Development and Evaluation of Method for Simultaneous Determination of Phenobarbital and Diphenylhydantoin in Plasma by High-Pressure Liquid Chromatography

S. H. ATWELL *, V. A. GREEN *, and W. G. HANEY ˣ

Abstract □ A procedure for the simultaneous analysis of diphenylhydantoin and phenobarbital in plasma by high-pressure liquid chromatography was developed and evaluated. Separation is effected on a porous particle silicic acid column with chloroform–dioxane–isopropanol–acetic acid (310:9.7:1.0:0.1 by volume) and is monitored at 254 nm. Results of the procedure were compared with results of a GLC assay.

Keyphrases □ Phenobarbital–diphenylhydantoin—simultaneous high-pressure liquid chromatographic determination, plasma □ Diphenylhydantoin–phenobarbital—simultaneous high-pressure liquid chromatographic determination, plasma □ High-pressure liquid chromatography—simultaneous determination of phenobarbital and diphenylhydantoin in plasma, development and evaluation of method

The simultaneous determination of phenobarbital and diphenylhydantoin in plasma is of interest to pharmaceutical analysts because of the established efficacy of dosage manipulation based on their plasma concentration and the frequency with which they are concurrently administered as anticonvulsants.

Several techniques are available for this determination on a routine basis (1, 2), but the most widely accepted techniques involve GLC. The drugs may be subjected to direct GLC analysis, but several problems are often encountered. The extraction process may be involved (3), retention times of the drugs are usually long (4), and the drugs may reversibly or irreversibly adsorb to the solid support, causing peak tailing (5) or nonlinear calibration curves (6).

To overcome the disadvantages of direct GLC analysis, volatile derivatives of the drugs have been formed and determined. While initial procedures for the formation of anticonvulsant derivatives were complex (7), on-column methylation (8) overcame this objection. However, several uncertainties have arisen regarding the reproducibility of this technique. Column packing material is rapidly degraded by the methylating reagent (9). The recognized instability of phenobarbital in alkaline media results in degradation [and peaks called "early phenobarbital" (10)], the rate of which is erratic (11). Interpretation of a chromatogram of these degradation products is probably premature (11, 12). Pippenger (13), in reporting a retrospective study involving 5000 anticonvulsant samples, noted these and other uncertainties in anticonvulsant analysis by GLC.

High-pressure liquid chromatography (HPLC) has intrinsic advantages over GLC in this regard. The most obvious is that the volatility of the analyte is not a prerequisite to analysis, and problems related to heat instability or volatile derivative formation are thereby avoided. Also, the detection mechanism in HPLC affords possibilities for increased selectivity and thus a more direct extraction procedure. For example, an HPLC system may detect only molecules with appreciable absorption at 254 nm, whereas GLC with flame ionization forms an almost universal detection system (14).

Based on these considerations, a method for the simultaneous determination of phenobarbital and diphenylhydantoin in plasma by HPLC was developed and evaluated. The method was applied to the analysis of patient plasma, and results were compared with results of a GLC method. While these studies were in progress, a procedure for analysis of the two drugs by HPLC was reported (15). However, extraction and sample preparation were involved, no provision was made for use of an internal standard, and no data were presented relating results of phenobarbital analysis by HPLC to results obtained by other procedures.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a 254-nm detector was used. The column was 30 cm × 4 mm (i.d.) stainless steel packed with 10-μm porous particle silicic acid[2]. The gas chromatograph[3] was equipped as previously noted (16).

Materials—Phenobarbital and diphenylhydantoin were obtained as USP reference standards. The remaining drugs, drug relatives, and metabolites were obtained from commercial suppliers and used without further treatment. TLC plates[4] were 5 × 20-cm glass, precoated with silica gel (0.25 mm) and fluorescent indicator.

Chromatographic Solvent Selection—*TLC*—TLC plates were spotted with 5 μl each of diphenylhydantoin and phenobarbital solutions (5 mg/ml) in methanol. Plates were developed in an ascending manner in a chamber previously equilibrated with the developing solvent. Developed plates were visualized with UV light (shortwave), and R_f values were determined.

