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Simple and accurate high-performance liquid chromatographic method for the measurement of three antiepileptics in therapeutic drug monitoring

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ABSTRACT

A high-performance liquid chromatographic method for simultaneous determination of three antiepileptics, phenytoin, phenobarbital, and carbamazepine, in serum for therapeutic drug monitoring is described. The drugs were extracted and injected onto a silica-gel column using a syringe-type minicolumn, Extrashot-Silica™, packed with diatomaceous earth granules. We used dichloromethane for extraction–injection and *n*-hexane containing 0.2% acetic acid, 2% ethanol, and 15% dichloromethane for the mobile phase of a silica-gel HPLC. The eluent was monitored with a UV detector set at 240 nm. Linear relationships between the amount of drug and peak height were confirmed at 1–20 μg/ml in serum for carbamazepine and 5–40 μg/ml in serum for phenytoin and phenobarbital. When a 5-μl aliquot of serum was subjected to this method, the observed detection limits of the drugs were far less than therapeutic concentrations. Thus, our method was simple and accurate enough to be used in routine therapeutic drug monitoring and basic pharmacokinetic studies.

INTRODUCTION

It is commonly accepted that 75 per cent of all epileptic seizures can be prevented if epilepsy patients are properly treated with antiepileptics [1,2]. This involves oral administration of phenytoin (5,5-diphenylhydantoin, DPH), phenobarbital (PB), carbamazepine (CBZ), and, occasionally, sodium valproate in combination. It has been strongly recommended that individual maintenance doses of the antiepileptics should be determined on the basis of therapeutic drug monitoring (TDM) before patients are discharged from the hospital [3,4]. For this purpose, it is necessary to determine the blood concentrations of the drugs. Among the many methods practised, HPLC seems to be the best suitable for community hospitals because of its low cost for simultaneous multi-drug determinations. To simplify the method as much as possible, we used a syringe-type minicolumn [5] to perform extraction and injection simultaneously in the determination of DPH, PB, and

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CBZ. Our results were comparable to those obtained by the conventional enzyme immunoassay (EIA).

EXPERIMENTAL

Syringe-type minicolumn and chemicals

A syringe-type minicolumn (Extrashot-Silica) was obtained from Kusano Scientific (Tokyo, Japan). DPH, PB, and CBZ were purchased from Dainippon Pharm. (Osaka, Japan), Wako Pure Chem. (Osaka, Japan), and Sigma (St. Louis, MO, USA), respectively. Other analytical grade reagents and organic solvents were obtained from Wako Pure Chem.

Standard drug solutions

Stock solutions containing DPH, PB, or CBZ (100 μg/ml) were prepared in dichloromethane containing 1% ethanol. These solutions were further diluted with dichloromethane to produce the desired concentrations. All solutions were stored at 4°C until analysis.

HPLC apparatus

Our HPLC system consisted of a continuous-flow delivery system (BIP-I, Jasco, Tokyo, Japan), a UV detector (Uvidec-100V, Jasco), a syringe-loading sample injector with a 100-μl loop (Model 7125, Rheodyne, Cotati, CA, USA), and a single-pen recorder (RC-150, Jasco). Detector wavelength was set at 240 nm and at 0.01 or 0.005 AUFS. A conventional analytical column, 125 mm × 4 mm I.D., was packed with silica gel (LiChrosorb Si60, particle size 5 μm, Merck, Darmstadt, Germany).

Serum samples

Twenty-seven serum specimens were collected from epilepsy patients who were treated with a combination therapy using DPH, PB, and/or CBZ. Epileptic seizures were controlled successfully in all patients. Serum samples were separated by centrifugation at 1500 *g* for 10 min and stored at −40°C until use.

Optimization of extraction–injection solvent

The solvent composition for extraction–injection was optimized by monitoring the effluent from a diatomaceous earth column with an inner volume of 4.0 ml, which was 100-fold larger than that of the minicolumn. The column inlet was connected to the pump and the outlet to the detector through a line filter. Details of the optimization procedure have been described in our previous paper [6].

A 0.5-ml aliquot of distilled water was injected onto the column through the injection plug with a syringe. An appropriate mixture of dichloromethane and *n*-hexane presaturated with water was introduced as mobile phase and the column was preconditioned at a flow-rate of 1 ml/min before drug injection. A 10-μl volume of mobile phase solvent spiked with 10 μg of each drug was injected into the column through the injection plug. The eluent was monitored at 240 nm at a flow-rate of 1 ml/min. The solvents used were 30, 35, 40, 50, 60, 70, 80, and 90% dichloromethane in *n*-hexane (v/v), and dichloromethane. From the resulting chromatograms, we determined the most appropriate composition to achieve frontal extraction. Then, this composition was applied to the minicolumn using serum specimens to confirm the ability of the extraction–injection solvents to give the highest peak height and the best reproducibility in analytical chromatograms obtained with a mobile phase consisting of *n*-hexane containing 0.2% acetic acid, 2% ethanol, and 15% dichloromethane at a flow-rate of 1 ml/min.

