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Journal of Chromatography B, 693 (1997) 407–414

JOURNAL OF CHROMATOGRAPHY B

Simultaneous rapid high-performance liquid chromatographic determination of phenytoin and its prodrug, fosphenytoin in human plasma and ultrafiltrate

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Received 28 May 1996; revised 19 December 1996; accepted 31 December 1996

Abstract

A reversed-phase high-performance liquid chromatographic assay for the simultaneous determination of phenytoin and fosphenytoin, a prodrug for phenytoin, in human plasma and plasma ultrafiltrate is described. For plasma, the method involves simple extraction of drugs with diethyl ether and evaporation of solvent, followed by injection of the reconstituted sample onto a reversed-phase C_{18} column. Plasma ultrafiltrate is injected directly into the HPLC column. Compounds are eluted using an ion-pair mobile phase containing 20% acetonitrile. The eluent is monitored by UV absorbance at 210 nm. The fosphenytoin standard curves are linear in the concentration range 0.4 to 400 μg/ml for plasma and 0.03 to 80 μg/ml for ultrafiltrate. Phenytoin standard curves are linear from 0.08 to 40 μg/ml for plasma and from 0.02 to 5.0 μg/ml for ultrafiltrate. No interferences with the assay procedure were found in drug-free blank plasma or plasma ultrafiltrate. Relative standard deviation for replicate plasma or ultrafiltrate samples was less than 5% at concentrations above the limit of quantitation for both within- and between-run calculations.

Keywords: Phenytoin; Fosphenytoin

1. Introduction

Fosphenytoin, the disodium phosphate ester of 3-hydroxymethyl-5,5-diphenylhydantoin, is a newly developed prodrug for the parenteral administration of phenytoin [1]. Following either intramuscular (i.m.) injection or intravenous (i.v.) infusion, fosphenytoin is converted to phenytoin by blood and tissue phosphatases with approximately 100% bioavailability (Fig. 1) [2,3]. The greater water solubility of fosphenytoin alleviates many of the problems associated with phenytoin administration. Fosphenytoin offers improved compatibility with commonly used i.v. fluids, decreased irritation and phlebitis at the infusion site, reduced complications following extravasation and administration at infusion rates up to three times the maximum rate for phenytoin (150 mg-PHT equivalent/min for FOS vs. 50 mg PHT/min) [1,4]. In contrast to the prolonged and erratic absorption of intramuscularly administered phenytoin, fosphenytoin is completely and

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PII S0378-4347(97)00057-1

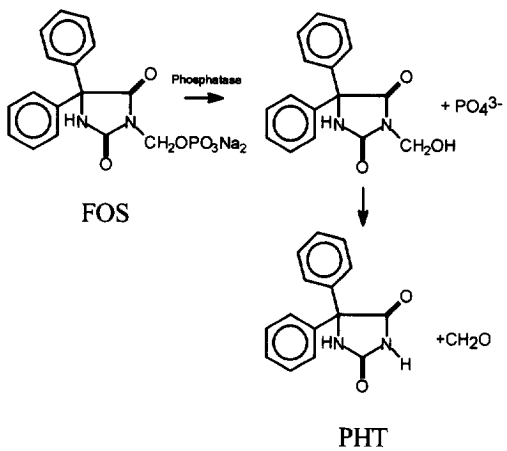

Fig. 1. In vivo conversion of fosphenytoin to phenytoin.

reliably absorbed following i.m. injection, producing minimal pain or irritation at the injection site [1].

Because fosphenytoin has no significant pharmacological activity of its own, the clinical effects following its administration are due to the concentration of derived phenytoin [1–4]. There are two considerations in monitoring plasma phenytoin concentrations during fosphenytoin therapy: (1) the potential of circulating fosphenytoin to interfere with the commonly used immunoassays for phenytoin and (2) the displacement of phenytoin from plasma protein binding sites by fosphenytoin [3–5]. To solve the first problem, an alternative method to immunoassays should be used to monitor phenytoin levels. The second problem can be solved by monitoring not only total plasma phenytoin concentration, but also the concentration of unbound phenytoin during the period when there are significant concentrations of fosphenytoin.

