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MICROBORE LIQUID CHROMATOGRAPHIC DETERMINATION OF CADRALAZINE AND CEPHALEXIN IN PLASMA WITH LARGE-VOLUME INJECTION

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SUMMARY

The application of microbore systems (15 cm × 1 mm I.D. columns filled with Nucleosil C_{18}, 5 μm particle size) to the determination of cephalexin and cadralazine in plasma was investigated. Factors such as mobile phase flow-rate, detector flow-cell volume and injection volume were examined with regard to the needs of routine drug analysis. Mobile phase flow-rates of 50–60 μl/min were used. A flow cell with an optical path length of 6 mm and an intermediary volume (2.4 μl) was selected for UV detection in order to obtain sufficient sensitivity. Large volumes of non-eluting solvent containing the drug were injected on to the column. The addition of an ion-pairing reagent to samples containing cephalexin and cefroxadin prior to the injection was found to improve the chromatographic performance. The blood sample size required for analysis with microbore columns was smaller than that with conventional columns. The analysis time was similar and the limit of quantitation was also similar, provided that large sample volumes were injected on to the microbore column.

INTRODUCTION

Recently, microbore columns (1 mm I.D. or less) have been attracting much attention in liquid chromatography owing to their advantages over conventional 4.6 mm I.D. columns. The consumption of mobile phase and expensive packing material can be reduced in proportion to the ratio of the squares of the column inner diameters. Enhanced sensitivities can be obtained in proportion to the same ratio of column diameters as less solute dilution occurs in microbore columns [1]. However, microbore columns have been little used in routine analysis because they suffer from drawbacks such as difficulties in packing efficient columns and limited sample volume for injection.

The application of microbore columns to the assay of cephalexin and cadralazine in plasma is reported in this paper. Conventional high-performance liquid

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chromatographic (HPLC) methods previously published for cephalexin [2] and cadralazine [3] were modified. These procedures were chosen because they permitted the application of microbore columns in conjunction with two different modes of sample preparation: plasma protein precipitation for cephalexin [2] and extraction from plasma for cadralazine [3]. Factors that affect the efficiency of the microbore system such as mobile phase flow-rate, detector flow-cell volume, injection volume and composition of the sample injected were examined with cadralazine or cephalosporins as test compounds.

EXPERIMENTAL

Materials and reagents

Ceftizoxime (sodium salt, $C_{13}H_{12}N_5O_5S_2Na$, molecular mass 405.38), cefadroxil ($C_{16}H_{17}N_3O_5S$, molecular mass 363.39) and cefroxadin (dihydrate, $C_{16}H_{19}N_3O_5S \cdot 2H_2O$, molecular mass 401.44) were obtained from Ciba-Geigy (Basle, Switzerland) and cephalexin (monohydrate, $C_{16}H_{17}N_3O_4S \cdot H_2O$, molecular mass 365.40) from Eli Lilly (Saint-Cloud, France). Cadralazine ($C_{12}H_{21}N_5O_3$, molecular mass 283.33) and CGP 24 751 ($C_{13}H_{23}N_5O_3$, molecular mass 297.35) as the internal standard were obtained from Ciba-Geigy. The structures of these compounds are shown in Fig. 1.

Phosphoric acid (85%), sodium dihydrogenphosphate, sulphuric acid (95–97%), sodium hydroxide (32%), perchloric acid (70%), hydrochloric acid (30%), potassium chloride, carbon tetrachloride, chloroform, acetonitrile and trichloroacetic acid (TCA) were purchased from E. Merck (Darmstadt, F.R.G.). Methanol and ethanol were purchased from Prolabo (Paris, France) and sodium pentane-, heptane- and octanesulphonates from Eastman-Kodak (Rochester, NY, U.S.A.). All chemicals were of analytical-reagent grade and were used without further purification.

Nucleosil C_{18}, 5 μm particle size (Macherey-Nagel, Düren, F.R.G.), and Co:Pell ODS, 30–38 μm particle size (Whatman, Clifton, NJ, U.S.A.), were used as packing materials.

Apparatus

The chromatographic system consisted of a Model 114M pump (Beckman, Berkeley, CA, U.S.A.), a Model 773 UV detector (Kratos, Ramsey, NJ, U.S.A.) from which the heat exchanger has been removed, set at 254 nm and equipped with 0.5-, 2.4- or 8-μl flow cells, and an automatic injector. The injector was either a WISP Model 710 B system (Waters, Milford, MA, U.S.A.) or a Model 231-401 system (Gilson, Villiers-le-Bel, France) equipped with a Rheodyne Model 7010 injection valve (with loops of different volumes). A stainless-steel tube, 45 cm $\times$ 0.25 mm I.D. $\times$ 1.6 mm O.D., was fitted to the detector outlet to provide a back-pressure and thereby avoid the formation of air bubbles in the flow cell. Chromatograms were recorded on a Sefram (Paris, France) recorder.

