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Note

High-performance liquid chromatography of anticonvulsants — micro-assay for phenytoin and phenobarbital

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Currently available high-performance liquid chromatographic (HPLC) methods for the analysis of anticonvulsant drugs such as phenytoin and phenobarbital have mostly employed ODS-bonded phases [1—7]. The application of this mode of HPLC for the assay of anticonvulsants in biological fluids has a number of disadvantages which include: the requirement for special equipment such as a temperature block [1—5], the dependence of the separation on pH [5, 7], coelution of the hydroxylated metabolite of phenytoin and phenobarbital [4, 5], and in our experience the frequent loss of column performance due to build up of impurities on top of the column bed. In addition, no detailed studies have been performed to determine the best mode of chromatography for the separation of phenytoin compounds and phenobarbital.

In this paper we describe in detail the chromatography of several anticonvulsants by both normal-phase partition and reversed-phase modes of HPLC. Also included is a micro-assay for phenytoin and phenobarbital in plasma employing a polar-bonded phase of the cyano type.

EXPERIMENTAL

Chemicals

Phenytoin was obtained from Parke Davis and Co. (Detroit, MI, U.S.A.). 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin (HPPH) and 5-(*p*-methylphenyl)-5-hydantoin (MPPH) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Phenobarbital was a gift from Smith, Kline and French Labs. (Philadelphia, PA,

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U.S.A.). HPLC grade solvents were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), and phosphoric acid was from J.T. Baker (Phillipsburg, NJ, U.S.A.).

High-performance liquid chromatography

HPLC was carried out with a reciprocating pump (Model 6000A, Waters Assoc., Milford, MA, U.S.A.) coupled to a variable-wavelength UV detector (Model 450, Waters Assoc.). Anticonvulsants were detected at either 200 nm or 195 nm.

The column packings were either a cyano-propyl bonded (Zorbax-CN) or ODS-bonded (Zorbax-ODS) microparticulate silica purchased from DuPont (Wilmington, DE, U.S.A.). These were packed into columns (250 mm × 4.6 mm I.D.) by the suppliers. Samples were dissolved in the mobile phase and injected with an automated injection system (Model 710A, Waters Assoc.).

Procedure

To 50—100 μl plasma in a tapered tube were added 200 μl of 50% acetonitrile in propan-2-ol containing MPPH (10 mg/l) as internal standard. The contents of the tube were mixed thoroughly on a vortex mixer and then centrifuged (4000—4500 *g*) for 2 min.

The supernatant (30—100 μl) was injected directly into the chromatograph and eluted with a mobile phase of 7 m*M* phosphoric acid—methanol—acetonitrile (6:3:1) and a flow-rate of 1.5 ml/min.

Quantitation

Plasma phenytoin and phenobarbital were determined by comparison of the peak height ratios of drug to the internal standard with those given by standard solutions containing from 2.0 to 30.0 mg/l phenytoin, 5—50 mg/l phenobarbital and 10 mg/l internal standard (MPPH). Over this range the peak height ratio of drug to internal standard was related linearly to the concentration of phenytoin and phenobarbital.

RESULTS

Normal-phase partition HPLC

The chromatography of phenytoin and related compounds was examined on a commercial packing in which a cyanopropyl phase was bonded to 6-μm porous silica particles. Although a mobile phase mixture of 15% propan-2-ol in hexane resolved phenytoin from HPPH, the separation of phenytoin from MPPH was not possible. The addition of 5% acetonitrile to a mobile phase of hexane—propan-2-ol enabled the resolution of all test compounds (Fig. 1).

Although for the separation of phenytoin compounds and phenobarbital, normal-phase partition chromatography offered lower operating pressures and improved stability of the column bed compared to reversed-phase HPLC, this mode of chromatography was found unsuitable for the assay of phenytoin in plasma because injection of semi-aqueous extracts of serum resulted in poor peak shape.