Recovery and calibration

Since serum samples were as small as 5 μl, addition of an internal standard was neither possible nor necessary prior to the analysis. Direct peak-height calibration was successfully achieved by optimization of the extraction–injection solvent. Linearity of the concentration–peak height curve was tested over the range 5–40 μg/ml for DPH and PB, and 1–20 μg/ml for CBZ. These test drug concentration ranges were selected in accordance with their toxic, therapeutic, and trough concentrations usually observed in clinical treatment. The recovery of each drug from serum specimens was calculated by comparing peak heights obtained by direct injection and by injection through the minicolumn. Also, the accuracy of our quantifications was confirmed by

comparing our results with those obtained by conventional EIA.

RESULTS

Optimization of extraction–injection solvent

The elution profiles of the three antiepileptics from the diatomaceous earth column with an inner volume of 4.0 ml are shown in Fig. 1. The top of the extraction peak and the peak-width on the baseline were near a theoretical frontal elution curve when the dichloromethane fraction in the mobile phase solvent increased to over 90%. The theoretical plate numbers of the drugs at frontal elution were almost equal to those assessed with benzene as the substrate, *i.e.* around 100. Then, we tested the reproducibility of DPH, PB, and CBZ simultaneous extraction–injection at various concentrations using 5-μl serum specimens and an Extrashot attached to the HPLC system. Repeated peak-height measurements at 1–20 μg/ml for CBZ and 5–40 μg/ml for DPH and PB showed good reproducibility, with the highest peak height occurring when 130 μl of dichloromethane not containing *n*-hexane was used as solvent. This solvent volume, which was larger than that of the loop capacity of 100 μl, was explained by the fact that the excess solvent remained in the Extrashot with a void volume of *ca.* 30 μl. The relative standard deviation of extraction–injection recoveries as assessed by the HPLC peak-height ratio between direct injection and injection through the Extrashot was less than 6.5% at any concentrations tested and less than 4% at therapeutic concentrations (Table I). Inter-day variation was somewhat larger than the intra-day variation described in Table I. However, the inter-day variation could be eliminated by correcting the calibration curve at the test day before the analytical run.

Analytical procedures

The Extrashot was pre-conditioned by passing 200 μl of ethanol and dichloromethane through the whole device before use. Dichloromethane remaining in the device was pushed out with 500 μl of air, using a syringe. A 5-μl serum specimen was introduced onto the surface of the support material with a microsyringe. The Extrashot was then attached to the injector of HPLC system (Fig. 2). Dichloromethane (130 μl) was introduced gently into the injector through the Extrashot and HPLC was conducted in the usual manner with the mobile phase solvent, *i.e. n*-hexane containing of 0.2% acetic acid, 2.0% ethanol, and 15% dichloromethane, at a flow-rate of 1 ml/min. The UV detector was set at 240

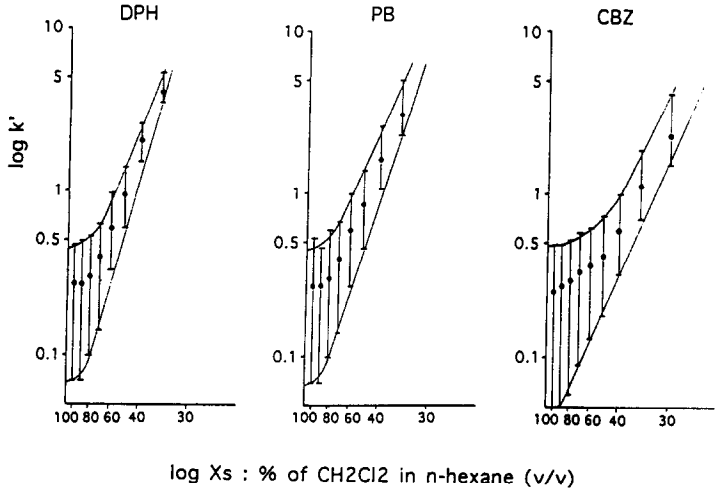

Fig. 1. Top of the extraction peak and peak width at various solvent compositions of dichloromethane and *n*-hexane at a flow-rate of 1 ml/min. The diatomaceous earth column used had an inner volume of 4 ml and a void volume of 3.5 ml. The theoretical plate number of the column, roughly estimated by a frontal peak of benzene, was approximately 100.