HPLC—Solvent mixtures to be evaluated in the high-pressure system were pumped through the column for 6 hr (1.0 ml/min) for equilibration. Solutions of diphenylhydantoin and phenobarbital (10 μg/ml, 1.0 ml) in methanol were evaporated to dryness, reconstituted with the chromatographic solvent (50 μl), and injected into the chromatographic system. The flow rate was adjusted so that both peaks emerged in less than 10 min. Resolution was calculated by the formula $R = 2d/(w_1 + w_2)$, where d is the distance between peak centers, and w_1 and w_2 are the widths of each peak.

Chromatographic Conditions—The solvent system used for HPLC analysis was composed of chloroform–dioxane–isopropanol–acetic acid (310:9.7:1.0:0.1 by volume). The flow rate was 1.5 ml/min, and the pressure required to maintain this flow rate was approximately 70 kg/cm² (1000 psi). GLC operating conditions were as previously noted (16).

Preparation of Calibration Curves—Solutions (2.0 ml) of

[1] ALC 202, Waters Associates, Milford, Mass.
[2] μPorasil, Waters Associates, Milford, Mass.
[3] GC-4BP, American Instrument Co., Silver Spring, Md.
[4] Analabs, North Haven, Conn.

Table I—R_f Values of Phenobarbital and Diphenylhydantoin on Silicic Acid Gel TLC

Solvent Mixture	Solvent Composition (Parts by Volume)	R_f Values: Phenobarbital	R_f Values: Diphenylhydantoin	ΔR_f
1	Chloroform–ether (85:15)	0.391	0.318	0.073
2	Chloroform–ether (60:40)	0.688	0.594	0.094
3	Chloroform–isopropanol (90:10)	0.947	0.947	0.000
4	Chloroform–ethanol (90:10)	0.856	0.856	0.000
5	Chloroform–methanol–water (75:25:1)	0.675	0.688	0.013
6	Chloroform–ether–acetonitrile (48:9:3)	0.461	0.384	0.077
7	Chloroform–cyclohexane–acetonitrile (20:50:25:5)	0.102	0.082	0.020
8	Chloroform–cyclohexane–ether–acetonitrile (20:50:25:5)	0.382	0.274	0.108
9	Chloroform (100)	0.106	0.088	0.018
10	Dioxane (100)	0.822	0.801	0.021
11	Chloroform–dioxane (50:50)	0.676	0.640	0.036
12	Chloroform–dioxane (97:3)	0.466	0.300	0.166
13	Dioxane–methanol–ammonium hydroxide (84:10:5)	0.635	0.700	0.065
14	Dioxane–*n*-butanol–ammonium hydroxide (9:9:2)	0.462	0.613	0.151
15	*n*-Butanol–cyclohexane (10:200)	0.338	0.423	0.085
16	*n*-Butanol–ether–ammonium hydroxide (10:90:10)	0.100	0.262	0.162
17	Isopropanol–cyclohexane–ammonium hydroxide (65:25:10)	0.350	0.514	0.164

phenobarbital and diphenylhydantoin in chloroform containing 25.0, 37.5, 50.0, 62.5, 87.5, and 137.5 μg/ml were mixed with an aliquot (2.0 ml) of the internal standard solution [5-(4-methylphenyl)-5-phenylhydantoin, 50.0 μg/ml], and the solution was evaporated to dryness in a stream of dry nitrogen. At the time of analysis, the residue was reconstituted with chloroform (0.5 ml) and an aliquot (1–3 μl) was injected into the chromatograph. Ratios of the peak height of the drug to that of the internal standard were determined at each concentration.

Extraction and Evaluation of Extraction Efficiency—Drug-free plasma was spiked with sufficient quantities of the two drugs to give concentrations of 5.0, 7.5, 10.0, 12.5, 17.5, and 27.5 μg/ml. Centrifuge tubes containing the internal standard (5.0 μg) were prepared by evaporating an aliquot of a chloroform solution of the internal standard. A portion of spiked plasma (0.5 ml) was added to the tube with pH 6.8 phosphate buffer (0.5 ml), distilled water (0.5 ml), and methylene dichloride (5.0 ml). This mixture was placed on a vortex-type mixer for 1 min and centrifuged. The organic layer was separated, dried with sodium sulfate, and evaporated to dryness in a stream of dry nitrogen. The residue was reconstituted in chloroform (50 μl), and an aliquot (1–3 μl) then was injected into the HPLC system.

