ELSEVIER

Journal of Chromatography B, 695 (1997) 349–353

JOURNAL OF CHROMATOGRAPHY B

Extrashot-ODS, a syringe-type minicolumn sample injector for a reversed-phase high-performance liquid chromatographic column Application to antiepileptics in human sera

Yukimitsu Kouno[a], Chiyoji Ishikura[a], Masato Homma[b], Kitaro Oka[b,*]

[a]*Bohsei Pharmacy Co., 2-1-28 Sakuradai, Isehara, Kanagawa 259-11, Japan*

[b]*Department of Clinical Pharmacology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1, Horinouchi, Hachioji, Tokyo 192-03, Japan*

Received 16 January 1997; revised 3 March 1997; accepted 3 March 1997

Abstract

Extrashot-ODS (EXS-ODS) is a syringe-type minicolumn developed for sample injection into reversed-phase high-performance liquid chromatographic columns. EXS-ODS consists of (a) a stainless-steel needle fitted to an ordinary syringe-loading sample injector for HPLC, (b) a 45-μl minicolumn tube made of polytetrafluoroethylene (PTFE) and packed with ODS-silica and (c) a minicolumn holder made of polystyrene, which is connected to the needle on one side and the other side is shaped so as to be fitted with a solvent syringe. Using the device, we simultaneously analyzed three antiepileptics in 20 μl of human sera. First, we introduced a 20-μl serum specimen diluted with 100 μl of buffer solution into the device and, second, 100 μl of distilled water. Then the device was attached to the HPLC injector and 130 μl of methanol were introduced into the HPLC column through the device. Then, reversed-phase HPLC was conducted in the usual manner, with the chromatogram reading at a wavelength of 210 nm for the assays of 5,5-diphenylhydantoin, phenobarbital and carbamazepine. The results obtained by direct peak-height calibration were comparable to those given by the immunological method. © 1997 Elsevier Science B.V.

Keywords: Extrashot-ODS sample injector; 5,5-Diphenylhydantoin; Phenobarbital; Carbamazepine

1. Introduction

We have proposed a direct sample injection technique for practical use of Extrashot-Silica (EXS-Silica, Kusano Scientific, Tokyo, Japan), which is a syringe-type minicolumn consisting of 45 μl diatomaceous earth granules [1–4]. With column size minimized, an organic extract can be transferred directly into an ordinary syringe-loading sample injector for normal-phase high-performance liquid chromatography (HPLC). Clean-up of biofluids becomes much easier and more accurate when organic extraction–injection solvent is optimized to be of minimal polarity for the target substance(s). Optimization of solvent polarity was achieved satisfactorily by the rapid-flow fractionation (RFF) technique, which is high-performance column extraction using naked diatomaceous earth granules as the support material [1,4].

One drawback found in the practical use of EXS-Silica was that the device did not work at all for reversed-phase chromatography. This is because of

*Corresponding author.

PII S0378-4347(97)00169-2

the water-immiscible organic solvent for extraction–injection, resulting in phase-destruction in the aqueous mobile phase of reversed-phase HPLC. To overcome these difficulties, we used ODS-silica instead of naked diatomaceous earth as the support material in minicolumns. A new device, called Extrashot-ODS (EXS-ODS), consists of a stainless-steel needle, a 45-μl minicolumn tube packed with ODS-silica and a minicolumn holder. We applied the new device to determining low concentrations of three antiepileptics in human sera from epilepsy patients by HPLC. The results of our method were reconfirmed by comparison with conventional enzyme immunoassays (EIA).

2. Experimental

2.1. EXS-ODS apparatus

Fig. 1A provides a full view of Extrashot-ODS (EXS-ODS). It is composed of three parts: (a) A stainless-steel needle fitted to an ordinary syringe-loading sample injector for HPLC, (b) a 45-μl minicolumn tube made of polytetrafluoroethylene (PTFE) and packed with 50 μm ODS-silica (Asahi Chemicals, Tokyo, Japan) and (c) a minicolumn holder made of polystyrene, which is connected to the needle on one side and the other side is shaped to fit a tuberculin test glass syringe for solvent delivery. Both ends of the support material, ODS-silica, are capped with cellulose filter tips. The sizes of these parts are illustrated in Fig. 1A. The packed tube is for clean-up of diluted serum specimens by retaining sample extracts on the surface of the ODS-silica. The needle is for introducing the sample extracts to the syringe-loading sample injector for HPLC.

