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Note

Tissue assay of phenobarbital, phenytoin and *p*-hydroxyphenytoin by high-performance liquid chromatography

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Phenobarbital (PB) and phenytoin (DPH) are anticonvulsants extensively employed in clinical medicine and numerous chromatographic methods have been described for their determination in serum and plasma[1–6]. The information gained from plasma concentration monitoring serves as a valuable aid in adjusting drug dosages, as a narrow range of concentrations is associated with optimal seizure control. Despite the extensive number of investigations of the pharmacokinetics of PB and DPH in plasma, there remains a paucity of data on their concentrations in brain and other tissues.

The anticonvulsants produce their pharmacological effects within the central nervous system and therefore have sufficient lipid solubility to distribute beyond the vascular system. The teratogenic consequences of maternal phenytoin use are well recognized[7] and presumably fetal drug accumulation is extensive. Clearly, there is a need for suitable analytical methods for the quantitation of anticonvulsant tissue concentrations. In papers describing the application of high-performance liquid chromatography (HPLC) to tissue analysis for PB or DPH there is minimal information on quality control[8–10]. Therefore, we have developed a precise and reproducible HPLC assay to measure PB, DPH and its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenytoin (HPPH), in several tissues of the rat. This method should allow the determination of anticonvulsant concentrations in tissues following single or multiple drug doses in animals.

EXPERIMENTAL

Apparatus

Chromatography was performed with an IBM Instruments (Wallingford, CT, U.S.A.) liquid chromatograph fitted with a Rheodyne 7126 injection valve (Rheodyne, Cotati, CA, U.S.A.) and a 50-μl sample loop. The column was a 25 cm × 4.6 mm I.D. IBM octadecyl (C_{18}) with a 5.0-μm particle size. A stainless-steel IBM

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column prefilter containing a 2-μm fritted disk was also used. The disk was routinely replaced every 3–4 weeks. An IBM 9523 variable-wavelength UV detector was operated at 254 nm and 0.03 a.u.f.s. Chromatograms were recorded and peak areas integrated using an IBM 9541 chromatography data system.

Reagents and chemicals

Analytical-reagent grade monobasic and dibasic potassium phosphate, tribasic sodium phosphate and perchloric acid and HPLC-grade methyl *tert.*-butyl ether, methanol and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Phenobarbital, sodium 5,5-phenytoin and 5-(*p*-hydroxyphenyl)-5-phenytoin were obtained from Sigma (St. Louis, MO, U.S.A.). Water was passed through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) before use.

Mobile phase and buffers

The mobile phase was a 32:68 (v/v) mixture of acetonitrile and pH 5.0 phosphate buffer. A flow-rate of 1.2 ml/min (100 bar) was used. The phosphate buffer used in the mobile phase was prepared by adding 400 μl of 1.0 *M* monobasic potassium phosphate to 1 l of distilled water and adjusting the pH to 5.0 with 1.0 *M* dibasic potassium phosphate. The buffer used for plasma and whole blood extractions was prepared by adjusting 0.1 *M* monobasic potassium phosphate to pH 5.9 with 1.0 *M* dibasic potassium phosphate. For tissue homogenate extractions, the buffer was prepared by adjusting 0.1 *M* tribasic sodium phosphate to pH 11.4 with 1.0 *M* phosphoric acid. The concentration of perchloric acid used for tissue homogenations was 0.34 *M* and contained EDTA ($1 \cdot 10^{-5}$ *M*).

Standards for calibration graphs

Stock solutions of PB and DPH (10.0 μg/μl) and HPPH (5.0 μm/μl) were prepared by dissolving the appropriate amounts in methanol. "Spiking" solutions containing 0.1 and 2.0 μg/μl of PB and DPH and 0.05 and 1.0 μg/μl of HPPH were prepared by diluting the stock solutions.

