Journal of Chromatography, 515 (1990) 321–327
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1815

Direct sample injection into the high-performance liquid chromatographic column in theophylline monitoring

YUKIMITSU KOUNO and CHIYOJI ISHIKURA
Bohsei Pharmacy Co., Isehara, Kanagawa 259-11 (Japan)
NORIYUKI TAKAHASHI
Department of Pharmacy; Tokyo Medical College Hospital, Shinjuku-ku, Tokyo 160 (Japan)
and
MASATO HOMMA and KITARO OKA*
Division of Clinical Pharmacology, Tokyo College of Pharmacy, Hachioji, Tokyo 192-03 (Japan)

ABSTRACT

A small diatomaceous earth column was used for direct sample injection into a high-performance liquid chromatographic (HPLC) column for theophylline monitoring. The original design of this device has been improved so as to make it easily usable and disposable following use. It consists of a diatomaceous earth-packed polystyrene tube, the inner surface of which is coated with PTFE, having a capacity of *ca.* 5 μl of plasma or serum sample, and a stainless-steel needle for introducing an extract into the HPLC sample injector. Using this device together with an optimized injection solvent mixture, an accurate determination of theophylline can be carried out at low cost. The results obtained were comparable to those given by an immunological method, such as fluorescence polarization immunoassay.

INTRODUCTION

A direct sample injection technique for high-performance liquid chromatography (HPLC) developed by Pinkerton *et al.*[1] has been applied to the determination of drug concentrations in human plasma[2–4]. Because of its complete elimination of clean-up procedures, it seems difficult to improve void-peak spreading for baseline separation. Disposable clean-up tools, such as Sep-Pak cartridges developed by Waters Assoc. (Milford, MA, U.S.A.) and Extrelut (Merck, Darmstadt, F.R.G.), have been widely used for conventional HPLC. However, no method has generally been available that could provide optimum conditions for extraction. Recently, a diatomaceous earth column has been developed for the extraction of various compounds in human biofluids. Optimizing the solvent polarity of binary mixtures for extraction[5–7] and compound determination by silica gel chromatography have become much easier and more accurate[8–13]. Recently, we have reported a syringe-type minicolumn for direct injection of plasma into HPLC columns[14]. This device, which we named Ex-

0021-9673/90/$03.50

trashot, rendered tedious sample pretreatment unnecessary. This paper describes an improved Extrashot which is designed to be disposable following use. It can be easily applied to therapeutic drug monitoring such as theophylline in asthmatic patients. The results obtained so far show close agreement with those given by conventional immunoassay.

EXPERIMENTAL

Extrashot apparatus

The newly designed Extrashot, shown in Fig. 1A, consists of the following components: 1, stainless-steel needle fitted to an ordinary syringe-loading sample injector such as a Rheodyne type; 2, minicolumn holder made of polystyrene; 3, minicolumn tube made of PTFE; 4, filter-papers; and 5, 45-μl minicolumn containing diatomaceous earth granules. The diatomaceous earth granules are prepared from

