Rapid and Micro High-Pressure Liquid Chromatographic Determination of Plasma Phenytoin Levels

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Abstract ◻ A rapid and simple high-pressure liquid chromatographic microanalytical method was developed for the determination of clinically encountered plasma phenytoin levels. This method is accurate down to about 1 μg of phenytoin/ml of plasma and requires as little as 10 μl of sample. Total analysis time is about 10 min. The method involves deproteinizing with acetonitrile followed by monitoring the deproteinized sample at 254 nm. Phenytoin's primary metabolite in humans, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, also can be quantitated when present in moderately high clinically encountered concentrations. Plasma profiles of phenytoin and its metabolite were followed with time after an intravenous bolus injection to a rabbit.

Keyphrases ◻ Phenytoin—high-pressure liquid chromatographic analysis in plasma ◻ High-pressure liquid chromatography—analysis, phenytoin in plasma ◻ Anticonvulsants—phenytoin, high-pressure liquid chromatographic analysis in plasma

Phenytoin is one of the most frequently prescribed anticonvulsants and also is used to treat various psychoses, trigeminal and related neuralgias, and various cardiac arrhythmias. It is considered the drug of choice in treating all forms of epilepsy except absence seizures (1).

BACKGROUND

Plasma concentrations of phenytoin may be related closely to its therapeutic as well as toxic effects (2). Phenytoin exerts a useful therapeutic effect at plasma concentrations of 10–20 μg/ml (1–3). Plasma levels higher than 20 μg/ml often are associated with adverse effects. The importance of plasma level monitoring also is increased by phenytoin's nonlinear elimination pharmacokinetics (4, 5).

No method of phenytoin measurement was available before 1956 (2). Since then, GLC (6–8), TLC (9, 10), mass fragmentographic (11), and high-pressure liquid chromatographic (HPLC) (12–15) methods have been used to determine phenytoin alone or in combination with other agents. Radioimmunoassay, enzyme immunoassay, spectrophotometric, and GLC determinations of phenytoin have been compared (16). All proposed methods suffer from one or more of the following requirements: (*a*) an excessive amount of time, (*b*) chemical derivatization, (*c*) extraction (either single or multiple steps) to "clean" the sample, (*d*) sample dilution, and (*e*) a fairly large plasma sample (1–2 ml).

The developed method has none of these drawbacks. Quantitation can be achieved within 10 min after obtaining the plasma sample. No derivatives are made nor extractions used. This method is ideally suited for assays of commonly encountered plasma phenytoin concentrations using as little as 10 μl of plasma. In addition, when present in moderately high plasma concentration, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (I), the major metabolite of phenytoin in humans, also may be quantitated.

EXPERIMENTAL

Reagents—Phenytoin[1], phenytoin sodium[2], 5-(*p*-hydroxyphenyl)-5-phenylhydantoin[3], and acetonitrile[4] were used.

Instruments—All determinations were made using an HPLC pump with an injector[5], an absorbance detector[6] with a 254-nm filter, a recorder[7], and a 30-cm × 3.9-mm (i.d.) reversed-phase column[8]. The system ran at ambient room temperature. The sensitivity was 0.005 absorbance unit full-scale deflection.

Standard Curve and Sample Preparation—A standard curve was prepared for phenytoin and I in the following manner. Aliquots of 200 μl of pooled human plasma were spiked with various quantities of concentrated acetonitrile–water stock solutions of phenytoin and I. The stock solutions were kept refrigerated and tightly sealed until use. A blank plasma and seven plasma samples with concentrations ranging from 1 to 40 μg of phenytoin/ml and from 0.1 to 4 μg of I/ml were vortex mixed[9] for about 5 sec. These samples were deproteinized by addition of 500 μl of acetonitrile, vortex mixed for about 5 sec, and centrifuged for 1 min at 2500 rpm.

Aliquots of 25 μl of the clear liquid fraction were chromatographed. Peak heights were used for quantitation. The flow rate was 2.0 ml/min with a mobile phase of acetonitrile–water (30:70 v/v), acidified with concentrated phosphoric acid to pH 2.65–2.69. Samples from *in vivo* experiments were analyzed similarly.