We present an HPLC assay for the simultaneous determination of fosphenytoin and phenytoin in human plasma and plasma ultrafiltrate. The method is simple, fast and applicable to both patient monitoring and pharmacokinetic studies.

2. Experimental

2.1. Materials

Fosphenytoin (FOS, Lot No. PD135711-0015B), was a gift from Parke-Davis (Ann Arbor, MI, USA). Phenytoin (PHT) and 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH), the internal standard (I.S.), were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile, HPLC grade, phosphoric acid, analytical reagent grade and ethyl ether, reagent A.C.S., anhydrous were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tetrabutylammonium hydrogen sulfate (TBA), AR was from Sigma (St. Louis, MO, USA). Deionized water was obtained as needed from a Barnstead Nanopure II system (Fisher Scientific). TDx fluorescence polarization immunoassays for phenytoin and free phenytoin were obtained from Abbott Laboratories (North Chicago, IL, USA). Centrifree micropartition devices with YMT membranes (Amicon, Beverly, MA, USA) were used to obtain plasma ultrafiltrate. Blood was collected from normal volunteers into Vacutainer (Becton Dickinson, Rutherford, NJ, USA) tubes containing EDTA.

2.2. Standard solutions

2.2.1. Plasma

Because of the conversion of FOS to PHT by phosphatases present in plasma, separate calibration curves and controls were prepared for each compound in order to monitor any degradation during storage or workup. The stock solution of FOS (4.0 mg/ml) was prepared in deionized water. Aliquots of 5 ml of pooled human blank plasma collected into tubes containing EDTA were spiked with various quantities of stock solution. Plasma standard samples were vortex mixed and stored at −20°C until use. The standard curves of FOS in plasma were made up of 11 calibrators with concentrations ranging from 0.4 to 400 μg/ml.

PHT stock solution (400 μg/ml) was prepared in acetonitrile. A 1-ml volume of this solution was evaporated to dryness under a stream of nitrogen gas at 40°C and reconstituted with 10 ml of pooled drug-free human plasma by shaking for 1 h on a horizontally reciprocating shaker at 120 cycles per min (cpm) and then sonicating for 30 min to guarantee full dissolution. Standards were prepared by serial dilution of this sample and stored at −20°C until use. The PHT standard curves in plasma were composed of 10 calibrators ranging from 0.08 to 40 μg/ml.

The internal standard was prepared by diluting 3.0

ml of MPPH stock solution (1.0 mg/ml in acetonitrile) to 100 ml with deionized water and kept refrigerated until use.

2.2.2. Ultrafiltrate

Because of the difficulty in obtaining large amounts of protein free human plasma water, standards for ultrafiltrate samples used to determine the protein binding of FOS and PHT were prepared in Krebs buffer which approximates the contents of plasma ultrafiltrate. They were prepared in the same manner as the plasma standards. Krebs' buffer consisted of 5.19 g NaCl (88.8 m*M*), 0.35 g KCl (4.73 m*M*), 0.185 g $CaCl_2 \cdot 2H_2O$ (1.27 m*M*), 0.34 g KH_2PO_4 (2.50 m*M*), 0.286 g $MgSO_4 \cdot 7H2O$ (1.18 m*M*) and 2.09 g $NaHCO_3$ (24.9 m*M*), dissolved in 950 ml deionized water, adjusted to pH 7.4 with 0.1 mol/l HCl and brought to 1000 ml with deionized water. The standard curves of FOS in Krebs' buffer consisted of 11 calibrators from 0.03 to 80 μg/ml. PHT curves consisted of 9 calibrators ranging from 0.02 to 5.0 μg/ml.