Standards were prepared by spiking 1.0-ml aliquots of plasma, whole blood or tissue homogenate with stock spiking solutions. The final concentrations ranged from 1.0 to 50.0 μg/ml for PB and DPH and from 0.5 to 25.0 μg/ml for HPPH. For PB, 5 μl of 1.0 μg/μl stock HPPH solution was added as an internal standard to each standard and sample, whereas for DPH and HPPH, 10 μl of 1.0 μg/μl PB solution was added as an internal standard. Calibration graphs of the recovered standards were prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system. Graphs were constructed of the peak-area ratio of each compound to internal standard against drug concentration.

Extraction procedure for plasma and whole blood

To 1.0 ml of plasma or whole blood in a polypropylene tube, 2.0 ml of pH 5.9 buffer were added and the tubes were mixed in a Vortex-type mixer. Methyl *tert.*-butyl ether (1.5 ml) was then added and the tubes were placed on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 10 min. After centrifuging for 10 min at 220 *g*, the ether layer was transferred into a clean polypropylene tube and evaporated to dryness under nitrogen in an N-EVAP evaporator (Organomation, Worces-

TABLE I

RECOVERY OF PHENOBARBITAL, PHENYTOIN AND *p*-HYDROXYPHENYTOIN FROM RAT TISSUES AND WITHIN-RUN ASSAY PRECISION

Tissue	*Concentration (μg/ml)*	*n*	*Recovery of phenobarbital (%)*	*CV (%)*	*Recovery of phenytoin (%)*	*CV (%)*	*Recovery of p-hydroxyphenytoin (%)*	*CV (%)*
Brain	0.50	5	92.5	5.1	95.9	7.6	89.8	7.5
	1.00	5	93.1	7.1	96.9	6.1	91.9	2.4
	5.00	5	86.8	4.9	87.2	3.1	81.7	2.9
	10.00	10	96.1	4.7	97.5	3.4	90.3	4.2
	25.00	10	81.0	4.0	81.7	4.7	79.1	5.9
	50.00	5	80.9	2.6	83.4	2.7	80.3	1.3
Plasma	10.00	5	82.0	3.6	86.3	2.7	79.3	3.6
Liver	10.00	5	88.0	3.2	88.2	3.2	84.0	3.1
Fetal tissue	10.00	5	95.9	5.1	104.3	1.5	98.3	1.9
Whole blood	10.00	5	85.4	5.7	98.8	6.4	87.0	6.3

ter, MA, U.S.A.). Samples and standards were reconstituted with 100–500 μl of methanol, depending on the concentration, and 10 μl were injected on to the HPLC column.

Extraction procedure for tissue homogenates

Rat tissues (brain, liver or fetal tissue) were weighed and homogenized in 10 ml of 0.34 *M* perchloric acid per 1.6 g of tissue. To 1.0 ml of each tissue homogenate in a polypropylene tube, 2.0 ml of pH 11.4 buffer were added and the tubes were vortexed. The remaining part of the extraction procedure beginning with the addition of methyl *tert*-butyl ether was identical with the procedure described for plasma and whole blood.

Recovery and assay validation

To determine the recovery and within-day precision, batch plasma, blood and tissue homogenates were spiked with known amounts of each drug and 5–10 aliquots (1.0 ml) were assayed per day. Recoveries were determined by comparing the peak areas from the extracted samples with those obtained from a direct injection of the same amount of each drug in methanol. To determine the between-day variability, batch homogenates were spiked and two 1.0-ml aliquots were assayed per day for 10–12 days and drug concentrations were quantified by using a calibration graph that was prepared daily. Quantitation was performed by calculating the peak-area ratio of each compound to the internal standard. A linear regression analysis for each of the calibration graphs was performed, resulting in the calculation of the slope, intercept and correlation coefficient.

RESULTS

The chromatographic procedure separated PB, DPH and its major metabolite with a short overall run time. Retention times for HPPH, PB and DPH were 5.1, 6.1 and 11.4 min, respectively. Fig. 1 illustrates typical chromatograms of a methanol stock solution, extracts from blank and spiked tissue and tissue obtained from a drug-treated animal.

Table I illustrates the recovery and within-run precision data obtained by chromatographing spiked batch plasma and tissue homogenates. Recoveries were greater than 85% in most instances and the within-run variability coefficients were less than 5% in 20 out of 30 cases. Table II the shows day-to-day variability for calibration graphs and aliquots of spiked brain homogenate assayed at two per day for 10–12 days. The variability of the slope of the calibration graphs was less than 6.0% and the spiked sample variability was less than 9.5% for each compound.