Drug Recovery Study—Three samples each of 100 μl of water and pooled human plasma were spiked with 4 μl of a stock solution of phenytoin and I to give a final concentration of 10 μg/ml. To each sample and plasma blank was added 250 μl of acetonitrile, and all were chromatographed as described.

Drug Interference Study—Many compounds were tested to determine if they would interfere: primidone[10], mephenytoin[11], salicylic acid[12], mephobarbital[13], trimethadione[14], ethotoin[14], phenacemide[14], methsuximide[15], ethosuximide[15], mephobarbital[16], phenobarbital sodium[17], hexobarbital[18], and heptabarbital[19]. Aliquots of 25 μl of 200-μg/ml acetonitrile–water stock solutions of each compound were injected and monitored at 254 nm.

***In Vivo* Study**—The solution for injection contained 20 mg of phenytoin sodium/ml in basified water. The right marginal ear vein of a 3.4-kg male albino rabbit was catheterized[20]. Phenytoin sodium, 40 mg, was injected *via* this route over 6–7 sec. Venous blood samples were collected in heparinized vials and were centrifuged within 30 min at 2500 rpm for 4 min to obtain plasma fractions. These fractions were stored frozen until analysis.

RESULTS AND DISCUSSION

Phenytoin and I standard curves were linear in the concentration ranges studied. For example, the phenytoin plot linear regression line was $y = 0.1431x - 0.0965$ ($\gamma = 0.9992$) and that for I was $y = 0.4407x - 0.0705$ ($\gamma = 0.9931$), where y is peak height in centimeters and x is concentration in micrograms per milliliter.

With the conditions of this analysis, the minimum concentrations that can be measured accurately by the procedure are about 1.0 μg of phenytoin and 1.0 μg of I/ml of plasma. This value for phenytoin is well below the therapeutic level. Lower concentrations give poor accuracy because of error in peak height measurement and, in the case of I, plasma interference. Retention times for I and phenytoin are 3.6 and 8.2 min, re-

[1] Prepared from phenytoin sodium.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Model M-6000A, U6K injector, Waters Associates, Milford, Mass.
[6] Model 440, Waters Associates, Milford, Mass.
[7] Ominscribe, Houston Instruments, Austin, Tex.
[8] μBondapak C_{18}, Waters Associates, Milford, Mass.
[9] Vortex-Genie, Scientific Industries, Springfield, Mass.
[10] Ayerst Laboratories, New York, N.Y.
[11] Sandoz Pharmaceuticals, Hanover, N.J.
[12] Merck Sharp & Dohme, West Point, Pa.
[13] Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[14] Abbott, North Chicago, Ill.
[15] Parke-Davis and Co., Detroit, Mich.
[16] Eli Lilly Laboratories, Indianapolis, Ind.
[17] J. T. Baker Chemical Co., Phillipsburg, N.J.
[18] Gane's Chemical Works, Corlstadt, N.J.
[19] Geigy Pharmaceuticals, Ardsley, N.Y.
[20] Radiopaque/Teflon, 22-gauge, 1 in., Jelco Laboratories, Raritan, N.J.

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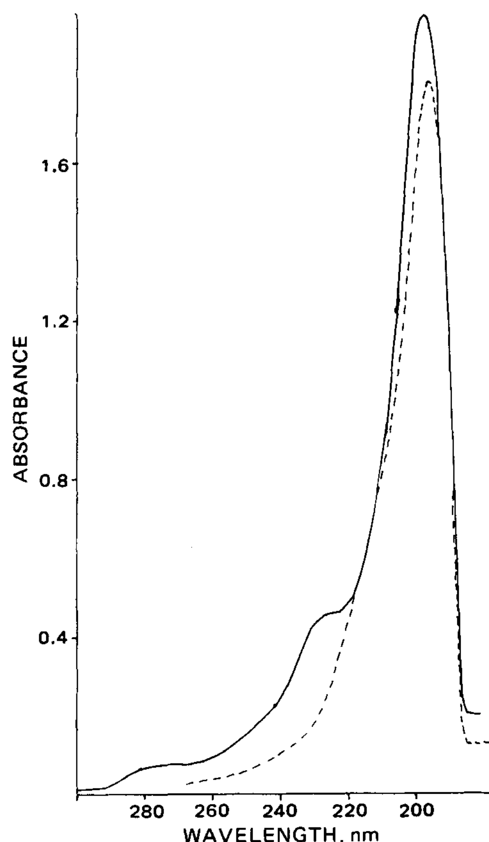

Figure 1—*UV spectra of phenytoin (- - -) and I (—), both at 1 mg % in acetonitrile–water (30:70 v/v) acidified to pH 2.65–2.69 with phosphoric acid. Spectra were obtained using 1-cm cells.*

spectively. The 2.0-ml/min flow rate and 25-μl injection volume were optimal.