Extraction efficiency was evaluated by comparing peak height ratios obtained after extraction of spiked plasma to those obtained from corresponding concentrations in the preparation of calibration curves.

Analysis of Phenobarbital and Diphenylhydantoin in Patient Plasma—An aliquot (0.5 ml) of plasma was added to a centrifuge tube containing the internal standard (5.0 μg). The plasma sample was treated as described, and a portion of the reconstituted extract was injected into the HPLC system. An aliquot of a standard solution equivalent to 10 μg/ml of plasma for each of the drugs was injected immediately afterward. The ratio of the peak height of the drug to that of the internal standard was determined, and concentration of the drugs in the unknown sample was determined by the single-point standard method (17).

RESULTS AND DISCUSSION

Initially, R_f values for the two drugs were determined (Table I) on silicic acid plates in numerous solvent mixtures to select mixtures likely to effect a HPLC separation. On the basis of these data, Solvent Mixtures 12, 14, 16, and 17 appeared to merit investigation. When the latter three mixtures were applied to the HPLC system, however, the peak area of phenobarbital in each was approximately five times that of diphenylhydantoin when equimolar quantities were injected. Each of these three mixtures contains ammonium hydroxide, and the increased peak area is likely related to the stronger 254-nm absorption of phenobarbital in alkaline media (18). The therapeutic plasma level for these two drugs as anticonvulsants is still the subject of some controversy (19), but it is generally thought to be in the range of 10 μg/ml for phenobarbital (20) and 10–15 μg/ml for diphenylhydantoin (21). Because of these similarities, the large peak area difference in these three solvents was objectionable. Further HPLC investigations were based on Solvent Mixture 12, containing a preponderance of chloroform with dioxane.

The chloroform–dioxane mixture was modified in an attempt to optimize the system for separation of the two drugs. The ratio of chloroform to dioxane was increased until a decrease in the resolution was noted (35:1). A small amount of isopropanol served to increase the resolution, as did acetic acid. The relative amounts of these solvents in the solvent mixture were changed until maximum resolution was noted. Results of these alterations as indicated by relative resolution factors are presented in Table II. Thus, a solvent mixture of chloroform–dioxane–isopropanol–acetic acid (310:9.7:1.0:0.1 by volume) was selected for the remaining HPLC studies.

In the next phase of the study, retention volumes in the system were determined and resolution factors were calculated (Table III) for some structurally related drugs and drug relatives as well as for the two principal metabolites, 5-(4-hydroxyphenyl)-5-phenylhydantoin (22) and 5-ethyl-5-(4-hydroxyphenyl)barbituric acid (23).

Table II—Effect of Solvent System Changes on Resolution of Phenobarbital and Diphenylhydantoin by HPLC

Solvent Mixture, ml: Chloroform / Dixoane	Resolution	Solvent Mixture, ml: Chloroform / Dioxane	:	Isopropanol / (Chloroform + Dioxane)	:	Acetic Acid / (Chloroform + Dioxane)	Resolution
4.50	1.32	32.00	:	1.00	:	1.00	2.16
5.67	1.37	32.00	:	1.00	:	0.10	2.56
7.33	1.99	32.00	:	0.10	:	0.10	2.93
9.00	2.26	32.00	:	0.10	:	0.05	2.99
10.11	2.34	32.00	:	0.05	:	0.05	2.84
13.28	2.60	32.00	:	0.10	:	0.02	3.19
19.00	2.74	32.00	:	0.10	:	0.01	3.37
32.00	2.93						
35.00	2.74						

Table III—Retention Volumes and Resolution Factors for Selected Drugs and Metabolites