DISCUSSION

HPLC methods have been described previously for the determination of the anticonvulsants considered here in serum and plasma. A major objective of this study was to develop a method that could also be applied to tissue samples. The most frequently employed solvents for the extraction of anticonvulsants are chloroform, methylene chloride, ethyl acetate and diethyl ether[11]. These solvents were tested in

TABLE II

BETWEEN-DAY VARIABILITY AND REPRODUCIBILITY OF CALIBRATION GRAPHS FOR PHENOBARBITAL, PHENYTOIN AND *p*-HYDROXYPHENYTOIN IN RAT BRAIN

Compound	*n**	*Actual concentration (μg/ml)*	*mean measured concentration (μg/ml)*	*CV (%)*	*Slope***	*CV (%)*	*Intercept*	*Correlation coefficient*
Phenobarbital	10	15.0	15.03	3.9	0.076 (10)	3.8	$1.7 \cdot 10^{-3}$	0.99
Phenytoin	11	15.0	14.46	5.8	0.097 (6)	5.7	$5.0 \cdot 10^{-2}$	0.99
p-Hydroxyphenytoin	9	7.5	8.14	9.3	0.282 (6)	5.6	$6.4 \cdot 10^{-2}$	0.99

* n = number of days with two samples per day. Standards were prepared daily in the ranges 1.0–50.0 μg/ml for PB and DPH and 0.5–25.0 μg/ml for HPPH.

** The numbers in parentheses give the number of calibration graphs used to determine the values given.

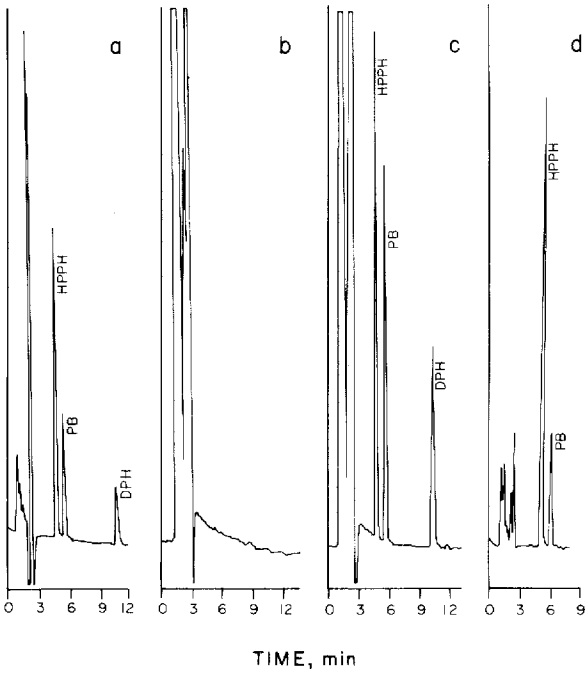


Fig. 1. (a) Chromatogram of a 2-μl injection of a methanol solution containing 50 ng/μl each of phenobarbital, phenytoin and *p*-hydroxyphenytoin. (b) Chromatogram of a 10-μl injection of the extract of 1 ml of blank brain homogenate reconstituted with 100 μl of methanol. (c) Chromatogram of a 10-μl injection of the extract from 1 ml of blank brain homogenate spiked with 10 μg of phenobarbital and phenytoin and 5 μg of *p*-hydroxyphenytoin and reconstituted with 200 μl of methanol. (d) Chromatogram of a 10-μl injection of the extract of 1 ml of brain homogenate from a rat previously treated with phenobarbital. HPPH was included as an internal standard. The residue was reconstituted with 200 μl of mobile phase. The concentration of phenobarbital was 24.5 μg/g.

an attempt to obtain the highest recoveries and the cleanest chromatograms. Chloroform and methylene chloride extracted PB and DPH efficiently from plasma and brain homogenates but failed to extract HPPH sufficiently (recovery $<20\%$). Both methyl *tert.*-butyl ether and ethyl acetate would have been suitable solvents, as they both extracted the compounds efficiently and produced clean chromatograms; however, the boiling point of methyl-*tert.*-butyl ether (55.2°C) is lower than that of ethyl acetate (77.0°C), making it easier to evaporate. Methyl *tert.*-butyl ether was selected instead of diethyl ether because of its higher flash point and lesser tendency to form harmful peroxides.

