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Sensitive GLC Procedure for Simultaneous Determination of Phenytoin and Its Major Metabolite from Plasma following Single Doses of Phenytoin

K. K. MIDHA [x], I. J. McGILVERAY, and D. L. WILSON

Abstract □ An improved GLC procedure was developed for the simultaneous determination of phenytoin and its metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, in plasma and urine following enzyme hydrolysis. After extraction, the drug, the metabolite, and the internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin, are measured by GLC with flame-ionization detection as their respective methyl derivatives following flash-heater methylation with trimethylanilinium hydroxide. The drug and metabolite give well-resolved symmetrical peaks on a phenyl methyl silicone column, and the method has a sensitivity of 150 ng/ml of phenytoin and 125 ng/ml of the metabolite. GLC–mass spectral evidence is presented for the formation and intact determination of methyl derivatives of the drug, its metabolite, and the internal standard.

Keyphrases □ Phenytoin and major metabolite—simultaneous GLC analysis, plasma □ GLC—simultaneous analysis, phenytoin and major metabolite, plasma □ Anticonvulsant agents—phenytoin, GLC analysis, plasma

Phenytoin is a commonly prescribed anticonvulsant drug. The various procedures used for drug level measurements were reviewed (1). It is one of the few drugs for which plasma concentrations are routinely monitored, and many GLC methods (2–14) are suitable for the assay of steady-state levels. The dose-dependent biotransformation of phenytoin and variations in its metabolism in treated subjects require that assay methods for determining plasma concentrations of the unchanged drug and its principal metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (15), suitable for single-dose pharmacokinetic studies be developed.

Chang and Glazko (8) reported the preparation of trimethylsilyl derivatives applied to the GLC assay of phenytoin in plasma and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin in urine. However, when this procedure is used routinely, traces of moisture cause problems and the sensitivity is limited. Derivatization with diazomethane was applied in procedures based on GLC estimation of 3-*N*-methyl derivatives (2, 4).

The safer tetramethylammonium hydroxide reagent also was used in a flash-heater methylation procedure (6), while trimethylanilinium hydroxide was described as a methylating agent for analysis of phenytoin and its metabolite (13). However, in the assay procedure for the latter, acid hydrolysis was used to degrade the conjugate and the method required two internal standards. Although the unconjugated metabolite could be measured (13), the plasma levels were not given for the metabolite and no variation or sensitivity limit was reported.

Recently, tetramethylammonium hydroxide was used for methylating phenytoin and its metabolite along with subsequent quantitative estimation by GLC with temperature programming (14). This reported procedure is cumbersome and is only applicable when plasma concentrations of phenytoin and the metabolite are around 1 μg or more. In this report, a modified method is described for the GLC analysis of plasma phenytoin and total 5-(*p*-hydroxyphenyl)-5-phenylhydantoin by formation of methyl derivatives with trimethylanilinium hydroxide. The procedure is of sufficient sensitivity to allow the quantitation of 0.15 μg/ml of phenytoin and 0.125 μg/ml of the metabolite. In addition, GLC–mass spectral evidence is presented for the formation and determination of methyl derivatives of the drug and the metabolite used in the quantitation.

EXPERIMENTAL

Reagents—Ether[1] was glass distilled prior to use. Stock solutions containing 100 μg/ml of phenytoin were prepared by dissolving appropriate amounts of the sodium salt of phenytoin[2] in distilled water. Stock solutions containing 100 μg/ml of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin[3] were prepared by dissolving the appropriate amount in 0.01 *N* NaOH. Appropriate dilutions of the drug (0.15–8.0 μg/ml) and the metabolite (0.125–4.0 μg/ml) were prepared as required.

Aqueous solutions (100 μg/ml) of the internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin[3], were prepared by dissolving an appropriate amount of the internal standard in 0.01 *N* NaOH and

[1] Ether (anhydrous), Mallinckrodt Chemical Works Ltd., Montreal, Canada.
[2] Diphenylhydantoin-Na, Parke-Davis & Co., Brockville, Ontario, Canada.
[3] Aldrich Chemical Co., Milwaukee, Wis.