At a concentration of 14.8 μg/ml, seven replicate analyses of phenytoin gave essentially identical peak heights. Similarly, at the same concentration and for the same number of assays, the coefficient of variation for I was only 1.9%. Under the same concentration conditions, four replicate analyses over 4 days gave interassay coefficients of variation of 2.2% for phenytoin and 1.2% for I. Placement of 50 ng each of phenytoin and I on-column resulted in signal-to-noise ratios of 12.5 and 76, respectively. The UV spectra of phenytoin and I are shown in Fig. 1. Although phenytoin and I showed maximal absorption at around 197 nm, detection at 254 nm using a mercury light souce had adequate sensitivity and an excellent signal-to-noise ratio.

The recovery study was done to show whether some of the drug was being lost during deproteinization. Recovery was essentially complete. Specifically, phenytoin was 100% recovered and I was 96% recovered, as calculated by relative average peak heights of plasma samples as compared to distilled water samples. There was some difficulty in I quantitation because of occasional minor blank interference, which had to be subtracted from peak height measurements. Had the small interfering peak height not been compensated for, I recovery would have been 107.4%, an overestimation of the plasma I concentration. No endogenous interference with the phenytoin signal was noted.

The drug interference study showed that phenobarbital sodium and ethotoin interfered with the I signal and that mephobarbital interfered with the phenytoin signal. The amounts injected were generally greatly in excess of plasma concentrations usually encountered clinically.

The rabbit data are graphically presented in Fig. 2. Plasma phenytoin levels declined biexponentially, with a terminal half-life of about 2 hr. At these levels, I can be monitored readily. Chromatograms of phenytoin and I in rabbit plasma are shown in Fig. 3. This preliminary rabbit study was done to show the ability of this procedure to follow plasma phenytoin and I concentrations.

The satisfactory amount of acetonitrile needed for deproteinization of the plasma was 2.5 times the plasma volume used. With this ratio, as little as 10 μl of plasma can be assayed to give good results. Acetonitrile proved excellent for plasma deproteinization, which obviates the need

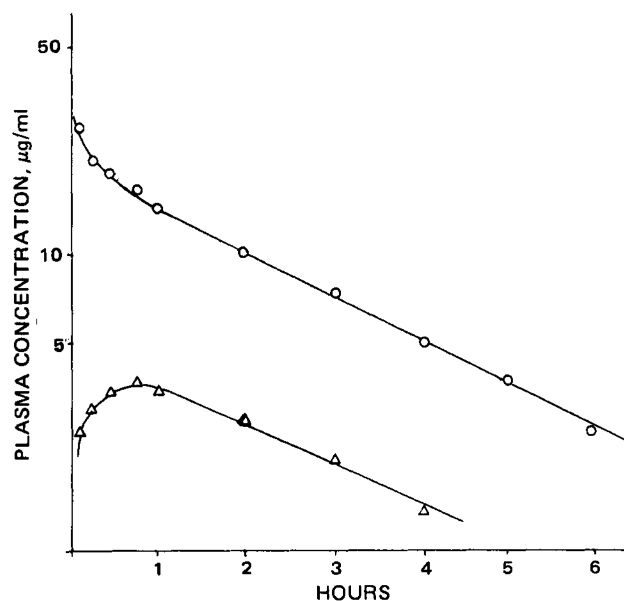

Figure 2—*Plasma concentration profiles of phenytoin (○) and I (△) in a rabbit after intravenous administration of phenytoin.*

for extraction. After centrifugation, the supernatant clear liquid may be poured into a screw-capped glass culture tube and refrigerated until the following morning if necessary; samples were stable overnight when stored in this manner. During storage or if a long break is taken between sample preparation and sample injection, the samples must be sealed from the air or volatile acetonitrile may evaporate and result in spuriously high drug concentration readings.