An extraction pH of 5.8–6.0 was maintained for both plasma and tissue homogenates. This value was high enough to eliminate the extraction of endogenous substances found in the biological matrix, yet low enough to prevent the compounds from being lost in the aqueous phase owing to ionization. The organic to aqueous phase ratio was varied but seemed to have little effect on recovery. We used 1.5 ml of ether for 3 ml of aqueous phase (1.0 ml of sample plus 2 ml of buffer).

In attempting to optimize the chromatographic conditions, several parameters were varied, including the strength and pH of the mobile phase and the flow-rate. Different mixtures of acetonitrile and water were evaluated at a flow-rate of 1.5

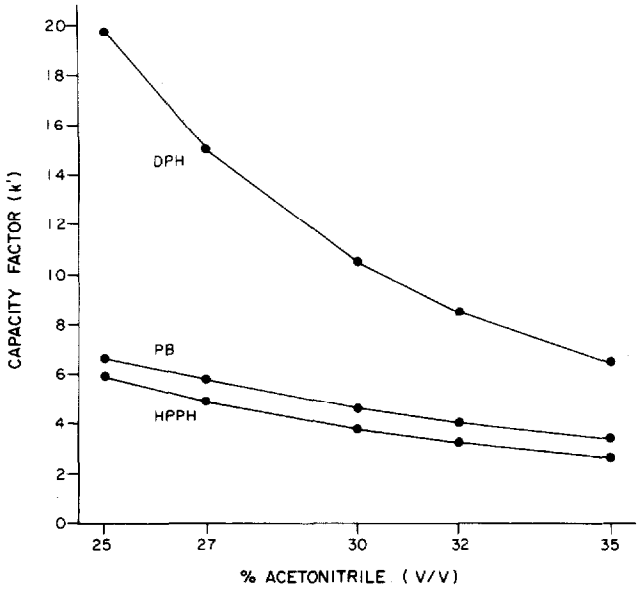


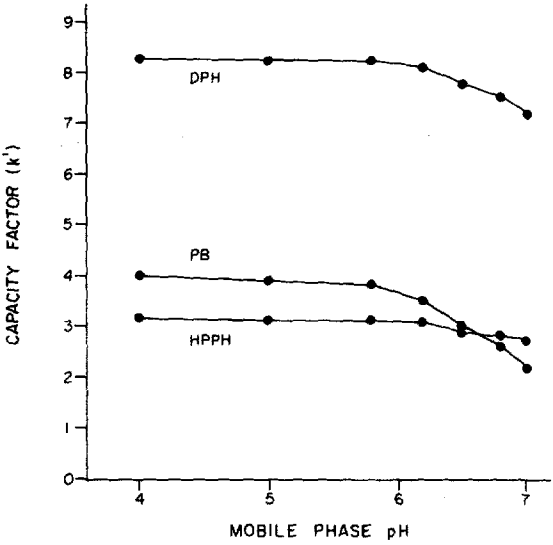
Fig. 2. Effect of acetonitrile concentration in an acetonitrile–phosphate buffer mobile phase on the capacity factor (k') of phenobarbital, phenytoin and *p*-hydroxyphenytoin at a flow-rate of 1.5 ml/min.

ml/min (Fig. 2). A 32:68 (v/v) mixture of acetonitrile and phosphate buffer produced a chromatogram with sharp, well separated peaks and with a short overall run time. The flow-rate was lowered to 1.2 ml/min in order to ensure re-establishment of the baseline after the matrix front. The capacity factor (k') was within the desired range of 1–10 for each component (HPPH = 3.3, PB = 4.0, DPH = 8.5) and the separation factor (α) for PB and HPPH was 1.2. The mobile phase pH was varied from 4.0 to 7.0 by the use of phosphate buffers (Fig. 3). The capacity factors remained almost constant at a pH of less than 5.8. At a pH greater than 5.8 the capacity factors decreased and at a pH greater than 6.6 the elution order of HPPH and PB reversed. Our results are in agreement with those published[11] for the dependence of k' for PB and DPH on the pH of the mobile phase; however, the previously published results did not included HPPH. As the pK_a of PB is 7.3 and that of DPH is 8.3, a mobile phase of pH 5.3 was used to suppress ionization and maintain consistent peak shapes and retention times[12].