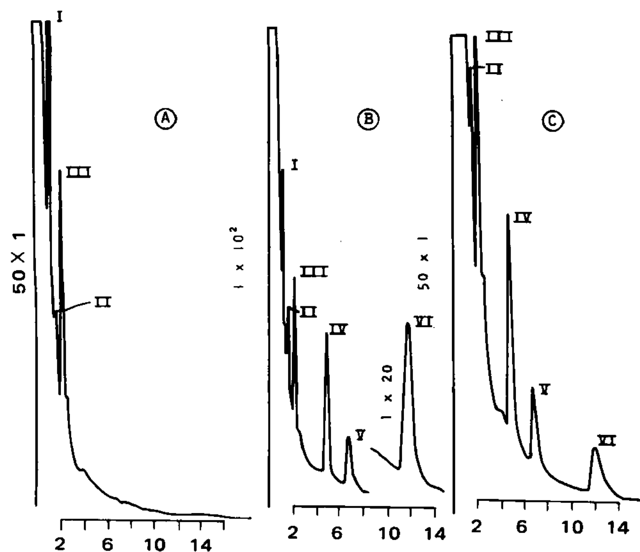

Figure 1—*Typical chromatograms of human plasma. Key: A, control plasma; B, plasma containing 2.0 μg/ml of phenytoin, 1.0 μg/ml of the metabolite, and 2.5 μg/ml of internal standard; C, plasma from a human volunteer who has been given 200 mg of phenytoin containing 2.04 μg of phenytoin and 1.03 μg of the metabolite; peak IV, methylated phenytoin; peak V, methylated internal standard; and peak VI, methylated metabolite. Peaks I–III are due to endogenous materials in plasma.*

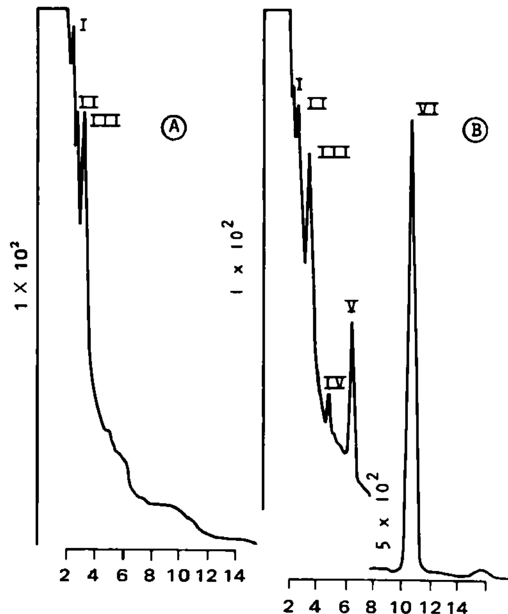

Figure 2—*Typical chromatograms of human urine. Key: A, control urine; B, urine (2 ml) from a total 48-hr collection from a human volunteer who has been given 300 mg of phenytoin containing 0.8 μg of phenytoin, 10.0 μg of internal standard, and 88.4 μg of the metabolite; peak IV, methylated phenytoin; peak V, methylated internal standard; and peak VI, methylated metabolite. Peaks I–III are due to endogenous materials in urine.*

diluting to 2.5 μg/ml with distilled water before use. Methanolic trimethylanilinium hydroxide was synthesized according to the method of Barrett (12). Plasma and blood were obtained from the Red Cross Blood Bank. All other chemicals were of the purest grade commercially available.

