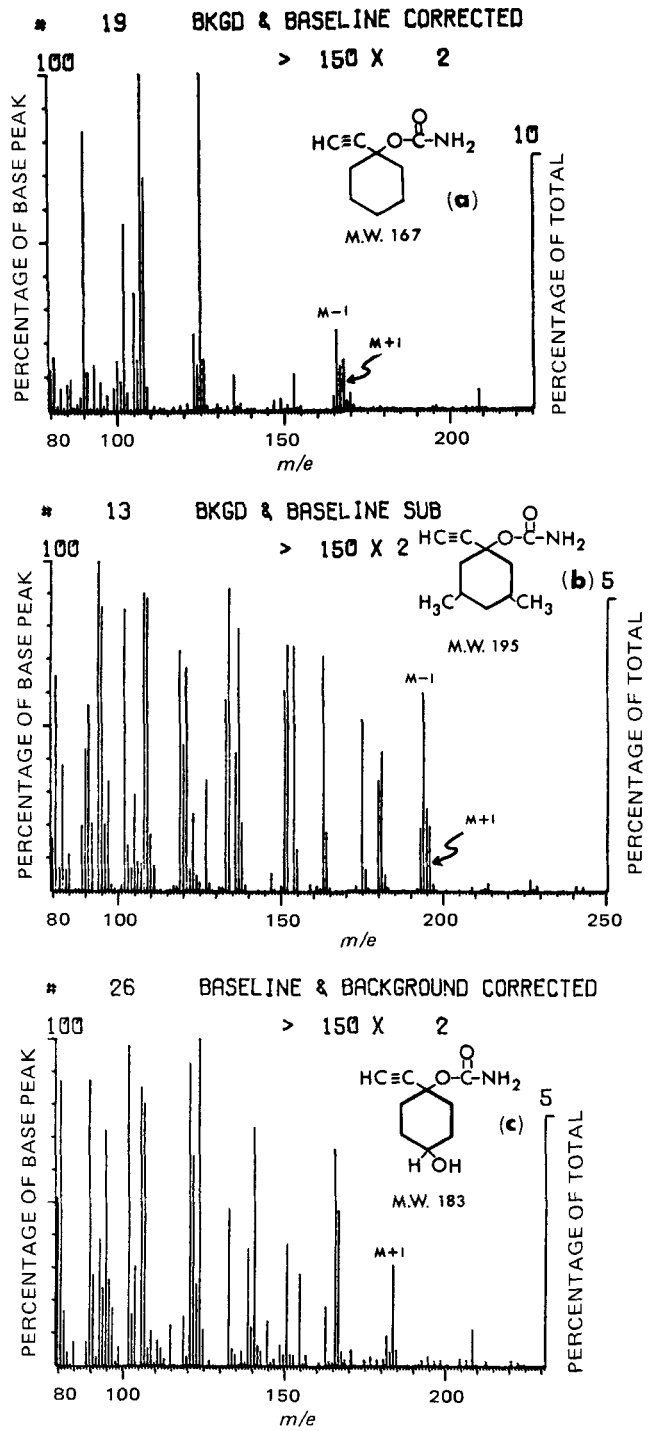

Figure 2—*Chemical-ionization mass spectra of I (a), II (b), and III (c) obtained after GLC. Methane was used as the reagent gas.*

and incubated overnight at 37° with 50 μl of β-glucuronidase solution[11]. The next day, 30 μl (6 μg) of the internal standard solution was added, and the procedure used for plasma was followed.

Following chromatography of the calibration standards, peak height ratios[12] of I and III to the internal standard were calculated. These ratios, plotted against concentrations in the 0.50–8.0-μg/ml range for I in plasma and in the 200–800-μg/ml range for III in urine, gave straight lines passing through the origin ($r = 1.0$). Mean slope values of 0.39 ± 0.02 and 0.012 ± 0.0007 were obtained for I and III, respectively.

A bioavailability study of different dosage forms of ethinamate dem-

[11] Glusulase, Endo Laboratories, Garden City, N.Y.

[12] Results were virtually unaffected when relative peak areas were used instead of relative peak heights.