2.3. Extraction procedure

To 0.1 ml of plasma sample (blank, standard, control or patient sample), add 0.1 ml of internal standard and 0.1 ml of 85% phosphoric acid. Vortex mix for 10 s and add 2 ml of diethyl ether. Shake on a horizontally reciprocating shaker for 20 min at 120 cpm. Centrifuge at 1300 *g* for 10 min. Transfer 1.5 ml of the supernate to a clean tube and evaporate under a stream of nitrogen gas at 40°C. Reconstitute the residue in 0.2 ml mobile phase and inject 50 μl into the HPLC system.

2.4. Free drug concentration

Centrifree micropartition system ultrafiltration membranes were used according to the manufacturer's instructions to separate free from protein bound drug. A 1-ml volume of plasma was added into the sample reservoir of the Centrifree system. The Centrifree system was then placed into a fixed angle centrifuge at 1000 *g* for 20 min at room temperature. Approximately 150 μl of ultrafiltrate was collected. A 50-μl volume was injected onto the HPLC column.

2.5. Instrumentation

The HPLC system consisted of an M510 pump controlled by a Model 680 gradient controller, WISP 712 autoinjector, a temperature control module and a Lambda Max Model 481 variable-wavelength detector (Waters Associates, Milford, MA, USA). Peak areas were calculated from the 0-1 V detector output using an HP-3359 laboratory automation system (Hewlett-Packard, Palo Alto, CA, USA).

The fluorescence polarization immunoassay (FPIA) for PHT was performed on a TDx analyzer (Abbott Laboratories) according to the manufacturer's instructions for both the plasma and free level concentrations.

2.6. Chromatography

Separations were performed using an HPLC column (150×3.9 mm I.D., 5 μm particle size, stainless steel) and Guard-Pak guard column packed with Resolve C_{18} (Waters Associates). The mobile phase was 20% acetonitrile in deionized water containing 0.005 *M* TBA as an ion-pair reagent. The measured pH of 1 l of mobile phase after addition of 0.8 ml of 85% phosphoric acid was between 2.2 and 2.5. Flow-rate was 2.0 ml/min. Column temperature was maintained at 30°C to assure reproducibility of retention times. Eluting peaks were monitored by UV absorbance at 210 nm.

3. Results and discussion

Concentration of PHT in patient samples is commonly measured by immunoassay. Because of the structural similarity of PHT and FOS, significant cross-reactivity occurs in the immunoassay, producing falsely elevated PHT levels when measured in the presence of FOS. This cross reactivity is nonlinear and the increase in measured PHT concentration will depend on the amount of both PHT and FOS present. Fig. 2A shows a graph of plasma concentrations of a patient who received FOS. FOS and PHT were measured by HPLC. The concentration of PHT measured by FPIA is also presented. The PHT concentration measured by FPIA is observed to be elevated compared to the HPLC concentration until 2 h after discontinuing FOS adminis-

