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Short communication

Phenytoin free fraction determination: comparison of an improved direct serum injection high-performance liquid chromatographic method to ultrafiltration coupled with fluorescence polarization immunoassay

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Abstract

Recent developments in restricted-access media (RAM) liquid chromatography make the simultaneous determination of total and free phenytoin concentrations possible by direct injection of drug-containing serum samples. A comparison of phenytoin free fraction determination by ultrafiltration coupled with fluorescence polarization immunoassay (TDX) to an improved direct injection RAM-HPLC method is presented. Our improved method differs from those previously reported with regard to column type, mobile-phase composition, and column temperature. Replicate samples analyzed by each method yielded similar values for serum phenytoin free fraction.

1. Introduction

Phenytoin is a phenylated hydantoin derivative used as an anti-epileptic to treat a multitude of seizure disorders. Total serum concentrations of phenytoin correlate with therapeutic efficacy as well as toxicity [1,2]. It is, however, the free (unbound) phenytoin moiety that is responsible for both the activity and toxicity of the agent. Routine monitoring of phenytoin serum concentration is performed in order to maintain therapeutic efficacy (seizure control) while minimizing such dose-related toxicities as ataxia, nystagmus, blurred vision, dysarthria, and drowsiness.

Phenytoin, a weak acid, is bound primarily to albumin in the plasma. The extent of protein binding is approximately 90% in volunteers with normal serum albumin concentration and in healthy patients on phenytoin monotherapy [3,4]. Conditions which affect serum albumin concentration also affect the degree of protein binding [5]. Alteration of phenytoin binding in uremia has also been described [6,7]. Recognition of diminished protein binding has led to increased

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monitoring of free phenytoin concentrations in this patient population as dosage adjustments based on total serum concentration may lead to higher free concentrations and potential toxicity [4,5,8,9].

Current analytical methods for determining phenytoin free fraction (equilibrium dialysis, ultrafiltration) require that separate analyses be performed to determine total and free concentrations [10]. Many laboratories involved in the routine clinical monitoring of phenytoin utilize ultrafiltration in conjunction with automated fluorescence polarization immunoassay (FPIA) [11]. While ultrafiltration-FPIA is a rapid, accurate, and established method, it nevertheless requires a separate centrifugation step and special filtration units to produce an ultrafiltrate containing the unbound species.

The recent development of restricted-access media (RAM) HPLC offers a unique alternative to ultrafiltration and equilibrium dialysis. RAM-HPLC permits the direct injection of drug-containing serum [12–14] or plasma [15] without damage to the analytical column. This is accomplished with a novel stationary phase that combines aspects of exclusion and reversed-phase chromatography [16,17]. A novel feature of RAM-HPLC is that bound and unbound drug can be determined simultaneously following direct injection of drug-containing serum. This technique has been used to determine serum free fractions for phenytoin [18,19], imirestat [18], warfarin [20], and indomethacin [20]. In our laboratory, however, scrupulous duplication of the methods by Pinkerton et al. [18] and Oshima et al. [19] yielded poor resolution of bound and unbound phenytoin peaks which in turn resulted in underestimation of phenytoin free fraction.

In this report we describe an improved direct injection RAM-HPLC method for determining phenytoin free fraction in normal human serum and compare it to an established ultrafiltration-FPIA procedure. Our improved method differs from those previously reported with regard to column type, mobile-phase composition, and column temperature.

2. Experimental

2.1. Materials

Sodium phenytoin was purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol and tetrahydrofuran were purchased from Burdick and Jackson (Muskegon, MI, USA). Deionized water was obtained from a Milli-Q Plus ultra pure water system purchased from Millipore (Bedford, MA, USA). Drug-free human serum was obtained with informed consent from healthy volunteers.

2.2. Instrumentation and chromatographic conditions

A component HPLC system (Shimadzu Scientific, Columbia, MD, USA) consisted of a LC-600 solvent delivery system, a Model 7125 manual injector with 1-ml sample loop, and a Model SPD-6A UV absorbance detector operated at 254 nm. A prepacked 15 cm × 4.6 mm I.D. RAM-HPLC column and guard column each with 5 μm diameter silica particles containing monomeric glycine-phenylalanine-phenylalanine as the internal surface bonded phase (GFF-II-S5-80) (Regis, Morton Grove, IL, USA) was operated with a mobile phase consisting of phosphate buffer (0.0125 *M*, pH 7.4) and tetrahydrofuran (99:1, v/v). The mobile phase was continuously sparged with helium and delivered at a flow-rate of 1.0 ml min. Column temperature was maintained at 37°C with a Model CTO-6A column oven (Shimadzu). Detector output was recorded and chromatograms analyzed by a CR-601 Chromatopac recorder/integrator (Shimadzu).