Table II—Dissolution Profiles of Ethinamate Tablets and Capsules[a]

Minutes	Ethinamate in Solution, $\bar{X}$% (Range, *RSD*) Tablet	Capsule
5	58.6 (9.3, 6.2)	41.9 (21.4, 18.8)
15	78.0 (11.6, 5.7)	74.3 (29.2, 14.2)
20	84.7 (6.2, 3.4)	89.4 (26.4, 19.4)
60	94.1 (9.5, 3.6)	101.9 (15.4, 5.6)

[a] Mean of six tablets or capsules.

Table III—Ethinamate in Plasma Data

Data Set	Mean ± *SE* Tablets	Capsules	Mean Difference[a]
Area under curves, 0–24 hr, μg hr/ml	23.63 ± 12.57	25.24 ± 12.84	−1.61
Mean peak plasma concentrations, μg/ml	9.60 ± 4.17	9.12 ± 4.60	+0.48
Mean peak times, hr	0.60 ± 0.38	0.75 ± 0.57	−0.15

[a] Not significant at $p < 0.05$.

onstrated the utility of this method. Ethinamate tablets and capsules[13], 500 mg each, were administered to 12 subjects in a two-way crossover study. Each subject fasted for 8 hr before receiving a therapeutic dose of two ethinamate tablets or capsules. Blood samples were drawn at 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, and 24 hr. Urine was collected for 24 hr.

Dissolution characteristics of the ethinamate tablets and capsules were determined using the USP–NF apparatus. Tablets or capsules were placed in 500 ml of pH 1.2 buffer at 37° and stirred at 50 rpm. The ethinamate content of the buffer solution was assayed colorimetrically[14] using an automated analysis system.

RESULTS AND DISCUSSION

To estimate the precision and accuracy of the assay, plasma samples with known amounts of I and urine samples with known amounts of III were assayed. The results are summarized in Table I.

Chemical-ionization mass spectra[15] of I–III were obtained after GLC (Figs. 2*a*–2*c*). These spectra verify that these compounds pass through the chromatographic column intact and are the compounds measured by the detector.

The dissolution profiles (Table II) show that I is rapidly made available for absorption from both the tablet and capsule formulations.

The mean plasma ethinamate concentrations after administration of tablets and capsules are shown in Fig. 3. These plasma data were evaluated by an analysis of variance on the basis of the area under the plasma concentration–time curves, peak times, and the peak plasma concentrations (Table III). All parameters showed the tablets and capsules to be similar. The mean recoveries of the metabolite in the 24-hr urines were 359.6 ± 119.1 mg (32.8% recovery) for the tablets and 490.4 ± 193.5 mg

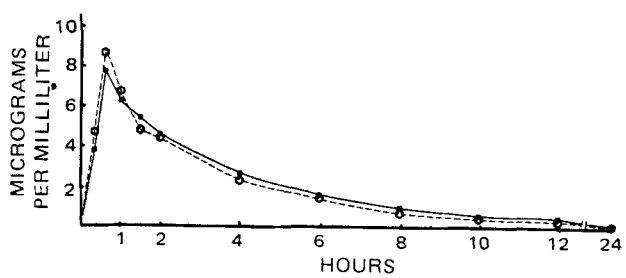

Figure 3—*Plasma ethinamate concentrations after administration of two 500-mg tablets (○) or two 500-mg capsules (■) of ethinamate to 12 subjects.*

[13] Valmid (ethinamate, Dista), Pulvules 399, 500 mg.

[14] Sulfuric acid is used to hydrolyze the amide portion of the molecule. The liberated ammonia is complexed with sodium phenolate. This complex is oxidized with sodium hypochlorite to yield a chromophore absorbing at 590 nm.

[15] A Finnigan model 3100 Quadrupole mass spectrometer (Sunnyvale, Calif.) was used with the column and conditions used in this procedure. Methane was the reagent gas.

(44.6% recovery) for the capsules. The mean difference of 130.8 mg of hydroxyethinamate in the 24-hr urine from the tablets and capsules was significant. However, this significant difference can be explained largely by the unusually low recovery of 77 mg of III from one subject. Without this subject, the average 24-hr urine recovery of III was 385 mg for the other 11 subjects. The mean difference of 105 mg of III recovered in the 24-hr urine from the two dosage forms was not significant.

The half-life of ethinamate in plasma was determined from a semilogarithmic regression analysis. The $t_{1/2}$ of ethinamate in plasma from these products was 1.9 ± 0.3 hr.