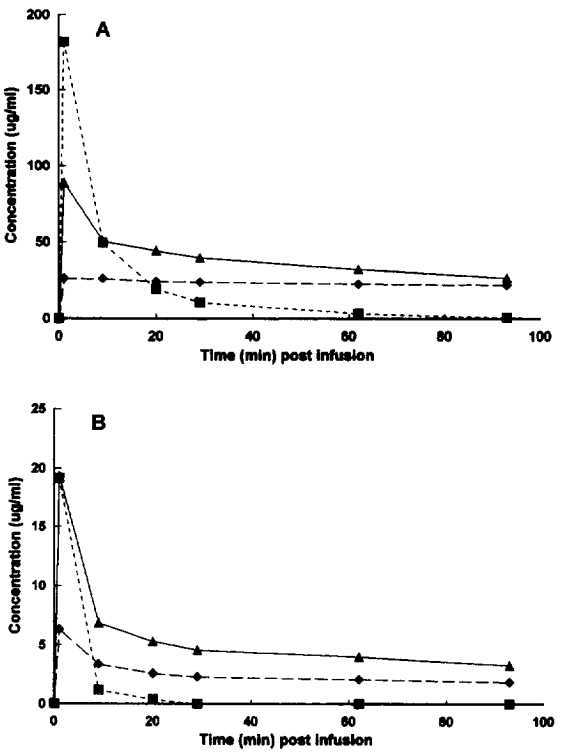

Fig. 2. (A) Plasma concentration of FOS measured by HPLC (■) and PHT measured by HPLC (♦) or FPIA (▲) after i.v. administration of fosphenytoin, 20 mg PHT-equivalent/kg at an infusion rate of 50 mg/min. (B) Ultrafiltrate concentration of FOS measured by HPLC (■) and PHT measured by HPLC (♦) or FPIA (▲) taken from the same patient.

tration. Kugler et al. observed similar results for the FPIA and to a lesser extent for the EMIT2000 immunoassays [5].

One other method is reported for the determination of FOS and PHT in human plasma [6]. It requires 1 ml of plasma which is extracted shortly after collection to prevent conversion of FOS to PHT. Separate extraction procedures and HPLC systems are required for FOS and PHT.

The method presented here requires 0.1 ml of plasma and can determine both FOS and PHT in a single analysis. Plasma samples can be frozen and stored for later analysis when blood is collected into tubes containing EDTA. Conversion of FOS to PHT was less than 1% at room temperature for 24 h or frozen at −20°C for 21 days.

A simple one-step extraction with diethyl ether followed by evaporation and reconstitution in mobile phase was used to analyze plasma samples. Absolute recovery of drug from plasma was determined by comparing extracted samples containing known amounts FOS and PHT to blank plasma spiked with the drugs after extraction. Recovery was 83% for FOS and 89% for PHT.

The ratio of peak area of drug to that of internal standard was used to quantitate both FOS and PHT. A weighted linear regression (weighting factor, 1/concentration) was used to fit peak-area ratio–plasma concentration data. Correlation coefficients were all greater than 0.999 for the individual curves of either FOS or PHT. The FOS calibration curve could be defined by the line $y=(0.0111\pm0.0005)x+(0.00764\pm0.005)$, while PHT had the line $y=(0.0315\pm0.0004)x+(0.0106\pm0.016)$.

The limit of detection ($S/N>2$) was 0.1 μg/ml for FOS and 0.02 μg/ml for PHT. The limit of quantita-

Table 1
Calculated concentration of fosphenytoin quality control samples prepared in human plasma

	Target concentration (μg/ml)			
	300	60.0	12.0	[a]0.403
Day 1	284	60.9	13.3	0.356
	272	62.2	13.9	0.354
	289	62.3	13.8	0.362
Day 2	290	62.6	13.3	0.348
	293	63.1	13.1	0.378
	292	61.2	12.7	0.378
Day 3	279	60.1	14.1	0.423
	286	60.3	13.9	0.432
	279	59.7	13.1	0.396
Day 4	278	59.5	13.5	0.318
	279	57.8	13.0	0.343
	279	55.6	13.0	0.358
Day 5	278	65.5	13.6	0.408
	284	63.0	13.7	0.387
	280	59.9	13.7	0.393
	280	62.6	13.4	0.391
	283	61.9	13.6	0.409
n	17	17	17	17
Mean	283	61.1	13.5	0.378
S.D.	5.8	2.3	0.4	0.031
R.S.D. (%)	2.0	3.7	2.9	8.1
Accuracy (%)	94.2	101.8	112.1	93.9

[a] These values were determined on separate days from the remaining controls.

tion was the lowest concentration at which percent error and R.S.D. were <20%. For FOS this was 0.4 μg/ml and for PHT 0.08 μg/ml.