2.3. Sample preparation

Stock solutions of sodium phenytoin (1 mg/ml) were prepared in methanol. Drug-free human serum was spiked with sodium phenytoin to achieve phenytoin concentrations of 10, 15, 20, and 30 μg/ml. Aliquots of 1 ml were vortex-mixed for 30 s and maintained in a water bath for 1 h at 37°C before analysis. Ten replicate samples

were prepared for each concentration. Each 1-ml sample was divided in half and phenytoin free fraction determined by both ultrafiltration-FPIA and RAM-HPLC. Owing to the large injection volumes utilized in this assay, each aliquot of 500 μl serum was centrifuged for 3 min at 15 600 *g* rpm using an Eppendorf Model 5414 microcentrifuge (Brinkman, Westbury, NY, USA) to prevent the introduction of particulate matter into the LC system. A 400-μl volume was manually injected onto the GFF-II column. For RAM-HPLC, free fraction was calculated from the quotient of free and total (free + bound) phenytoin peak areas. Standard curves covering the concentration range of 5 to 40 μg/ml were prepared in drug-free human serum and analyzed by RAM-HPLC. Peak-area sums (bound + free) were used for quantitative computations. Calibration curves were calculated by least-squares linear regression analysis using a commercial software package (DeltaGraph, Monterey, CA, USA). Accuracy and precision of the method were determined by replicate analysis of four known concentrations equally divided over the calibration curve.

2.4. Ultrafiltration and fluorescence polarization immunoassay (FPIA)

The other 500-μl aliquot of each divided sample was analyzed for total and free phenytoin by an automated fluorescence polarization immunoassay system (TDX System, Abbott Diagnostics, North Chicago, IL, USA). Separation of bound and unbound phenytoin fractions was accomplished by centrifugation (Biofuge Model 17-R, Baxter Scientific Products, McGaw Park, IL, USA) at 1726 g and 37°C for 10 min using a fixed-angle rotor and the Centrifree micropartition system (Amicon, Beverly, MA, USA). The serum ultrafiltrate was then analyzed for free phenytoin. Instructions for the analysis of total and free phenytoin as outlined by the TDX Assay Manual (Abbott Diagnostics) were scrupulously followed. Free fraction was calculated from the quotient of free and total phenytoin concentration. Percent deviation in free fraction determination for the two methods was calculated using the following formula:

$$\%\,\text{Error} = \frac{ff_{(\text{RAM-HPLC})} - ff_{(\text{ultra-FPIA})}}{ff_{(\text{ultra-FPIA})}} \cdot 100$$

where $ff_{(\text{RAM-HPLC})}$ is the free fraction determined by RAM-HPLC and $ff_{(\text{ultra-FPIA})}$ is the free fraction determined by ultrafiltration-FPIA.

2.5. Statistical analysis

Mean phenytoin free fractions for each concentration were compared using a two-tailed *t*-test. *P*-values less than 0.05 were considered significant.

2.6. RAM-HPLC separation mechanism for bound/free phenytoin

Perry has recently detailed the chemistry and development of restricted-access media stationary phases [21]. The RAM stationary phase used in this study consists of porous silica particles whose outer surface has been rendered hydrophilic and thus will not adsorb proteins; moreover, the particle pore size is sufficiently small (5.2 nm diameter) to exclude most serum proteins. A covalently bound layer of monomeric glycine-phenylalanine-phenylalanine (designated GFF-II) produces a hydrophobic inner surface (pores) which remains accessible to low-molecular mass analytes. The result of RAM's unique chemistry is that drug-containing serum samples can be directly injected without harming the column. Large molecular mass serum proteins, including albumin, are excluded from the stationary phase and elute with the void volume. A wide variety of low-molecular mass analytes are retained by the hydrophobic interior and elute subsequent to the serum proteins. Serum volumes as great as 750 μl can be injected, repeatedly, without serious consequence. Furthermore, direct serum injection virtually eliminates the need for extensive sample pretreatment required for assays employing conventional reversed-phase columns. Separation of peaks cor-

responding to bound and free phenytoin is principally a function of injection volume, although mobile-phase composition and column temperature also contribute to peak resolution. Upon injection, serum samples containing phenytoin are diluted by the mobile phase and bound phenytoin is released. As the injection volume increases, the dilution effect is delayed and bound phenytoin is released later. The newly released phenytoin interacts with the reversed-phase moiety for a shorter period of time resulting in a retention time less than that of the original free fraction. This discrepancy in the period of stationary phase interaction gives rise to separate peaks corresponding to previously bound and free phenytoin.