Compound	R_v[a]	Resolution[b]
Glutethimide	3.37	—[c]
Phensuximide	3.45	—
Trimethadione	3.49	—
Mephobarbital	3.90	—
Hexobarbital	4.24	—
Mesantoin	4.74	—
Butalbital	5.11	2.38
Secobarbital	5.50	2.33
Cyclobarbital	5.74	1.97
Ethotoin	5.86	1.87
5-Allyl-5-(2-cyclopenten-1-yl)barbituric acid	5.94	1.75
Amobarbital	6.12	1.56
Pentobarbital	6.24	1.51
Butethal	6.31	1.43
Allobarbital	6.37	1.26
Vinbarbital	6.42	1.23
Butabarbital	6.75	1.02
Bromisovalum	7.74	0.95
Primidone	8.50	1.40
5-(4-Methylphenyl)-5-phenylhydantoin	10.00	1.27
Phenacemide	13.00	1.94
Phenylethylmalonamide	24.60	—
5-(4-Hydroxyphenyl)-5-ethylbarbituric acid	26.00	—
5-(4-Hydroxyphenyl)-5-phenylhydantoin	39.00	—

[a] Flow rate was 1.5 ml/min. [b] Resolution *versus* phenobarbital (R_v = 8.02 ml). [c] Resolution factors not given were greater than 2.5. [d] Resolution *versus* diphenylhydantoin (R_v = 11.3 ml).

Since a resolution factor of 1.0 gives less than 3% error (24), only bromisovalum would interfere with the chromatographic interpretation. However, this finding does not preclude interference by metabolites of the products in Table III or by other structurally unrelated drugs. In addition to serving as an indication of potential interference, these data allowed a rational selection of the internal standard.

Standard curves for the two drugs were prepared seven times over 1 month. For diphenylhydantoin, the curve had a mean slope of 0.1255 ml/μg (2.14% *SD*), a mean correlation coefficient of 0.998, and a mean *Y*-intercept of −0.043. For phenobarbital, the mean slope was 0.1255 ml/μg (3.19% *SD*), the mean correlation coefficient was 0.999, and the mean *Y*-intercept was −0.070. These data indicate that the procedure is amenable to use of a single-point standard. There was no change in these parameters or in the number of theoretical plates during this study, indicating the stability of the column packing material.

Several investigators (6, 25) noted problems with standard solutions caused by the instability of these drugs in aqueous media and in organic solution. While degradation is slow (except at alkaline pH), standard solutions of the drugs and internal standard must usually be prepared fresh. To avoid this problem, the internal standard solution was prepared in chloroform, and aliquots were added to a number of extraction tubes and evaporated to dryness. In this state, no degradation was noted during the study. In addition, tubes containing aliquots of the standard solutions were prepared in the same manner, stored in the dry state, and reconstituted at the time of analysis. An aliquot of the reconstituted solution was injected, the solution was evaporated to dryness, and the tube was retained for later use. Since no more than 0.6% of the solution (3 μl of a 0.5-ml solution) was injected each time, this process could be repeated a number of times before new standards were required.

Table IV—Recovery of Phenobarbital and Diphenylhydantoin from Plasma as Determined by HPLC and GLC

Drug	Micrograms Added	Mean Recovery ± *SD*, % HPLC	GLC
Phenobarbital	5.0	101.60 ± 2.17	95.64 ± 6.42
Diphenylhydantoin	5.0	95.64 ± 2.44	97.23 ± 4.76
Phenobarbital	7.5	98.14 ± 1.50	92.16 ± 4.70
Diphenylhydantoin	7.5	101.5 ± 2.04	97.84 ± 5.30
Phenobarbital	10.0	96.74 ± 2.21	94.76 ± 3.76
Diphenylhydantoin	10.0	100.19 ± 1.13	102.41 ± 4.20
Phenobarbital	12.5	100.86 ± 2.84	100.34 ± 5.60
Diphenylhydantoin	12.5	99.55 ± 3.42	93.77 ± 4.91
Phenobarbital	17.5	96.74 ± 2.65	90.42 ± 7.74
Diphenylhydantoin	17.5	99.60 ± 3.27	94.93 ± 6.42
Phenobarbital	27.5	92.64 ± 4.97	87.26 ± 9.43
Diphenylhydantoin	27.5	97.45 ± 3.76	92.14 ± 6.07