This assay was performed without column thermoregulation, and this omission contributed to the slight variations in retention times of the compounds from day to day. If used for routine clinical monitoring of plasma phenytoin levels, a standard solution of phenytoin (and I if analyzed) should be injected into the chromatographic system prior to sample analysis to obtain an exact retention time value for any given day; ther-

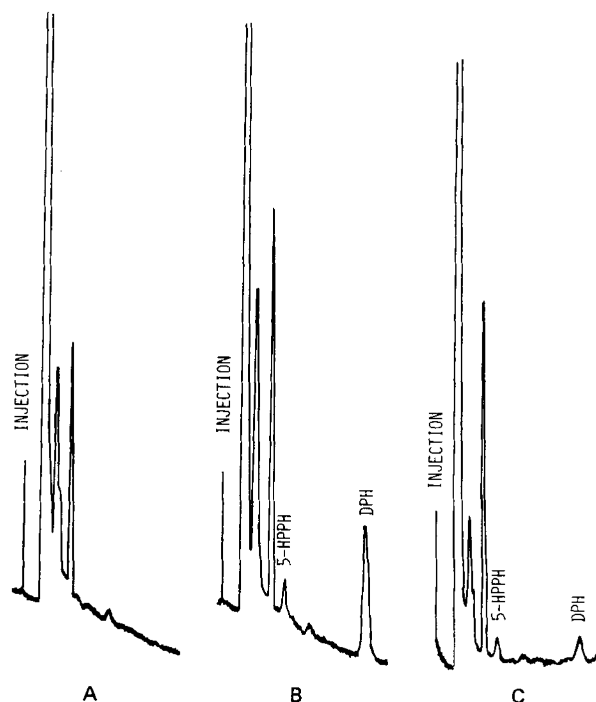

Figure 3—*Typical chromatograms of phenytoin (DPH) and I (5-HPPH) seen on analysis of rabbit plasma. Key: A, blank; B, 6 min after intravenous phenytoin administration; and C, 4 hr after intravenous phenytoin administration.*

moregulation of the column might also be desirable. The slight variations in retention times do not appreciably affect peak heights for quantitation.

Acidification of the mobile phase was shown to affect chromatography favorably. It shortened the retention times of plasma residues and lengthened retention times of phenytoin and I, thereby minimizing plasma interference with the assay.

The column gave good resolution of the compounds for at least 6 months of operation with systems of this sort, being used about 3 days/week.

In conclusion, this method should be useful for clinical monitoring of plasma phenytoin concentrations. Its metabolite, I, can be detected in moderately high concentrations, as shown by a preliminary *in vivo* rabbit investigation. The method is extremely rapid, economical of plasma and reagents, and simple.

REFERENCES

(1) D. M. Woodbury and E. Fingl, in "The Pharmacological Basis of Therapeutics," 5th ed., L. S. Goodman and A. Gilman, Eds., Macmillan, New York, N.Y., 1975, chap. 13.

(2) L. Lund, in "The Biological Effects of Drugs in Relation to Their Plasma Concentrations," D. S. Davies and B. N. C. Prichard, Eds., University Park Press, Baltimore, Md., 1973, chap. 18.

(3) "Physicians' Desk Reference," Medical Economics Co., Oradell, N.J., 1976, p. 1151.

(4) A. Richens and A. Dunlop, *Lancet*, **2**, 247 (1975).

(5) D. G. Lambie, R. H. Johnson, R. N. Nanda, and R. A. Shahir, *ibid.*, **2**, 386 (1976).

(6) K. K. Midha, I. J. McGilveray, and D. L. Wilson, *J. Pharm. Sci.*, **65**, 1240 (1976).

(7) R. J. Perchalski, K. N. Scott, B. J. Wilder, and R. H. Hammer, *ibid.*, **62**, 1735 (1973).

(8) J. MacGee, *Anal. Chem.*, **42**, 421 (1970).

(9) N. Wad, E. Hanifl, and H. Rosenmund, *J. Chromatogr.*, **143**, 89 (1977).

(10) G. E. Simon, P. I. Jatlow, H. T. Seligson, and D. Seligson, *Am. J. Clin. Pathol.*, **55**, 145 (1971).