3. Results and discussion

A 400-μl injection volume of phenytoin-containing human serum produced two well-resolved peaks with retention times of 14.5 and 17.5 min that corresponded to bound and free phenytoin, respectively (Figs. 1 and 2C). This finding is in agreement with previous studies of Pinkerton et al [18] and Oshima et al [19]. All endogenous serum components eluted prior to phenytoin (Fig. 2). The RAM-HPLC method was shown to be linear over the phenytoin concentration range of 5 μg/ml to 40 μg/ml in human serum, with a mean slope of 26.746 (S.D. = 0.611, $n = 5$) and a mean intercept of 2.769 (S.D. = 1.279, $n = 5$). Linear regression analysis of the sum of phenytoin peak areas versus spiked phenytoin serum concentration gave a correlation coefficient of 0.996 (range = 0.993 to 0.998, $n = 5$). Accuracy and precision of RAM-HPLC and ultrafiltration-FPIA methods for the determination of total phenytoin concentrations are presented in Table 1. Data from this table indicate that both methods were accurate and precise.

Several observations for achieving optimal peak separation were noted: (1) capacity (κ) was directly proportional to buffer concentration and indirectly proportional to organic modifier concentration, column temperature, and pH; (2) selectivity (α) was directly proportional to organic modifier concentration, column temperature, pH, and injection volume; (3) efficiency of

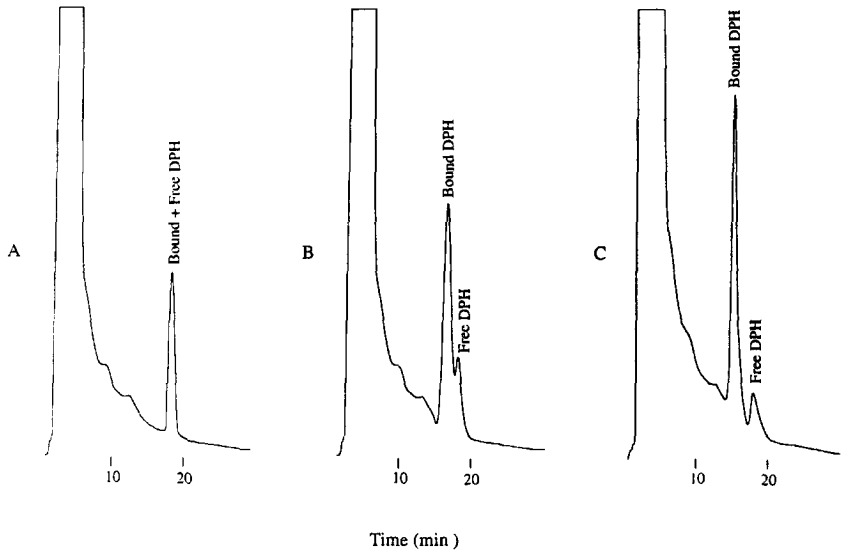

Fig. 1. Injection volume is the principal determinant in resolving bound and free drug fractions by RAM-HPLC. As injection volume increases, separate peaks corresponding to bound and free DPH become discernable. (A) Injection volume of 100 μl of 20 μg/ml DPH in human serum, (B) injection volume of 200 μl, (C) injection volume of 400 μl (AUFS = 0.04). DPH = phenytoin. AUFS = absorbance units full scale.

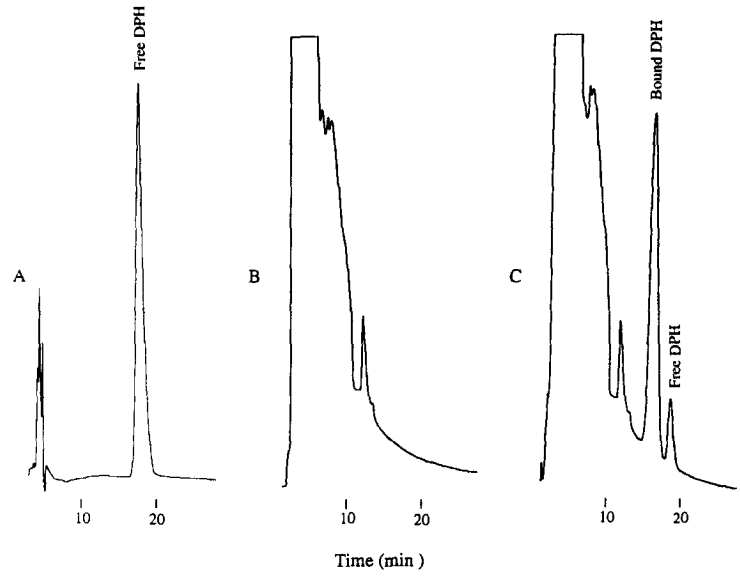

Fig. 2. By allowing drug-containing serum samples to be directly injected, RAM-HPLC precludes the need for extensive sample pretreatment and column-switching devices. (A) A 100-μl volume of 100 μg/ml DPH in mobile phase, (B) 400 μl blank human serum, (C) 400 μl of 30 μg/ml DPH in human serum (AUFS = 0.04). DPH = phenytoin. AUFS = absorbance units full scale.