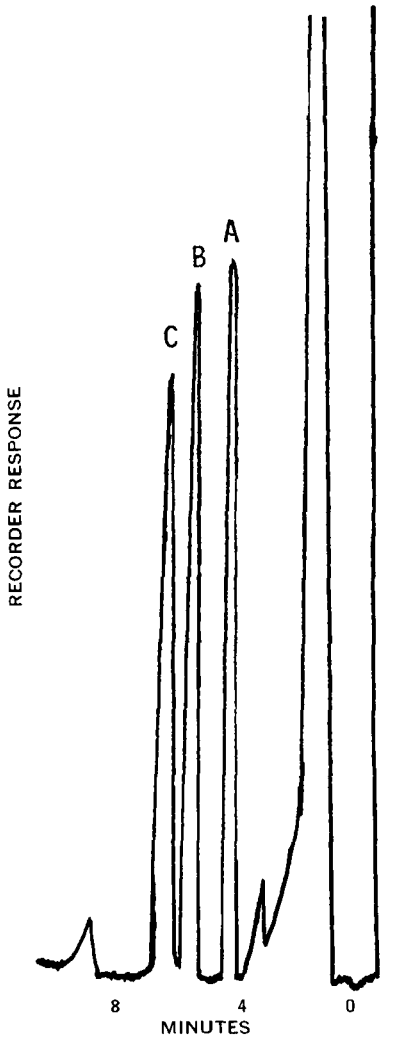

Figure 1—*Chromatographic trace of an extract of plasma found to contain 8.5 μg/ml of phenobarbital (A), 10.0 μg/ml of internal standard (B), and 9.2 μg/ml of diphenylhydantoin (C).*

Recovery of the two drugs from spiked plasma samples was determined (Table IV) at several concentrations by HPLC and by GLC. While mean results of the two procedures appear to be similar, the standard deviation of results obtained by GLC is larger. This increased spread of results may be attributed to the fact that the extraction process for GLC assay has several steps performed in an effort to clean up the chromatogram. However, determination is made in the HPLC assay by analysis of a concentrate of the initial chloroform extract since interpretation of the resulting chromatogram is sufficiently uncomplicated by extraneous peaks (Fig. 1). For both procedures, standard deviation increases at low and high concentrations of drug, reflecting the fact that precision is greatest in chromatographic methods when the drug and internal standard have the same peak area. In addition, precision is

Table V—Diphenylhydantoin and Phenobarbital Concentration in Plasma as Determined by HPLC and GLC

Sample	Phenobarbital, μg/ml		Diphenylhydantoin, μg/ml	
	HPLC	GLC	HPLC	GLC
1	21.4	22.5	20.3	20.1
2	14.9	15.5	11.0	11.2
3	24.0	23.4	5.0	5.3
4	11.8	12.3	17.9	17.8
5	24.5	26.0	6.2	6.0
6	7.9	7.8	2.3	2.4
7	9.3	9.7	4.8	4.8
8	15.2	14.8	8.9	9.3
9	27.3	27.7	7.9	8.1
10	7.0	7.2	23.6	24.0

greater for the analysis of diphenylhydantoin than for phenobarbital, since the internal standard is closely related to diphenylhydantoin and thus more effective in compensating for extraction and chromatographic irregularities.

Results of the analysis by GLC and HPLC of plasma of patients taking both drugs are presented in Table V. In light of the standard deviations of the results of the two procedures, the results are comparable. The HPLC procedure has since been used for determination of the two drugs in the plasma of more than 40 different patients, and no interferences have been apparent.

Several appealing features of this method may be noted. Sample preparation is rapid, no tedious pH adjustments are required, and extraction is quantitative. Internal standard and standard drug preparations are stable for long periods. The derived chromatogram is uncomplicated by extraneous peaks, and injections may be made every 7 min. Finally, the method requires only 0.5 ml of plasma, and analysis of smaller quantities of plasma should be possible by injecting larger volumes of the concentrated extract.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 5, 1974, from the *School of Pharmacy, University of Missouri–Kansas City, Kansas City, MO 64110*

Accepted for publication October 21, 1974.

Abstracted in part from a thesis submitted by S. H. Atwell to the Graduate School, University of Missouri–Kansas City, in partial fulfillment of the Master of Science degree requirements.

The authors acknowledge the cooperation of Mr. Louis Sartori of Waters Associates and the financial support of the Graduate School, University of Missouri–Kansas City.

* Children's Mercy Hospital, Kansas City, MO 64142

ˣ To whom inquiries should be directed.