(11) C. Hoppel, M. Garle, and M. Elander, *J. Chromatogr.*, **116**, 53 (1976).

(12) J. E. Evans, *Anal. Chem.*, **45**, 2428 (1973).

(13) R. F. Adams and F. L. Vandemark, *Clin. Chem.*, **22**, 25 (1976).

(14) K. S. Albert, M. R. Hallmark, M. E. Carroll, and J. G. Wagner, *Res. Commun. Chem. Pathol. Pharmacol.*, **6**, 845 (1973).

(15) P. M. Kabda, G. Gotelli, R. Stomfill, and L. J. Marton, *Clin. Chem.*, **22**, 824 (1976).

(16) V. Spiehler, L. Sun, D. S. Miyada, S. G. Sarandis, E. R. Walwick, M. W. Klein, D. B. Jordan, and B. Jessen, *ibid.*, **22**, 749 (1976).

Comparison of Adsorbed Films of a Polyvinylpyrrolidone Copolymer with Spread Monolayers

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Received November 28, 1977, from the *College of Pharmacy, Rutgers—the State University, Piscataway, NJ 08854.* Accepted for publication February 23, 1978.

Abstract ☐ The adsorption of a polyvinylpyrrolidone–polyvinyl acetate graft copolymer from solution was studied by surface pressure measurement. Adsorption from the dilute solutions was slow, limited, in part, by diffusion of polymer molecules to the surface. When adsorbed monolayers were compressed on a surface balance, the resulting surface pressure values paralleled those of a spread monolayer, strongly suggesting that the structures of adsorbed and spread monolayers are the same.

Keyphrases ☐ Polyvinylpyrrolidone copolymer—adsorption from solution studied by surface pressure measurement, films compared to spread monolayers ☐ Adsorption—polyvinylpyrrolidone copolymer from solution, studied by surface pressure measurement, films compared to spread monolayers ☐ Surface pressure—measurement used to study polyvinylpyrrolidone copolymer adsorption from solution, films compared to spread monolayers ☐ Polymers—polyvinylpyrrolidone–vinyl acetate copolymer, adsorption from solution studied by surface pressure measurement, films compared to spread monolayers

The surface properties of polymers are studied conveniently by spreading them as monolayers on a water surface. In such studies, polymer molecules are spread onto an aqueous substrate in small quantity so as barely to disturb the surface tension. Once the spreading process is complete, the surface area is reduced to smaller values (thereby concentrating the two-dimensional polymer systems) and the surface properties are measured as a function of the area available to each unit of polymer.

However, in systems of practical interest such as pharmaceutical dispersions, a polymer in the formulation is in solution and polymer molecules migrate to the interface and are adsorbed. The first few polymer molecules adsorbed encounter an uncrowded interface and have space in which to adopt the energetically most favorable orientation. But the interfacial region gradually becomes crowded as adsorption proceeds, and polymer molecules arriving later may be unable to spread completely. The conformation of molecules in concentrated adsorbed monolayers may thus be different from that in spread monolayers, so the properties of the two types of systems may differ.

BACKGROUND

Most studies comparing the structures of spread and adsorbed monolayers have been carried out on proteins. Yamashita and Bull (1) found that adsorbed films of lysozyme were thicker than spread monolayers. They suggested that the adsorbed protein molecules largely retain their native configuration at the surface while spread lysozyme molecules unfold more completely. Surface films of trypsin formed by applying the enzyme to a clean surface showed a complete loss of enzymatic activity (2–4). However, when the films were formed by adding more trypsin to an interface that already had some trypsin present, some enzymatic activity was retained. In this situation, complete spreading did not occur and the film properties depended on the method of film formation.

Musselwhite and Palmer (5) prepared monolayers of bovine serum albumin using two different techniques. In one experiment, the monolayers were spread in the usual way, and the film was concentrated by compression to a smaller surface area. The second approach involved concentration of the monolayer by maintaining the same surface area and adding more protein to the surface. The newly added protein molecules encountered a surface already partially occupied. This mode of increasing the surface concentration of the protein is similar to the process that occurs during adsorption. The force–area diagrams for the two techniques were quite different. These reports indicated that adsorbed and spread films of proteins are not equivalent.

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