The detection wavelength used was 254 nm. More sensitive results were obtained by using lower wavelengths of 195, 200 or 214 nm, but greater interferences then occurred. The use of 254 nm produced clean chromatograms for both plasma and tissue homogenates. As we were reconstituting the 1.0 μg/ml standard to 100 μl and injecting only 10 μl, the sensitivity could be improved by either reconstituting less or injecting a larger volume. We found that the peaks were sharper and more symmetrical with the smaller injection volume and that the higher concentration standards required a larger reconstitution volume (200–500 μl) in order to maintain a 10-μl injection volume. Cleaner chromatograms with less of a solvent front were obtained when the samples were reconstituted with mobile phase instead of methanol.

In assaying *in vivo* samples, no interferences were seen for the tissue homog-

enates. The chromatogram of an *in vivo* brain sample shown in Fig. 1d was representative of other tissue samples tested. It should be noted that even though the sample contained only PB, no peaks were detected in subsequent *in vivo* samples that would have interfered with DPH elution at 11.4 min. When assaying *in vivo* plasma and whole blood samples we detected an occasional interfering peak that eluted just prior to HPPH. We discovered that this peak resulted from the sodium heparin solution used to rinse the blood collection syringes before the rat blood was obtained by cardiac puncture. To obviate this problem, a slight change in the mobile phase was made when assaying plasma or whole blood samples. By using a 28:72 (v/v) mixture of acetonitrile and pH 5.0 phosphate buffer, the heparin and HPPH peaks were completely separated (Fig. 4) with only a 6.5-min increase in the overall run time (DPH 17.5 min). We also detected three peaks that did not interfere with HPPH, PB or DPH in the *in vivo* plasma and whole blood samples having retention times of 4.4, 11.9 and 13.0 min when using the modified mobile phase. These peaks were



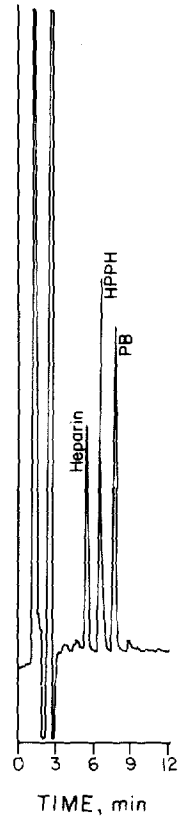


Fig. 3. Effect of the mobile phase pH on the capacity factors of phenobarbital, phenytoin and *p*-hydroxyphenytoin using a 32:68 (v/v) mixture of acetonitrile and phosphate buffer at a flow-rate of 1.2 ml/min.

Fig. 4. Chromatogram of a 10-μl injection of the extract of 1 ml of heparinized plasma from a rat previously treated with phenobarbital. The residue was reconstituted with 300 μl of methanol and was found to contain 13.2 μg/ml of phenobarbital. A 28:72 (v/v) mixture of acetonitrile and phosphate buffer was used as the mobile phase.

found to have originated from the sodium heparin venoject tubes used to collect the samples. These extraneous peaks would not be expected in the analysis of serum samples.

CONCLUSIONS

The accuracy, ease of sample preparation and short analysis time of the assay demonstrate that it is a useful method for the routine determination of PB, DPH and HPPH in plasma and various tissues of the rat. After 4 months of regular use in our laboratory, the assay has performed well. The same column has been used for assay development and sample analysis over a 7-month period with no apparent decrease in performance. Even though our developmental work has been limited to rat tissues, we feel that the assay could be easily adapted to other animal tissues on which pharmacokinetic studies are being conducted.

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