Quality control samples were prepared independently from calibrators by spiking blank plasma with known amounts of FOS or PHT. Tables 1 and 2 give the precision and accuracy for the determination of FOS and PHT extracted from quality control samples. The R.S.D. at higher concentrations for FOS ranged from 2.0 to 3.7% and accuracy from 94.2 to 112.1%. For PHT at higher concentrations, the precision ranged from 3.5 to 4.6% and accuracy from 95.7 to 98.5%.

With drugs such as PHT which are extensively bound to plasma proteins (~95% bound), small perturbations in bound fraction can cause significant changes in the concentration of the pharmacologically active free drug. Because circulating FOS displaces PHT from plasma proteins, standard measurement of total plasma PHT concentration does not give good indication of the active unbound PHT. Thus it is often relevant to monitor the unbound PHT [7]. The current assay is applicable to the determination of free FOS and PHT in plasma ultrafiltrate in addition to total plasma concentrations. Fig. 2B shows the unbound levels from the same samples as in Fig. 2A. FOS and PHT are both measured by HPLC. The higher unbound PHT levels in the earlier compared to later samples show the displacement of PHT from plasma proteins by FOS. The FPIA results for PHT are also presented. As observed for the total plasma concentrations, the immunoassay gives elevated levels of PHT.

The standard curve and quality control samples for plasma ultrafiltrate were prepared independently from each other in Krebs' buffer, which approximates plasma water. Free concentrations of PHT and FOS were determined in plasma ultrafiltrate obtained

Table 2
Calculated concentration of phenytoin quality control samples prepared in human plasma

	Target concentration (μg/ml)			
	29.4	14.7	7.3	[a]0.0795
Day 1	27.8	14.6	7.40	0.0764
	33.0	14.2	7.21	0.0778
	27.7	14.5	7.45	b
Day 2	27.5	15.0	7.57	0.0803
	28.0	15.2	7.53	0.0696
	28.2	14.5	7.64	0.0747
Day 3	27.6	13.9	7.22	0.0808
	28.0	14.8	7.14	0.0752
	28.3	13.9	7.08	0.0764
Day 4	27.0	14.6	7.01	0.0863
	27.6	14.1	6.95	0.0812
	28.3	14.0	7.21	0.0819
Day 5	27.5	13.9	7.11	0.0848
	27.8	13.8	6.89	0.0913
	28.3	13.7	7.10	0.0945
	28.0	13.8	6.98	0.0795
	27.8	13.5	6.79	0.0811
n	17	17	17	16
Mean	28.1	14.2	7.19	0.0807
S.D.	1.3	0.49	0.25	0.0063
R.S.D. (%)	4.6	3.5	3.5	7.8
Accuracy (%)	95.7	96.8	98.5	101.6

[a] These values were determined on separate days from the remaining controls.
[b] Sample processing error. Sample not included in statistical analysis.

Table 3
Calculated concentration of fosphenytoin quality control samples for plasma ultrafiltrate prepared in Krebs buffer

	Target concentration (μg/ml)			
	53.5	26.8	8.03	[a]0.0318
Day 1	54.0	27.1	8.15	0.0307
	54.0	27.1	8.14	0.0324
	54.2	27.1	8.15	0.0309
Day 2	54.0	27.1	8.15	0.0276
	54.0	27.1	8.15	0.0332
	54.0	27.1	8.14	0.0313
Day 3	54.0	27.1	8.15	0.0300
	54.0	27.1	8.14	0.0300
	54.0	27.0	8.09	0.0297
Day 4	54.6	27.3	8.26	0.0362
	54.6	27.3	8.29	0.0359
	54.7	27.3	8.26	0.0292
	54.8	27.4	8.26	0.0355
	54.7	27.4	8.29	0.0341
	54.7	27.4	8.22	0.0350
	54.6	27.3	8.30	0.0296
n	16	16	16	16
Mean	54.3	27.2	8.20	0.0320
S.D.	0.3	0.1	0.07	0.0027
R.S.D. (%)	0.6	0.5	0.8	8.5
Accuracy (%)	101.5	101.5	102.1	100.5

[a] These values were determined on separate days from the remaining controls.