the GFF-II column was much improved over the GFF type; (4) tetrahydrofuran was more effective than isopropanol or acetonitrile at resolving bound and free phenytoin.

Batch turnaround times for 10 samples were 30 min for ultrafiltration-FPIA and 4 h for RAM-HPLC. Comparison of phenytoin free fraction determinations by ultrafiltration-FPIA and RAM-HPLC are presented in Table 2. Values determined by RAM-HPLC differed significantly from those obtained by ultrafiltration-FPIA; however, this significance is not clinically relevant since means for therapeutic concentrations (10–20 μg/ml) were within the accepted normal range of 0.1–0.15 for determinations carried out at 37°C. In addition, predicted errors were less than 10%.

Changes in column stationary phase, mobile-phase composition, and column temperature distinguish our RAM-HPLC method from those previously published for determining phenytoin free fraction. Both Pinkerton et al. [18] and Oshima et al. [19] utilized RAM-HPLC columns whose internal surface was bonded with

Table 1
Accuracy and precision for the analysis of total phenytoin concentration in human serum

Theoretical concentration added (μg/ml)	HPLC			TDX		
	Measured[a] (μg/ml)	Accuracy (%)	R.S.D. (%)	Measured[a] (μg/ml)	Accuracy (%)	R.S.D. (%)
30.0	29.5 ± 0.71	98.3	2.41	29.7 ± 0.68	99.0	2.29
20.0	19.6 ± 0.81	98.0	4.13	19.7 ± 0.62	98.5	3.15
15.0	15.3 ± 0.99	102.0	6.47	15.1 ± 0.71	100.7	4.70
10.0	10.2 ± 0.80	102.0	7.84	9.9 ± 0.62	99.0	6.26

[a] Mean ± S.D., $n = 10$.

Table 2
Comparison of phenytoin free fraction as determined by ultrafiltration-FPIA and RAM-HPLC

Concentration added (μg/ml)	DPH free fraction[a]		Error (%)
	Ultrafiltration-FPIA	RAM-HPLC	
30.0	0.168 ± 0.005	0.152 ± 0.007[b]	− 10.52
20.0	0.111 ± 0.004	0.118 ± 0.004[b]	5.93
15.0	0.098 ± 0.007	0.106 ± 0.009[b]	7.55
10.0	0.095 ± 0.003	0.101 ± 0.005[b]	5.94

[a] Mean ± S.D. based on $n = 10$.
[b] $p \leq 0.05$, two-tailed t-test.

polymeric glycine-phenylalanine-phenylalanine (GFF). The recent development of monomeric glycine-phenylalanine-phenylalanine (GFF-II) as an internal surface bonded phase has greatly improved the retention and efficiency characteristics of these type of columns [21]. Our initial experience with the GFF type columns in determining phenytoin free fraction was that resolution of peaks corresponding to bound and free phenytoin was less than adequate, but upon switching to a GFF-II stationary phase both peaks were resolved. In addition, we found that peak resolution was best achieved on the GFF-II stationary phase with tetrahydrofuran as a mobile phase additive as opposed to acetonitrile [18] or isopropanol [19]. Furthermore, while previous investigators had performed separations at ambient temperatures, we found that operating the GFF-II column at 37°C not only improved efficiency and peak shape but reduced the retention of all eluted peaks, thus shortening sample run time. Finally, by determining phenytoin free fraction for both RAM-HPLC and ultrafiltration-FPIA at the physiological temperature of 37°C values are considered to be more clinically relevant [22].

4. Conclusion

An improved direct injection restricted-access media HPLC method for the simultaneous determination of bound and free phenytoin is presented. This novel method is comparable to ultrafiltration-FPIA for determining phenytoin free fraction. RAM-HPLC, although slower than FPIA in terms of batch sample turnaround time, has the potential to be a universal method for determining drug free fraction. In the future, RAM-HPLC may provide an alternative method to ultrafiltration and equilibrium dialysis in determining drug free fraction, especially for compounds that exhibit strong binding affinities for filtration and dialysis membranes.

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