GLC—A gas chromatograph[4], equipped with a metal sleeve injection port and a flame-ionization detector, was employed. The column was of coiled glass tubing, 1.83 m (6 ft) long by 3.0 mm i.d., packed with a 5% phenyl methyl silicone fluid[5] (OV-7) on acid-washed, dimethylchlorosilane-treated, high performance Chromosorb W[5] support, 80–100 mesh. The column was conditioned by maintaining the oven at 340° for 18 hr with a low nitrogen flow. Operating conditions were: injection port, 310°; column oven, 215°; and detector, 280°. The flow rate of nitrogen was 60 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Procedure—To 2-ml plasma or urine samples in screw-capped[6] centrifuge tubes (15 ml) were added 0.2 ml of 1 *M* acetate buffer (pH 5.0) and 50 μl of β-glucuronidase[7]. Then the samples were incubated for 4 hr at 37°. To the hydrolyzed samples were added 1 ml of the internal standard (2.5 μg/ml) and 2 ml of 1 *N* HCl. The samples were then extracted with 5 ml of ether by shaking[8] for 10 min at 50 rpm followed by centrifugation at 2500 rpm for 10 min.

Four-milliliter portions of the ether layer were transferred into a centrifuge tube (20 ml) containing 5 ml of 0.2 *M* phosphate buffer (pH 11.2). The tubes were mixed for 10 min followed by centrifugation for 10 min, and the organic extracts were then discarded. The remaining aqueous phosphate solution was acidified with 2 ml of 2 *N* HCl and extracted twice with 5-ml portions of ether (mixed for 10 min and centrifuged for 10 min).

Four milliliters of the first extract and 5 ml of the second extract were transferred into an evaporating tube[9] (16), and the combined ether extracts were evaporated to dryness at 50° under a stream of dry nitrogen. The dried extracts were dissolved by mixing[10] with 25 μl of methanolic trimethylanilinium hydroxide (0.2 *M*), and aliquots (1–2 μl) were injected into the gas chromatograph.

Calibration Curves—Peak height ratios were calculated by dividing the height of the peak from the drug or the metabolite by the height of the peak from the internal standard. Calibration curves were assembled from the results of spiked control plasma or urine by plotting the peak height ratios against the concentrations of the drug or the metabolite.

RESULTS AND DISCUSSION

Flash-heater methylation of phenytoin, the internal standard, and the metabolite with trimethylanilinium hydroxide gave sharp peaks with retention times of 4.9, 6.9, and 11.9, respectively (peaks IV–VI, Fig. 1B), under the described conditions. The structures of the methyl derivatives of the drug, the internal standard, and the metabolite formed under these conditions were established by combined GLC–mass spectrometry[11].

The mass spectrum of methylated phenytoin (peak IV, Fig. 1B) suggested the dimethylated derivative, namely, 1,3-dimethyl-5,5-diphenylhydantoin. The mass spectrum gave a molecular ion at *m/e* 280 and other diagnostic ions at *m/e* 251, 223, 203, 194, 165, 152, 146, and 118. This observation was in agreement with literature reports (6, 14), except that flash-heater methylation was carried out by means of tetramethylammonium hydroxide in previous studies.

The GLC–mass spectrum of the internal standard (peak V, Fig. 1B) showed a molecular ion at *m/e* 294 and other major ions at *m/e* 279, 265, 251, 237, 217, 208, 203, 194, 165, 132, 118, and 91. These ions suggested that the methylated 5-(*p*-methylphenyl)-5-phenylhydantoin had the structure 1,3-dimethyl-5-(*p*-methylphenyl)-5-phenylhydantoin, *i.e.*, methylation taking place at 1-*N* and 3-*N* atoms of the hydantoin ring.

GLC–mass spectrometry of the metabolite 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (peak VI, Fig. 1B) indicated that three methyl groups had been introduced in the molecule. It had a molecular ion at *m/e* 310 and other characteristic ions at *m/e* 280, 233, 224, 203, 148, and 118. These ions suggested that the methylated metabolite had the structure 1,3-dimethyl-5-(*p*-methoxyphenyl)-5-phenylhydantoin. This observation was in agreement with those of previous investigators (13, 14).