2.2. HPLC apparatus

Our HPLC system consisted of a continuous-flow delivery pump (BIP-1, Jasco, Tokyo, Japan), a UV detector (Uvidec-100V, Jasco), a manual injection valve (Model 7125, Rheodyne, Cotati, CA, USA) with a 100-μl loop, and a single pen recorder (RC-150, Jasco). The wavelength of the detector was set at 210 nm and the absorbance unit full scale detection was set at 0.01 or 0.005. The analytical

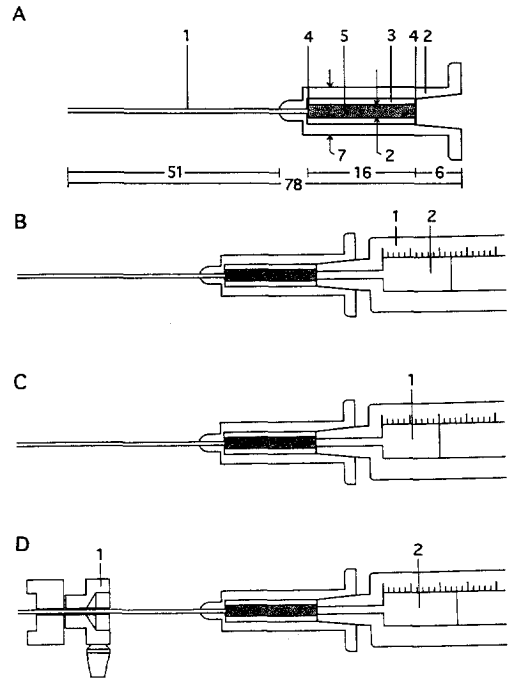

Fig. 1. (A) Overall view of the EXS-ODS™. 1=Application needle; 2=minicolumn holder made of polystyrene; 3=minicolumn tube made of PTFE; 4=filter; 5=support material, ODS-silica. Sizes are in mm. (B) Diluted serum sample introduction with a tuberculin test glass syringe. (C) Minicolumn washing with distilled water. (D) Injection into the HPLC system with the EXS-ODS™.

column was packed with ODS-silica gel (Hibar LiChrosorb RP-18, particle size 7 μm, column size 250×4 mm I.D., Merck, Darmstadt, Germany).

2.3. Chemicals and materials

5,5-Diphenylhydantoin (DPH), phenobarbital (PB) and carbamazepine (CBZ) were purchased from Dainippon Pharmaceutical (Osaka, Japan), Wako Pure Chemicals (Osaka, Japan) and Sigma (St. Louis, MO, USA), respectively. Other inorganic reagents and organic solvents were of analytical grade and were obtained from Wako Pure Chemicals. Phosphate buffer solutions were prepared by dissolving KH_2PO_4 in distilled water, the pH being adjusted with phosphoric acid. EIA kits (EMIT) for the three antiepileptics were obtained from Daiichi Pure Chemicals (Tokyo, Japan).

2.4. Standard drug solutions

Stock solutions containing DPH, PB or CBZ (100 μg ml^{-1}) were prepared in methanol. These solutions were further diluted with methanol to produce the desired concentrations at 1–40 μg ml^{-1}. These concentrations ranged from non-effective to toxic concentrations of the antiepileptics at standard dosages. All solutions were stored at 4°C until analysis.

2.5. Serum samples

Twenty-eight peripheral blood samples, collected from epilepsy patients being treated with DPH, PB and/or CBZ combination therapy, were used to test the efficacy of the new device for practical use. Serum specimens of each blood sample were obtained by centrifugation at 1500 *g* for 10 min and stored at −40°C until use.

2.6. Procedures

First, the HPLC system was conditioned in the usual manner with a mobile phase solvent of methanol–acetonitrile–phosphate buffer, pH 4.4 (21:14:65, v/v) at a flow-rate of 1 ml/min at room temperature. The UV detector was set at 210 nm and 0.005 or 0.01 a.u.f.s., depending on sample concentrations. Second, a 20-μl volume of the serum specimen was diluted with 100 μl of phosphate buffer solution (pH 3.7) and the mixture was vigorously shaken using an electric mixer for 10 s. The resulting solution was introduced into the EXS-ODS using a tuberculin test glass syringe (Fig. 1B), followed by washing with 100 μl of distilled water (Fig. 1C). In the next stage, the EXS-ODS was attached to a syringe-loading sample injector for HPLC analysis and 130 μl of methanol were introduced gently (Fig. 1D). When we started our usual HPLC operation, the device was detached from the apparatus.

2.7. Recovery and calibration

The recovery rate of each antiepileptic from the serum specimens was calculated by the ratio of peak-heights obtained from direct injection of standard drug solutions and injection of serum specimens containing the same volume of standard drug solutions through the EXS-ODS. Direct peak-height calibration was successfully achieved from the optimized pH values of the buffered serum solutions and the type of extraction–injection solvent. Addition of an internal standard was not necessary prior to analysis. Linearity of the concentration–peak height curves was tested in the 1–40 μg ml^{-1} range for the three antiepileptics. The accuracy of our quantifications was confirmed by comparing our results with those obtained by conventional EIA.