Table 4
Calculated concentration of phenytoin quality control samples for plasma ultrafiltrate prepared in Krebs buffer

	Target concentration (μg/ml)			
	3.52	1.06	0.282	[a]0.0199
Day 1	3.53	1.06	0.290	0.0194
	3.54	1.06	0.288	0.0191
	3.54	1.07	0.295	0.0212
Day 2	3.55	1.07	0.292	0.0216
	3.54	1.07	0.292	0.0203
	3.54	1.06	0.293	0.0215
Day 3	3.55	1.07	0.291	0.0199
	3.54	1.07	0.290	0.0215
	3.53	1.06	0.295	0.0213
Day 4	3.51	1.05	0.296	0.0213
	3.52	1.05	0.281	0.0209
	3.51	1.06	0.282	0.0185
	3.52	1.05	0.283	0.0203
	3.51	1.05	0.28	0.0204
	3.52	1.05	0.283	0.0200
	3.52	1.05	0.281	0.0213
n	16	16	16	16
Mean	3.53	1.06	0.288	0.0205
S.D.	0.01	0.01	0.006	0.0010
R.S.D. (%)	0.4	0.8	2.0	4.6
Accuracy (%)	100.3	100.0	102.2	103.2

[a] These values were determined on separate days from the remaining controls.

using Centrifree micropartition filters. No binding of FOS or PHT to these filters was observed. Tables 3 and 4 give the precision and accuracy for the determination of FOS and PHT in quality control samples of human plasma ultrafiltrate. FOS R.S.D. in these samples ranged from 0.5 to 0.8% and accuracy from 101.5 to 102.1%. For PHT, R.S.D. ranged from 0.4 to 2.0% and accuracy from 100.0 to 102.2%. The limit of detection is 0.015 μg/ml for FOS and 0.01 μg/ml for PHT. The limit of quantitation is 0.03 μg/ml for FOS and 0.02 μg/ml for PHT. The R.S.D. of filtered samples of plasma containing both FOS and PHT is 0.4% for FOS and 0.3% for PHT.

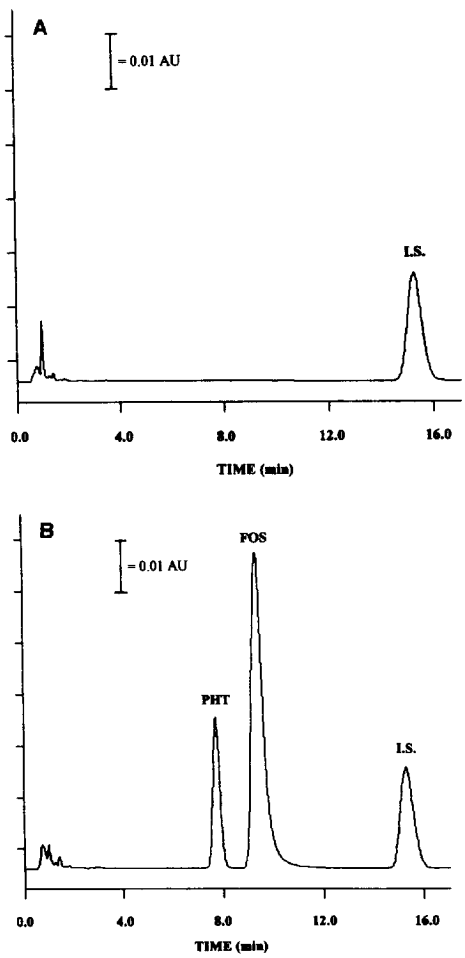

Fig. 3. Chromatograms of extract of patient plasma (A) before beginning fosphenytoin infusion and (B) 1 min after infusion taken from the patient shown in Fig. 2 (FOS concentration 182 μg/ml; PHT concentration 26.2 μg/ml).