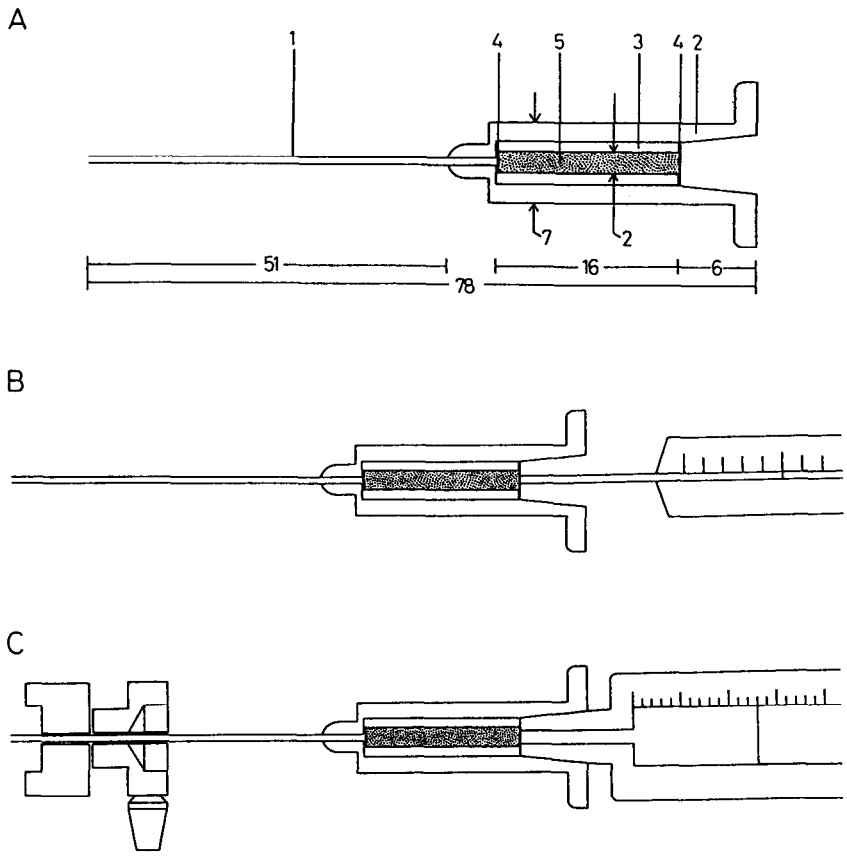

Fig. 1. (A) Overall view of the Extrashot. 1 = Application needle; 2 = minicolumn holder made of polystyrene; 3 = minicolumn tube made of PTFE; 4 = filter; 5 = support material. Sizes in mm. (B) Sample loading by a microsyringe. (C) Solvent introduction into the HPLC system through the Extrashot.

Celite powder No. 545 (Johns Manville, Denver, CO, U.S.A.) by precipitation in distilled water. The granule particle size is 50–100 μm and 1 ml of granules weighs about 0.58 g.

Sample loading

A 5-μl aliquot of plasma or serum is loaded onto the surface of the packed material through the filter-paper by a microsyringe as illustrated in Fig. 1B. The aqueous sample is absorbed and continuously retained by the granules. The sample as an aqueous phase occupies about 11% of the dead volume of the Extrashot.

Solvent introduction

After the sample has been loaded, the Extrashot was attached to an ordinary syringe-loading sample injector of the HPLC system (Fig. 1C), followed by a 130-μl portion of the extraction–injection solvent over a period of 5–10 s by a tuberculin test glass syringe.

HPLC

The HPLC system consisted of a continuous-flow solvent-delivery system (BIP-I; Jasco, Tokyo, Japan), a UV detector (Uvidec-100V; Jasco), a syringe-loading sample injector (Model 7125; Rheodyne, Cotati, CA, U.S.A.), and a single pen recorder (RC-150; Jasco). The sample injector was equipped with a 100-μl loop by which the extract from the device is loaded. The analytical column (125 mm × 4 mm I.D.) was packed with silica gel (LiChrosorb Si 60, particle size 5 μm) (Merck, Darmstadt, F.R.G.). This column was treated with 100 μl of 1% sulphuric acid and excess of distilled water until the effluent was neutral. Methanol and finally chromatographic solvent mixtures were then introduced. Sulphuric acid treatment was conducted once prior to first use so that the surface pH was slightly below 7, at which the theoretical plate number of acidic compounds such as theophylline exceeds 3000 per column. The solvent system for the theophylline determination was water–acetic acid–ethanol–dichloromethane (0.2:0.2:4:95.6, v/v) at a flow-rate of 1.0 ml/min. The UV detector was set at 275 nm for theophylline analysis.

Optimization of solvent for Extrashot

Binary solvent mixtures were used for extraction–injection by Extrashot. To attain critical frontal extraction of the target molecule, a strong solvent component such as ethanol and a non-polar component such as dichloromethane or *n*-hexane were used. The optimum solvent composition was determined by a preliminary experiment using a larger diatomaceous earth column of 4 ml inner volume and 0.5 ml of water, these dimensions being about 100 times those of the Extrashot. Using this column, a solvent composition *vs.* capacity factor curve was obtained to find a suitable solvent composition with minimum polarity by which the target molecule could be extracted and other polar compounds eliminated.

Calibration

Direct peak-height calibration was used. The amount of sample is small and, consequently, the addition of an internal standard is neither possible nor necessary prior to the analysis. Peak heights at various concentrations, such as 1, 5, 10, 20, and

30 μg/ml, gave the correlation equation $y = 0.0853x + 0.2040$ ($r = 0.9994$; $p < 0.001$), where y is the concentration of theophylline (μg/ml) and x is observed peak height (mm) at 0.02 a.u.f.s.