Figure 1A shows a typical chromatogram obtained by processing control blank plasma as already described but with the internal standard omitted. Extraneous peaks I, II, and III at retention times

[4] Model F-11, Perkin-Elmer, Montreal, Quebec, Canada.
[5] Chromatographic Specialties, Brockville, Ontario, Canada.
[6] Lined with Teflon (du Pont).
[7] Glusulase, Endo Laboratories, Garden City, N.Y.
[8] Roto-Rack, Fisher Scientific Co., Montreal, Quebec, Canada.
[9] Custom-made by Canadian Laboratory Supplies Ltd., Montreal, Quebec, Canada.
[10] Vortex Genie mixer, Fisher Scientific Co., Montreal, Quebec, Canada.
[11] Hitachi Perkin-Elmer model RMSU mass spectrometer coupled to a Perkin-Elmer model 990 gas chromatograph through a two-stage jet separator.

Table I—GLC Estimation of Phenytoin Added to 1 ml of Plasma

Phenytoin Added, μg	Mean Peak Height Ratio[a]	CV, %[b]
0.15	0.305	3.6
0.25	0.406	0.86
0.50	0.609	1.38
1.00	1.34	2.2
2.00	2.70	0.8
4.00	5.67	0.55
6.00	8.62	2.79
8.00	12.07	0.86

[a] $n = 9$. [b] Mean $CV = 1.63\%$ and $y = mx$, where $m = 1.4840$; $r^2 = 1.0$.

Table II—GLC Estimation of the Metabolite Added to 1 ml of Plasma

Metabolite Added, μg	n	Mean Peak Height Ratio	CV, %[a]
0.125	6	0.07	7.04
0.25	7	0.13	3.98
0.50	3	0.31	1.88
1.0	6	0.58	1.29
2.0	4	1.24	1.98
4.0	4	2.38	1.22

[a] Mean $CV = 2.90\%$ and $y = mx$, where $m = 0.599$; $r^2 = 1.0$.

Table III—Recovery of Phenytoin, the Metabolite, and the Internal Standard from Plasma Determined by GLC Assay

Micrograms Added	Mean Micrograms Recovered[a]	Mean Percent Recovery	CV, %
Phenytoin, 2	1.52	76	3.8
Metabolite, 1	0.64	64	3.5
Internal standard, 5	2.95	59	1.0

[a] $n = 3$.

1.2, 1.6, and 2.2 min, respectively, were observed in the chromatograms of all human plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 2.0 μg of phenytoin and 1.0 μg of the metabolite is shown in Fig. 1B, where it is clear that extraneous peaks I, II, and III do not interfere with the peaks due to the methylated drug (peak IV), the internal standard (peak V), and the metabolite (peak VI).

Figure 1C shows a chromatogram obtained from the plasma sample (2 ml) from blood withdrawn from a male volunteer (84 kg) at 8 hr after administration of 200 mg (two 100-mg tablets)[12] of phenytoin. An analysis time of 14 min was achieved.

Figure 2A shows a typical chromatogram obtained by processing control blank urine without the internal standard. The flow rate of the carrier gas was 65 ml/min. Extraneous peaks I, II, and III (Fig. 2A) were observed in the chromatograms of all human urine samples. A chromatogram obtained when the method was applied to a urine sample (2 ml) from total urine collected over 48 hr from a volunteer who received 300 mg (three 100-mg tablets)[11] of phenytoin is shown in Fig. 2B, where it is clear that extraneous peaks I, II, and III before 4 min do not interfere with the peaks due to methylated phenytoin (peak IV), the internal standard (peak V), and the metabolite (peak VI). By increasing the flow rate of the carrier gas, an analysis time of 12 min was achieved.

The response of the flame-ionization detector to phenytoin was linear with concentrations over the 0.15–8.00-μg/ml range. The ratio of peak height of phenytoin and the internal standard plotted against concentration in the 0.15–8.00-μg/ml range gave a straight line passing through the origin ($r^2 = 0.9989$). A mean slope value of 1.4840 was obtained for phenytoin. Similarly, the response of the flame-ionization detector to the metabolite was linear with concentrations over the 0.125–4.00-μg/ml range.