3. Results

3.1. Recovery rates

The tested drug concentration range of 1–40 μg ml^{-1} was in accordance with the non-effective, therapeutic, and toxic ranges usually observed in clinical treatments. As shown in Table 1, mean recovery rates of the drugs from serum specimens were high enough to be quantified. Also, there was no considerable difference in the recovery rates at different concentrations. The relative standard deviations of the recoveries (coefficient of variation) were

Table 1
Extraction recovery of antiepileptics from a serum specimen at various concentrations

Drug	Concentration given (μg ml^{-1})	*n*	Extraction recovery (%) (mean±S.D.)	Coefficient of variation (%)
PB	1	6	92.6±3.09	3.34
	5	6	92.4±3.14	3.40
	10	6	93.6±3.60	3.85
	20	6	93.5±3.32	3.55
	40	6	94.9±3.24	3.41
	Mean	–	93.4±3.28	3.51
DPH	1	6	90.8±3.10	3.41
	5	6	91.0±3.09	3.40
	10	6	91.7±3.19	3.48
	20	6	92.2±2.90	3.15
	40	6	91.5±2.98	3.26
	Mean	–	91.4±3.05	3.34
CBZ	1	6	89.4±1.55	1.73
	5	6	90.1±1.60	1.78
	10	6	87.8±1.68	1.91
	20	6	88.4±1.47	1.66
	40	6	92.3±1.45	1.57
	Mean	–	89.6±1.55	1.73

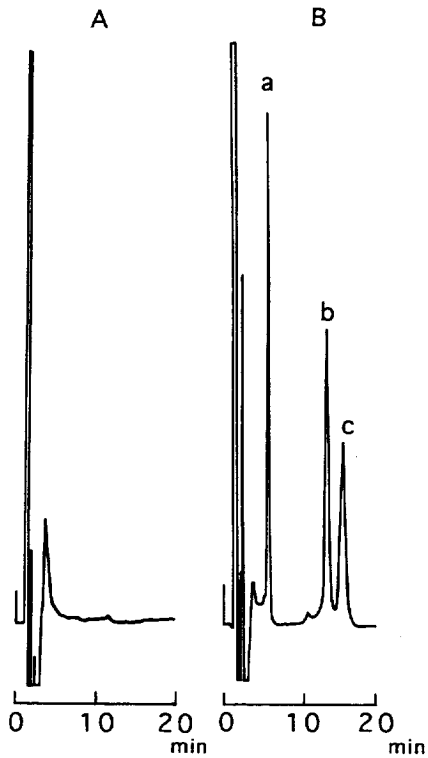

Fig. 2. Typical chromatogram of serum antiepileptics. Chromatogram A represents the result of blank analysis before drug administration and chromatogram B, the result following administration of three antiepileptics, their concentrations being; (a) PB (20.10 μg ml^{-1}); (b) DPH (19.81 μg ml^{-1}) and (c) CBZ (7.4 μg ml^{-1}).

less than 4%. As a result, we decided to conduct direct peak-height calibration rather than a method using an internal standard.

3.2. HPLC analysis

Fig. 2 depicts a typical chromatogram of a serum specimen taken from an epilepsy patient after simultaneous administration of PB, DPH and CBZ. The retention times of PB, DPH and CBZ were 6.5, 15.9 and 18.7 min, respectively. The capacity ratios, k', of the drugs (in the same order) were 4.17, 11.62 and 13.86. For direct peak-height calibration, we performed triplicate analyses of each standard serum specimen containing three antiepileptics at concentrations of 1–40 μg ml^{-1}. The calibration curves obtained were $y=14.64x-1.03$ for PB, $y=8.20x+0.22$ for DPH and $y=10.39x-0.71$ for CBZ, where y is the peak height (in mm at 0.01 a.u.f.s.) and x is the drug concentration (in μg ml^{-1} of serum). As shown in Fig. 2, we observed baseline distortions before peaks a and b. However, there was no difficulty achieving calibration of drug concentrations for clinical use. These distortions seemed to be from the relatively larger volume (130 μl) of injection solvent and not from contamination of the metabolites. To assess the accuracy of our HPLC assay, we compared our results with those obtained by EIA. The results illustrated in Fig. 3 showed a good correlation between the methods. This indicated that our simple