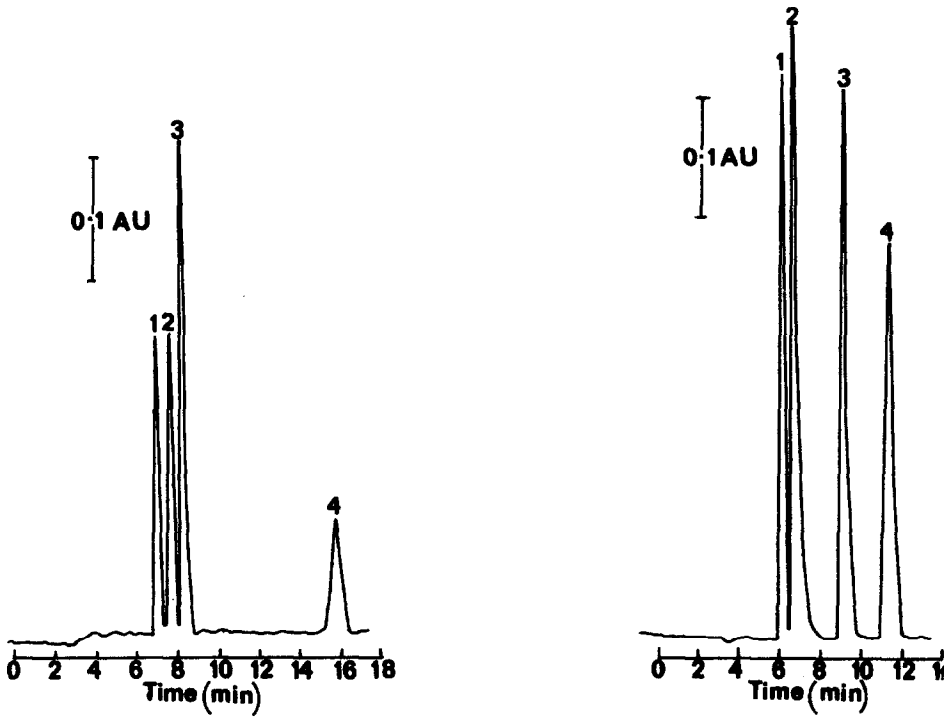

Fig. 1. Separation of anticonvulsants by normal-phase partition HPLC on Zorbax-CN with a mobile phase of 5% acetonitrile and 10% propan-2-ol in hexane and a flow-rate of 1.5 ml/min. Detection was at 200 nm. Peaks: 1 = MPPH; 2 = phenytoin; 3 = Phenobarbital; 4 = HPPH.

Fig. 2. Separation of anticonvulsants by reversed-phase HPLC on Zorbax-CN with a mobile phase of 10% acetonitrile and 30% methanol in 7 m*M* phosphoric acid and a flow-rate of 1.5 ml/min. Detection was at 195 nm. Peaks: 1 = phenobarbital; 2 = HPPH; 3 = Phenytoin; 4 = MPPH.

Reversed-phase HPLC

A typical resolution of phenytoin compounds and phenobarbital by reversed-phase HPLC (Zorbax-CN) employing mobile phase mixtures of phosphoric acid—methanol—acetonitrile is shown in Fig. 2. Although a similar separation could also be achieved on a non-polar bonded phase of Zorbax-ODS, this system retained phenytoin strongly and the HPPH weakly.

In general, for the resolution of phenytoin, HPPH, MPPH, and phenobarbital, it was found that to obtain reasonable capacity ratios, it was necessary to employ the Zorbax-CN column operated in the reversed-phase mode.

Assay of phenytoin and phenobarbital in plasma

Sample preparation and chromatography. Sample preparation was a simple protein precipitation described in the Experimental section. A mixture of 50% acetonitrile in propan-2-ol was used as an extractant because it gave more complete and faster protein precipitation than acetonitrile.

A typical chromatogram illustrating the assay of phenytoin and phenobarbital in plasma is shown in Fig. 3a. Extracts of drug-free plasma yielded no

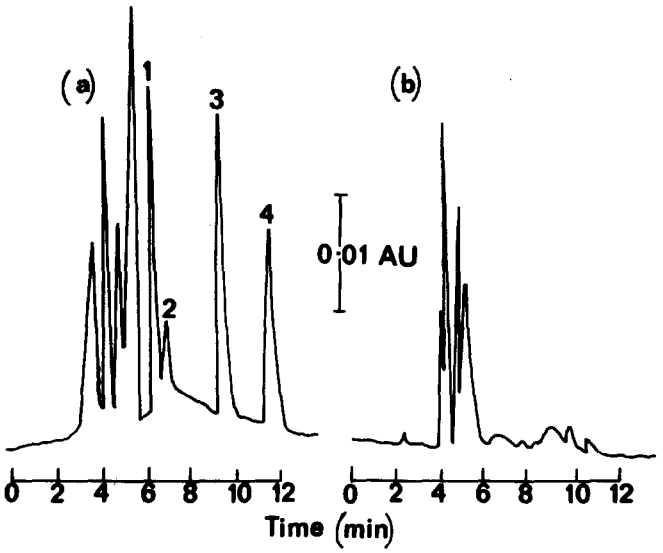

Fig. 3. (a) Chromatogram of a 50-μl injection of a serum sample from a patient on anticonvulsant therapy. Peaks: 1 = phenobarbital 10 mg/l; 2 = HPPH; 3 = phenytoin 15 mg/l; 4 = MPPH 10 mg/l. (b) Chromatogram of a drug-free serum sample. The chromatographic systems were the same as for Fig. 2.

significant interference from endogenous plasma components (Fig. 3b).