The ratio of peak height of the metabolite and the internal standard plotted against concentration in the 0.125–4.00-μg/ml range gave a straight line passing through the origin ($r^2 = 0.9995$). A mean slope value of 0.599 was obtained for the metabolite assay. The overall coefficients of variation for phenytoin and 5-(p-hydroxyphenyl)-5-phenylhydantoin were 1.63% (Table I) and 2.90% (Table II), respectively. The overall recoveries of the drug, the metabolite, and the internal standard from plasma were 76 ± 3.8, 64 ± 3.5, and 59 ± 1.0%, respectively (Table III).

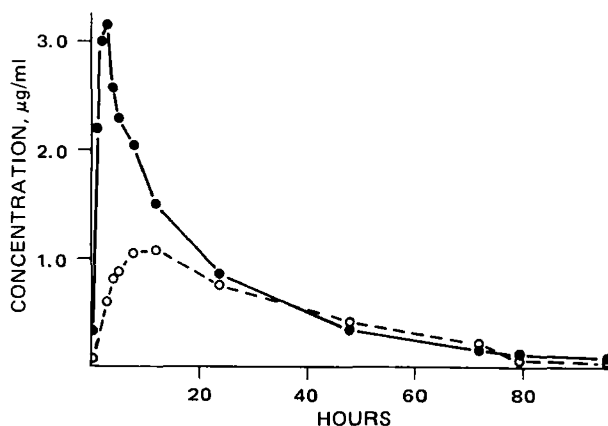

Figure 3—*Phenytoin (●) and the metabolite (○) concentrations in the plasma of a human volunteer (84 kg) following a single oral dose of 200 mg of phenytoin.*

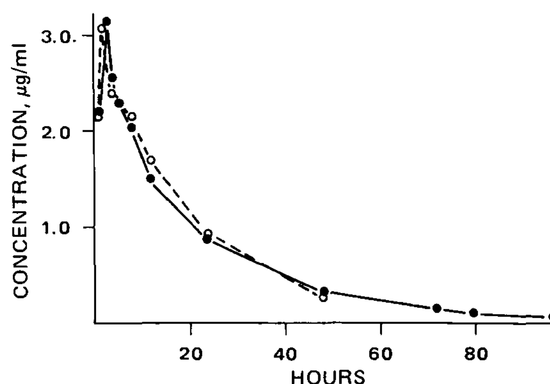

Figure 4—*Comparison of phenytoin plasma concentrations by the GLC method (●) and a UV method (○) following a single dose of two 100-mg tablets of phenytoin to a human volunteer (84 kg).*

The application of the present method to the plasma level determination of phenytoin and the metabolite is shown in Fig. 3. A 200-mg dose of phenytoin (two 100-mg tablets) was given to a healthy male volunteer (84 kg), blood was withdrawn at different time intervals over 96 hr, and aliquots of the plasma were assayed for phenytoin and the metabolite by GLC.

Figure 4 illustrates the comparison of the GLC assay for phenytoin with that of a UV procedure (17), as modified previously (18, 19), over 48 hr in the same volunteer (84 kg). The UV methods, which lacked sensitivity below 0.5 μg/ml, compared favorably for phenytoin. The slope of the line, two-variable linear regression, between the GLC plasma concentration (X) and the modified UV method (Y) was 0.927. This line did not include zero slope within the 95% confidence limits ($p = 0.05$, two tailed) and had a correlation coefficient of 0.987. The intercept was 0.176. When areas under the curves to 96 hr were compared, a difference of 1.80% was obtained.

In conclusion, the described GLC method is simple, sensitive, and specific for phenytoin and the metabolite 5-(p-hydroxyphenyl)-5-phenylhydantoin. It can be employed for single- as well as multiple-dose pharmacokinetic studies. It has the advantage that it measures the drug and its major metabolite simultaneously.