TABLE I

EXTRACTION RECOVERY OF ANTIEPILEPTICS FROM SERUM SPECIMEN AT VARIOUS CONCENTRATIONS ($n = 6$)

Drug	Concentration given (μg/ml)	Extraction recovery (%) (mean ± S.D.)	Coefficient of variation (%)
DPH	5	95.0 ± 6.17	6.49
	10	95.0 ± 3.22	3.39
	20	95.9 ± 3.01	3.14
	40	97.4 ± 1.57	1.61
Mean		95.9 ± 3.49	3.64
PB	5	98.3 ± 3.30	3.36
	10	97.8 ± 1.80	1.84
	20	96.9 ± 3.59	3.71
	40	97.2 ± 1.23	1.27
Mean		97.6 ± 2.55	2.61
CBZ	1	97.4 ± 3.85	3.95
	5	92.2 ± 1.28	1.39
	10	98.1 ± 2.29	2.33
	20	98.6 ± 1.99	2.02
Mean		96.6 ± 2.35	2.43

nm and 0.005 or 0.01 AUFS depending on the sample concentrations.

Calibration of serum concentrations

Fig. 3 depicts a typical chromatogram of a serum taken from an epilepsy patient after administration of DPH, PB, and CBZ. Retention times of PB, DPH, and CBZ were 7.2, 7.9, and 14.7 min, respectively. The capacity ratios, k', of the drugs, in the same order, were 4.81, 5.30 and 10.73. Complete baseline separation was achieved so that we were able to carry out direct peak-height calibration. Linear relationships between concentration and peak height were obtained at concentration of 1–20 μg/ml for CBZ and 5–40 μg/ml for DPH and PB. Triplicate analysis was carried out at each concentration to give calibration curves of $y = 3.02x - 1.89$ for DPH, $y = 4.21x - 2.05$ for PB, and $y = 11.62x - 1.89$ for CBZ, where y is peak height in mm at 0.01 AUFS and x, drug concentration in μg/ml of serum.

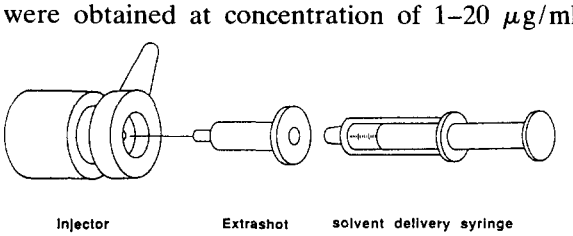

Fig. 2. An overall view of Extrashot-HPLC injection system. Extrashot, which had been pre-injected with a serum specimen, was attached to the injector and 130 μl of dichloromethane was introduced using a solvent delivery syringe for extraction–injection in combination.

Comparison between HPLC and EIA

To assess the accuracy of our HPLC assay, we compared our results with those obtained by EIA (EMIT, Daiichi Pure Chem., Tokyo, Japan). Results illustrated in Fig. 4 showed a good correlation between the methods. This indicated that our simple HPLC method was able to accurately determine serum drugs at any concentration observed in clinical specimens.

DISCUSSION

EIA and fluorescence polarization immunoassay (FPIA) have been the most practical methods for TDM, including antiepileptics. However, as TDM has spread far and wide, simple and cost-efficient HPLC procedures have become desirable in community hospitals. Sophisticated