Reproducibility. Data on within-run precision were obtained by analyzing pooled human plasma spiked with phenytoin and phenobarbital at concentrations ranging from 2.0—25 mg/l and 8.0—50 mg/l, respectively. The mean coefficient of variation of ten replicates at each concentration was 3.4% for phenytoin and 2.8% for phenobarbital (Table I). The mean between-run precision for the assay of phenytoin and phenobarbital in plasma from patients undergoing anticonvulsant therapy was 5.3% and 5.5%, respectively (Table II).

Background. To obtain data on the amount of background appearing at elution times corresponding to those of the drugs of interest, we processed several drug-free plasmas with added internal standard (MPPH) through the complete procedure. Background interference was calculated at values from 0.00 to 0.4 mg/l.

TABLE I

WITHIN-RUN PRECISION OF ASSAY FOR PHENYTOIN AND PHENOBARBITAL (n = 10)

Phenytoin			Phenobarbital		
Concn. added (mg/l)	Mean measured value (mg/l ± S.D.)	C.V. (%)	Concn. added (mg/l)	Mean measured value (mg/l ± S.D.)	C.V. (%)
2.0	2.1 ± 0.10	4.8	8.0	8.4 ± 0.15	1.8
10.0	9.9 ± 0.24	2.4	25.0	24.8 ± 0.98	3.9
25.0	25.1 ± 0.76	3.0	50.0	52.4 ± 1.40	2.7

TABLE II

BETWEEN-RUN PRECISION OF ASSAY FOR PHENYTOIN AND PHENOBARBITAL ($n = 10$)

Plasma sample	Phenytoin		Phenobarbital	
	Mean concn. (mg/l ± S.D.)	C.V. (%)	Mean concn. (mg/l ± S.D.)	C.V. (%)
1	3.3 ± 0.34	10.3	9.4 ± 0.70	7.4
2	12.5 ± 0.28	2.2	12.2 ± 0.20	1.6
3	18.1 ± 0.80	4.4	21.4 ± 1.4	6.5
4	22.4 ± 0.97	4.3	30.8 ± 2.0	6.5

Sensitivity. The sensitivity of the procedure is limited largely by the serum volume, extraction efficiency and background. With a 0.1-ml sample volume, the compounds can be detected in concentrations as low as 1 mg/l.

Comparison with gas chromatography

To compare the results of the HPLC assay with those of gas chromatographic (GC) analysis we analyzed 40 specimens containing phenytoin and 40 samples containing phenobarbital by both methods. For phenytoin the coefficient of correlation was 0.914; slope, 0.912; y-intercept, +1.9. The standard estimated error was 0.068. Results of similar calculations for phenobarbital gave a coefficient of correlation of 0.894 (slope, 1.094; y-intercept, −1.71; estimated error, 0.089.

DISCUSSION

We have examined the chromatography of phenytoin compounds by both normal-phase partition and reversed-phase modes of HPLC. Although both modes of chromatography enabled the separation of a test mixture consisting of phenytoin, HPPH, MPPH and phenobarbital, normal-phase partition chromatography offered greater selectivity for the separation of phenobarbital and HPPH.

The only significant difference observed in the chromatography of phenytoin compounds on the two systems of reversed-phase HPLC examined was the much greater retention of phenytoin than its hydroxylated metabolite on the ODS-phase compared to the cyano-phase.

For the assay of phenytoin and phenobarbital in plasma we chose the less retentive Zorbax-CN column because it offered several advantages over the ODS-bonded phase. These were the close elution of phenytoin and its major hydroxylated metabolite allowing for their simultaneous quantitation, the improved stability of the column bed and the ability to inject solvent extracts of plasma directly into the column without further purification and evaporation.

The analysis time and resolution of the compounds during reversed-phase HPLC was found to be related to the concentration of acetonitrile in the mobile phase and, in practice, this provided a considerable opportunity to make the analysis optimal. The acetonitrile concentration (10%, v/v) used in

the assay was chosen because it adequately resolved phenobarbital and the hydroxylated metabolite of phenytoin. Increasing the amount of acetonitrile in the mobile phase to 20% resulted in decreased retention of all compounds of interest with co-elution of HPPH and phenobarbital.

Although the results obtained by HPLC and GC on the same samples correlated well for phenytoin and phenobarbital the sample requirement for GC (0.5—1.0 ml) was greater than for the HPLC method (50—100 μl). This was an important consideration in the design of our assay because the volume of plasma that can be obtained from children is often limited.

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