Addition of TBA to the mobile phase causes the more polar FOS to elute after PHT and allows both compounds to be measured in a single injection. Retention of PHT and I.S. is unaffected by the presence of TBA. Retention times are 5.8, 8.8 and 11.3 min for PHT, FOS and I.S., respectively. A

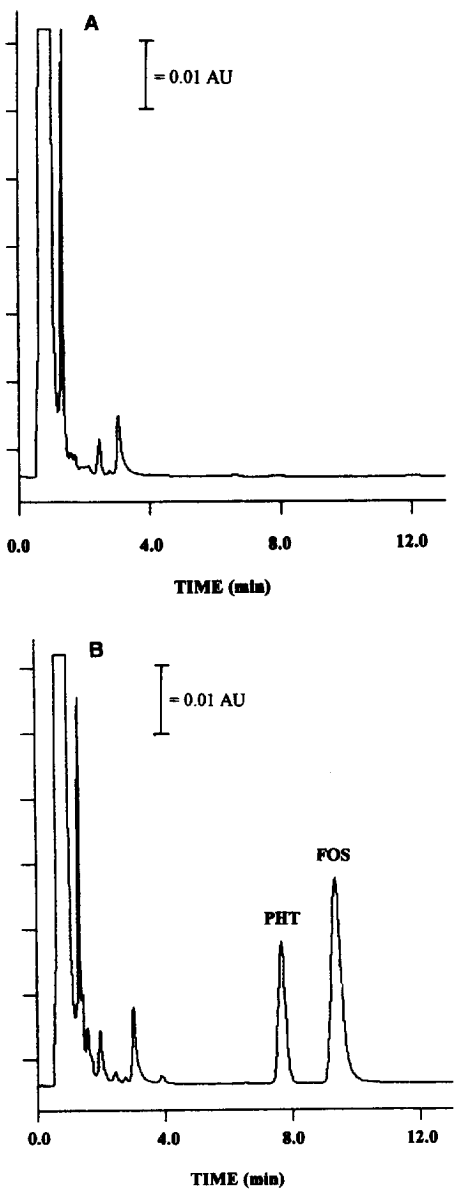

Fig. 4. Chromatograms of ultrafiltrate of patient plasma (A) before beginning fosphenytoin infusion (B) 1 min after infusion taken from the patient shown in Fig. 2 (FOS concentration 19.2 μg/ml; PHT concentration 6.3 μg/ml).

published method to determine FOS, PHT and degradation products in parenteral solutions has a retention time of 3 min for FOS and 25 min for PHT [8]. The current method has a run time approximately one half of this. Fig. 3 presents chromatograms of extracted blank plasma and a sample containing FOS and PHT, Fig. 4 presents chromatograms of the ultrafiltrate of these samples. Changes in mobile phase composition of up to 1% organic modifier, 0.3 pH units and 5% buffer concentration and changes in column temperature of up to 3°C do not affect the ability to separate FOS and PHT from interfering compounds.

No interferences were observed in extracted samples which contained any of the following drugs: carbamazepine, diazepam, digoxin, ethosuximide, gentamicin, lithium, lorazepam, phenobarbital, *N*-acetylprocainamide, primidone, procainamide, quinidine, theophylline and valproic acid.

4. Conclusion

The results show this method to be precise, accurate and specific for the simultaneous determination of phenytoin and its prodrug fosphenytoin in human plasma and plasma ultrafiltrate. The method is sufficiently sensitive for use in both patient monitoring and pharmacokinetic studies. The simple extraction step and short assay time allow use of this method for rapid screening of phenytoin samples after the administration of fosphenytoin when use of immunoassay techniques may be inappropriate. We have used this assay for several hundred samples over a period of four years and found the procedure to be simple, fast and easily transferable between instruments and analysts in our laboratory.

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