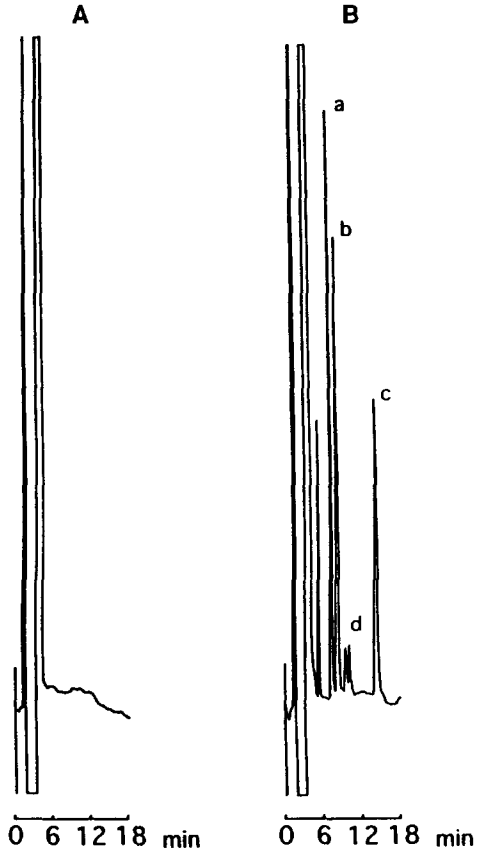

Fig. 3. Typical chromatogram of serum antiepileptics. Chromatogram A represents the result of blank analysis before drug administration and chromatogram B, the result following administration of three antiepileptics, their concentrations being: a, PB (21.10 μg/ml); b, DPH (19.01 μg/ml); c, CBZ (6.8 μg/ml); d, unknown peaks.

support materials developed first by Pinkerton *et al.*, can eliminate the tedious clean-up procedures used in biofluid treatment [7–9]. The syringe-type minicolumn we developed has also simplified the time consuming pretreatments [5].

One drawback of the Pinkerton column may be that baseline separation is difficult because of its void-peak spreading caused by complete elimination of the clean-up process. The most characteristic feature of our method is optimization of the extraction–injection solvent composition with the least polarity possible. In the present study, the retention characteristics of antiepileptics were assessed on a diatomaceous earth support in the extraction column. The changes in the capacity ratios and peak widths in accordance with the polarity of the water-insoluble mobile phase solvents gave the most appropriate solvent system for condensing the three antiepileptics into a minimum volume of solvent with the least polarity possible (Fig. 1). With such a solvent system, more polar components can be eliminated. The results shown in Fig. 1 clearly show that dichloromethane, or dichloromethane containing a small amount of *n*-hexane, is the extraction solvent with the least polarity possible. By using these solvents, an extraction peak-width of less than one k' unit at the frontal area was experimentally attained using a minimum volume of solvent. In the next stage, we examined the peak-height reproducibility of serum samples, deciding to use dichloromethane as the extraction–injection solvent.

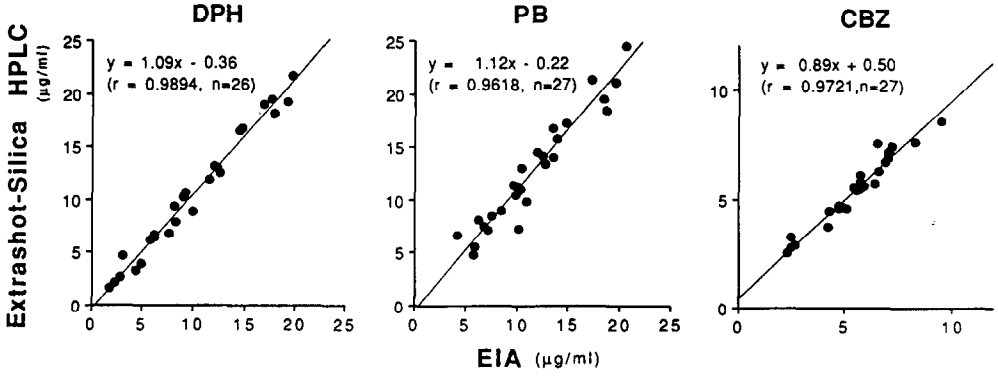

Fig. 4. EIA *vs.* Extrashot-Silica HPLC for the three antiepileptic serum concentrations in epileptic patients.

In many cases, the trial-and-error selection of an extraction solvent has been difficult. However, extraction monitoring by setting up a rapid-flow fractionation column system enabled us to select the most proper solvent for critical frontal extraction, eliminating other polar contaminants which disturb baseline separations. Using this technique, we have optimized the extraction solvents for glucocorticoids [6,10], testosterone [11], progesterone [12], estrogens [12], cyclosporin A [13], theophylline [5,14], and ketoprofen [15] so far. The present study and our previous ones [5] have clearly shown that the solvent system optimized by larger scale experiments can be reproduced in the syringe-type minicolumn with a one-hundredth inner volume of 45 μl. With this system, over 500 analyses have been performed without any noticeable change in analytical column efficiency.

The analytical accuracy of our method was finally confirmed by comparison with EIA. As shown in Fig. 4, serum concentrations of the three antiepileptics obtained by the two methods were in satisfactory correlation. Our method gave slightly higher concentrations of DPH and PB and slightly lower concentrations of CBZ as compared to EIA. However, there was no substantial difference between the two in the practical sense of clinical applications for TDM.

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