Solvents and reagents

Organic solvents and other reagents were of analytical-reagent grade and purchased from Wako (Osaka, Japan). Theophylline kit II (TDX) for fluorescense polarization immunoassay (FPIA) with an automated system was obtained from Dinabot (Tokyo, Japan).

RESULTS

The accuracy and efficiency of the device were assessed on the basis of plasma concentrations of theophylline. Test plasma mixtures at various concentrations were prepared from a blood sample from a healthy male volunteer.

Loading capacity of Extrashot

This parameter was found to be 5 μl when operating the device manually with a tuberculin test syringe for solvent delivery and varied according to the flow-rate, being higher at a slower rate. It was thus considered that, at an operating capacity of 5 μl, the aqueous phase would not leak out into the HPLC system.

Optimization of extraction solvent

The solvent composition *vs.* capacity factor curve shown in Fig. 2 was obtained using theophylline and a binary solvent mixture of ethanol and dichloromethane. Frontal extraction of theophylline from distilled water was successfully carried out when the ethanol content of the mixture exceeded 3%. Extraction–injection using plasma specimens containing theophylline at various concentrations was then observed. Repeated peak-height measurements at theophylline concentrations between 1 and 20 μg/ml showed good reproducibility with a 4% ethanol concentration. The relative standard deviation for peak height was less than 3.5% ($p < 0.001$) at any concentration, such as 1, 5, 10 and 20 μg/ml. A typical chromatogram is shown in Fig. 3.

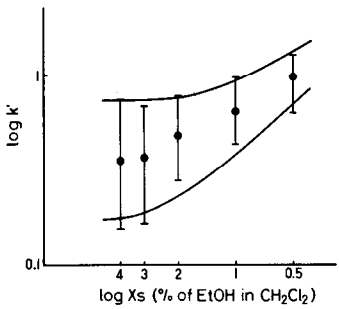

Fig. 2. Solvent composition (X_s; %, v/v) *vs.* capacity factors (k') and peak widths obtained with the diatomaceous earth column of 4 ml inner volume. The points indicate the top of the extraction peak and the bars the peak width. One k' unit corresponds to 4 ml of each solvent. EtOH = Ethanol.

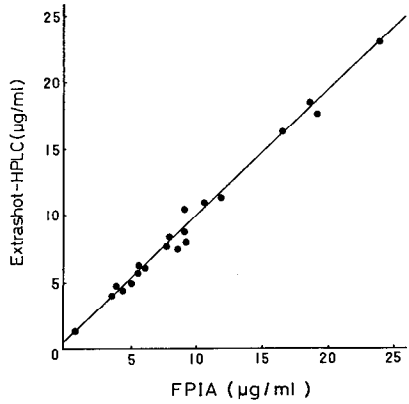

Fig. 3. Typical chromatogram of theophylline determination by Extrashot HPLC. The plasma specimen was obtained from an asthmatic patient. TP = theophylline corresponding to 5 μg/ml.

Fig. 4. Extrashot HPLC *vs.* FPIA for theophylline determination. $y = 0.9375x + 0.6240$ ($r = 0.9939$, $p < 0.001$).

Comparison of Extrashot HPLC with FPIA

This comparison showed the two methods to give essentially the same results, as is evident from Fig. 4. The results obtained by FPIA were about 1.07 times those given by Extrashot HPLC. In the therapeutic range of theophylline between 10 and 20 μg/ml, the results were comparable, FPIA being only about 3% higher.

DISCUSSION

Conventional experiments for selecting an extraction solvent for biofluids are usually performed on a trial-and-error basis. However, our extraction-monitoring procedure using a diatomaceous earth column constitutes a new method for optimizing a solvent system prior to HPLC determination[6]. A rapid-flow fractionation (RFF) column system set up by this extraction monitoring has been found to be clinically suitable for the monitoring of various drugs[8–13]. The use of combined extraction monitoring and RFF has been found to be ideal for optimizing extraction conditions such as solvent choice, control of solvent polarity, volume of solvent and extraction flow-rate.