[12] Commercial phenytoin (diphenylhydantoin) product.

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[x] To whom inquiries should be directed.

NOTES

Synthesis of 1,2-Dioleoyl-3-(α-^{14}C-1-adamantoyl)-*sn*-glycerol and 1-^{14}C-Adamantanecarboxylic Acid

ANTHONY J. VILLANI [x] and FRANCIS R. PFEIFFER

Abstract □ The pancreatic lipase inhibitor 1,2-dioleoyl-3-(α-^{14}C-1-adamantoyl)-*sn*-glycerol, with a specific activity of 8 mCi/mmole, was prepared by consecutive acylation of 1,2-isopropylidene-*sn*-glycerol with 1-^{14}C-adamantanecarboxylic acid chloride and oleoyl chloride. The ^{14}C-labeled acid was conveniently prepared by carboxylation of 1-adamantanol using ^{14}C-sodium formate in concentrated sulfuric acid.

Keyphrases □ 1,2-Dioleoyl-3-(α-^{14}C-1-adamantoyl)-*sn*-glycerol—pancreatic lipase inhibitor, synthesized □ 1-^{14}C-Adamantanecarboxylic acid—synthesized by carboxylation of 1-adamantanol using ^{14}C-sodium formate in sulfuric acid □ Pancreatic lipase inhibitor—1,2-dioleoyl-3-(α-^{14}C-1-adamantoyl)-*sn*-glycerol synthesized □ Inhibitors, pancreatic lipase—1,2-dioleoyl-3-(α-^{14}C-1-adamantoyl)-*sn*-glycerol synthesized

The investigation of biological applications of novel synthetic lipids has led to the study of pancreatic lipase inhibitors (1). The triglyceride 1,2-dioleoyl-3-(α-^{14}C-1-adamantoyl)-*sn*-glycerol (I) was found to be an inhibitor of pancreatic lipase when tested *in vitro* with isolated enzyme.

The mode of inhibition may involve covalent bonding of the 1-adamantoyl group of I to the enzyme. If the acylated enzyme could be isolated, then the role of the sterically hindered adamantoyl group in the mechanism of inhibition might be delineated.

The carbonyl carbon of the adamantoyl moiety of I was labeled with carbon-14 to test this theory. The synthesis of I is shown in Scheme I. *In vivo* biological data of I in rats will be reported elsewhere.

DISCUSSION

Since the literature procedure (2) for the carboxylation of 1-adamantanol was not readily adaptable to the semimicro radiosynthetic preparation, a modification was developed. In the present procedure, ^{14}C-carbon monoxide was generated *in situ* from ^{14}C-sodium formate and concentrated sulfuric acid. When this reaction was run in the presence of the adamantane cation, generated *in situ* from 1-adamantanol (II), the desired carboxylation was achieved and adamantanecarboxylic acid (III) was obtained in 82% yield. This procedure and the alternative method of Majerski *et al.* (3) provide convenient routes for the introduction of a carbon label in adamantane.

The carboxylic acid III was readily converted (4) to the acid chloride (IV) with thionyl chloride in refluxing benzene. The esterification of IV with 1,2-isopropylidene-*sn*-glycerol (5, 6) in the presence of pyridine was attended by the formation of the anhydride of adamantanecarboxylic acid (VI). The ratio of the ester V to the anhydride VI was identified (Fig. 1) as approximately 2:1 after 20 hr of refluxing in methylene chloride. The maximum yield of V could be obtained by the addition of excess isopropylideneglycerol and an increased reaction time.

Compound VI was also isolated and identified in cold runs and compared to an authentic sample of adamantanecarboxylic anhydride prepared by the procedure of Stetter and Rauscher (7).

The isopropylidene protective group was cleaved by mild acid to give the diol VII, which was used without purification. The acylation of VII with oleoyl chloride (8, 9) in the presence of pyridine gave crude I as a clear oil. After purification by magnesium silicate[1] column

[1] Florisil.