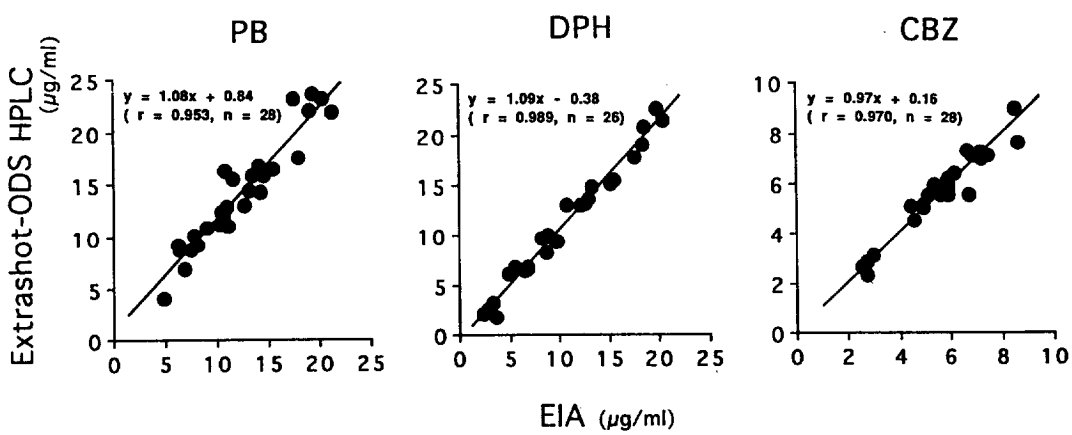

Fig. 3. EIA vs. Extrashot-ODS HPLC for serum concentrations of the three antiepileptics in epileptic patients.

HPLC method was able to accurately determine serum drugs at any concentration observed in clinical specimens. Finally, day-to-day variations in peak height can be easily corrected by injecting a standard drug solution once, prior to analytical runs.

4. Discussion

We have already developed EXS-Silica for direct serum (or plasma) injection into an ordinary syringe-loading sample injector of HPLC. Using this device, time-consuming pretreatment (or clean-up) procedures for normal phase HPLC have been improved dramatically, so that extraction and injection can be completed simultaneously, within 1 min. One drawback found in EXS-Silica was that the device could not be applied to reversed-phase HPLC. In this paper, we developed EXS-ODS by changing the support material from naked diatomaceous earth to ODS-silica granules. As a result, it became possible to use water-miscible solvents instead of water-immiscible ones for direct sample injection into HPLC columns operating with an aqueous mobile phase solvent system. Thus, the baseline distortion caused by phase-destruction due to solvent mismatch was minimized, allowing determination of the lowest concentrations with the highest reliability as shown in Figs. 2 and 3.

The newly developed EXS-ODS required optimization of experimental conditions for sample holding and sample release from the support material. There have been many studies dealing with disposable clean-up items, such as Sep-Pak cartridges, developed by Waters (Milford, MA, USA), and Extrelut, by Merck. Among them, coated silica gel works well for holding substrates in buffer solutions and then releasing them into aqueous organic solvents. The experimental conditions reported for many drugs could be applied to EXS-ODS procedures directly or after minor modification, if required. Generally, optimization of the conditions for EXS-ODS could be achieved by simply comparing observed peak heights between direct injection and injection through the device, as we have done in this paper with three antiepileptics.

EXS-ODS can be applied to any type of up-to-date HPLC model, with minor changes in the loop volume of the syringe-loading sample injector. Over 500 analyses have been performed with the EXS-ODS without any noticeable change in the efficiency of the analytical column.

The analytical accuracy of our method was finally confirmed by comparison with EIA, as shown in Fig. 3. Serum concentrations of the three antiepileptics obtained by the two methods correlated satisfactorily. Our method gave slightly higher concentrations of PB and DPH and slightly lower concentrations of CBZ, compared to EIA. However, there was no substantial difference between the two methods in the practical sense of clinical applications [5–7].

Acknowledgments

We are indebted to Kusano Scientific, Tokyo, Japan, for technical support.

References

[1] M. Homma, K. Oka, N. Takahashi, Anal. Chem. 61 (1987) 784–787.
[2] Y. Kouno, C. Ishikura, N. Takahashi, M. Homma, K. Oka, J. Chromatogr. 515 (1990) 321–327.
[3] M. Kanai, Y. Kouno, M. Homma, K. Yamada, K. Oka, J. Chromatogr. 567 (1991) 415–424.
[4] Y. Kouno, C. Ishikura, M. Homma, K. Oka, J. Chromatogr. 622 (1993) 47–52.
[5] A.G. Gilman, T.W. Rall, A.S. Nies and P. Talor, in Goodman and Gilman's The Pharmacological Basis of Therapeutics, Pergamon Press, New York, 8th Ed., 1990, pp. 436–462.
[6] L.Z. Benet, N. Massoud and J.G. Gambertoglio, Pharmacokinetic Basis for Drug Treatment, Raven Press, New York, NY, 1984.
[7] B. Johanson, J. Chromatogr. 381 (1986) 107–113.