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Solubility and Ionization Characteristics of Phenytoin

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Abstract □ The solubility of phenytoin was determined in pH 7.4 and 5.4 phosphate buffers at five temperatures; in hydroalcoholic solutions, 0–4% methanol; and in pH 4.8–8.4 buffer solutions. From the temperature data, the enthalpy and entropy of solution of this nonideal system were calculated and were similar at both pH values. The data obtained from the buffer solutions were used to calculate the apparent dissociation constant, pKa′, of phenytoin as 8.06. A GLC method with on-column methylation was used to quantitate phenytoin with 5-(*p*-methylphenyl)-5-phenylhydantoin as an internal standard. The assay uses chloroform for extraction of the drug from aqueous solutions. The ratio of peak heights was adjusted for weights of aqueous and organic layers, and results were calculated in micrograms per gram of sample and mole fraction of phenytoin. Although hydroalcoholic solutions enhanced drug solubility, there is a potentially significant disadvantage in using alcohol for clinical studies.

Keyphrases □ Phenytoin—solubility and ionization characteristics in various buffers and hydroalcoholic solutions □ Solubility—phenytoin in various buffers and hydroalcoholic solutions □ Ionization characteristics—phenytoin in various buffers and hydroalcoholic solutions □ Anticonvulsants—phenytoin, solubility and ionization characteristics in various buffers and hydroalcoholic solutions

Because of its regulating effect on the bioelectric activity of the nervous system, phenytoin is used widely as an anticonvulsant and cardiac antiarrhythmic (1). The therapeutic activity of the drug appears to be related to the concentration of free unbound drug in the plasma (2, 3). The present paper reports some fundamental physicochemical properties of the drug which have considerable relevance for pharmacokinetic studies. A GLC method was devised to achieve acceptable sensitivity (<5% error) from 0.5 to 100 μg of phenytoin/ml.

The apparent dissociation constant, pKa′, was 8.31 when determined by UV spectrophotometry and 8.33 when determined by potentiometric titration in hydroalcoholic solutions (4). A graph of pKa′ values as a function of the ethanol concentrations yields the apparent pKa′ value in water (0% alcohol). A pKa′ of 9.2 was determined by titration in 50% methanol (5), in good agreement with that for 50% ethanol (4).

Phenytoin is very slightly soluble in alkali at 26° (5), *e.g.*, 165 μg/ml at pH 9.1 (borate buffer) and 1.52 mg/ml at pH 10, but it is practically insoluble in water, *e.g.*, 14 μg/ml at pH 7 and 20 μg/ml at pH 7.4 at 24° (6). However, it is sparingly soluble in alcohol, 19.4 mg/g (7) or 1 g in about 60 ml, and about one part in 30 ml of acetone (8). It is only slightly soluble in ether and chloroform (9).

Shortly after the introduction of phenytoin as an anticonvulsant in 1937 (10), the first analytical method, a titrimetric procedure, was developed for the determination of quantitative plasma phenytoin levels (11, 12), but this method lacked sensitivity in the clinical microgram range. Several spectrophotometric procedures (13–18) had considerably improved sensitivity.

The most recent development in phenytoin assay procedures involves the application of GLC, a highly specific and sensitive analytical technique (19, 20). A procedure using trimethylsilyl derivatives of phenytoin and its metabolic products is sensitive, but these products are unstable in the presence of moisture (21, 22). The quaternary ammonium base, trimethylanilinium hydroxide (I), was used successfully as a methylating agent (23) to convert the hydantoins to nonpolar, volatile derivatives, using the injection port of a gas chromatograph as the reaction chamber. This flash-heater methylation reaction is complete; the 1,3-dimethylhydantoin products are stable (24) and were identified by mass spectrometry as the sole products of this reaction (25).

EXPERIMENTAL

Materials—Phenytoin (5,5-diphenylhydantoin) and 5-(*p*-methylphenyl)-5-phenylhydantoin (II) were used as obtained (99+%)[1]. The melting point and IR spectrum of phenytoin were determined as an index of purity (9). Chloroform[2] and all other chemicals employed were reagent grade.

[1] Aldrich Chemical Co., Milwaukee, WI 53233.

[2] J. T. Baker Chemical Co., Phillipsburg, N.J.