With regard to solvent polarity for extraction, it is of primary importance that the polarity of the solvent mixture be the minimum necessary for extracting the target molecule and eliminating other polar compounds[6]. Adhering to this principle, critical frontal extraction has been successfully carried out with a minimum volume of extract. Further, a solvent can be easily introduced into an RFF column by nitrogen at a pressure as low as 1 kg/cm^2. An appropriate flow-rate as fast as 1 ml/s is possible

through the use of a gauge control. Hence the time required for sample treatment is considerably shortened. With the minimum column size, the desired flow-rate can be secured by manually operating the solvent-delivery syringe. These considerations served as the basis for the design of this improved Extrashot[14]. The outstanding features of this device are its ease of use and disposability following use.

The plasma- or serum-loading capacity of the Extrashot is as much as 5 μl. This volume of blood from asthmatic patients administered theophylline daily contains only 50 ng of this drug. This amount is ten times greater than the detection limit of our HPLC system. Therefore, in this study, the UV detector was operated at a sensitivity of 0.01–0.02 a.u.f.s., permitting the analysis of more dilute samples.

Solvent polarity was optimized so that a quantitative amount of the drug could be extracted and introduced into the 100-μl loop of the HPLC injector. The volume of eluent from the Extrashot was about 90–100 μl following the administration of a 130-μl aliquot of solvent. The theophylline recovery was 94 $\pm$ 3% at 1 μg/ml and 97 $\pm$ 3% at 20 μg/ml, being higher than 95% at therapeutic concentrations. The drug was contained in a minimum volume of solvent with the least possible polarity. Using this solvent system, more polar components in plasma specimens are prevented from being extracted, so that their elimination is thus achieved. It is for this reason that the chromatograms obtained were much cleaner and more accurate than those obtained by the usual pretreatment.

Day-to-day variations in peak height can be easily corrected by injection an authentic specimen once prior to an analytical run. The relative standard deviations for determinations at various concentrations were less than 5% and the results were in good agreement with those given by FPIA, as shown in Fig. 3.

CONCLUSION

The improved Extrashot is a disposable device for direct plasma extraction–injection with conventional HPLC. The method is convenient and inexpensive for therapeutic drug monitoring at a community hospital. The Extrashot should also be applicable for determining concentrations of other drugs in blood provided that at least 1 μg/ml is present.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Takashi Nitta for assistance with Extrashot conditioning and Mrs. Sachiko O'hara for the preparation of the manuscript. Technical support from Kusano Scientific (Tokyo, Japan) is gratefully acknowledged.

REFERENCES

1 T. C. Pinkerton, T. D. Miller, S. E. Cook, J. D. Perry, J. D. Rateike and T. J. Szczerba, *Biomed. Chromatogr.*, 1 (1986) 96–105.
2 T. C. Pinkerton and I. H. Hagestam, *Anal. Chem.*, 57 (1985) 1757–1763.
3 T. C. Pinkerton, J. A. Perry and J. D. Rateike, *J. Chromatogr.*, 367 (1986) 412–418.
4 T. Nakagawa, A. Shibukawa, N. Shimono, T. Kawashima, H. Tanaka and J. Haginaka, *J. Chromatogr.*, 420 (1987) 297–311.

5 K. Oka, K. Minagawa, S. Hara, M. Noguchi, Y. Matsuoka, M. Kono and S. Irimajiri, *Anal. Chem.*, 56 (1984) 24–27.
6 K. Oka, N. Ohki, M. Noguchi, Y. Matsuoka, S. Irimajiri, M. Abe and T. Takizawa, *Anal. Chem.*, 56 (1984) 2614–2617.
7 K. Oka, T. Ijitsu, K. Minagawa, S. Hara and M. Noguchi, *J. Chromatogr.*, 339 (1985) 253–261.
8 K. Oka, S. Aoshima and M. Noguchi, *J. Chromatogr.*, 345 (1985) 419–424.
9 K. Oka, M. Noguchi, T. Kitamura and S. Shima, *Clin. Chem.*, 33 (1987) 1639–1642.
10 K. Oka, T. Hirano and M. Noguchi, *J. Chromatogr.*, 423 (1987) 285–291.
11 K. Oka, T. Hirano and M. Noguchi, *Clin. Chem.*, 34 (1988) 557–559.
12 K. Oka, K. Hosoda, T. Hirano, E. Sakurai and M. Kozaki, *J. Chromatogr.*, 490 (1989) 145–154.
13 X. Kang and K. Oka, *Yakugaku Zasshi*, 109 (1989) 274–279.
14 M. Homma, K. Oka and N. Takahashi, *Anal. Chem.*, 61 (1989) 784–787.