Columns

The column consisted of 15 cm $\times$ 1 mm I.D. $\times$ 3.2 mm O.D. glass-lined stainless-steel tube (S.G.E., Victoria, Australia) whose rigidity preserved the media

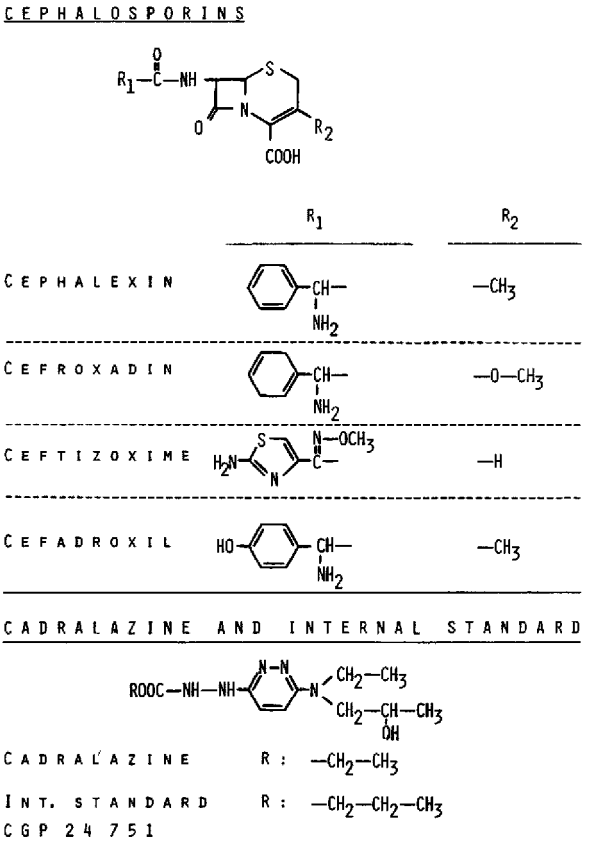

Fig. 1. Structures of the investigated compounds.

bed integrity. The column inlet and outlet were Valco zero dead volume union fittings into which 3.2 mm diameter stainless-steel frits with a porosity of 2 μm were inserted.

The slurry consisted of 0.2 g of Nucleosil C_{18} (5 μm) (previously dried for 2 h at 120°C) in 1.2 ml of carbon tetrachloride–methanol (95:5, v/v). It was placed in an ultrasonic bath for 10 min and then poured into a 7-ml reservoir connected to the top of the column previously filled with methanol. The unoccupied reservoir volume was filled with methanol, which was used as pressurizing solvent. A pressure of 700 bar, delivered by a Haskel pneumatic pump, was suddenly applied. The pressure was then raised to 800 bar and maintained for 10 min. The column was then equilibrated with the mobile phase at ambient temperature.

A pre-column (stainless-steel tube, 4 cm × 1 mm I.D. × 1.6 mm O.D.), tap-filled with Co:Pell ODS, was used for the determination of cephalexin. A 1.6 mm diameter stainless-steel frit was inserted at the top of the pre-column. The outlet of the pre-column was connected directly to the column with a Valco union fitting without a frit to separate the packing materials.

RESULTS AND DISCUSSION

Mobile phase flow-rate

The effect of mobile phase flow-rate on the efficiency of a 1 mm I.D. column was investigated with two cephalosporins as test compounds, cefadroxil (capacity factor, $k' = 4$) and ceftizoxime ($k' = 7$). The height equivalent to a theoretical plate (HETP) increased rapidly with increasing flow-rate over the range 15–130 μl/min (Fig. 2). For these drugs, a flow-rate of 50 μl/min led to a reduced plate number. It was nevertheless selected in this work as it corresponds to the same mobile phase linear velocity and consequently to the same time of analysis as with a 4.6 mm I.D. column operated at 1 ml/min.

The corresponding column top pressure with flow-rates of 50–60 μl was ca. 100 bar. Higher flow-rates could not be used to reduce retention times because the lifetime of the column was considerably reduced.

Composition and volume of the injected sample

Usually, sample volumes of 0.2–1 μl are injected on to 1 mm I.D. columns to avoid a loss of efficiency [4]. However, large volumes of sample dissolved in non-

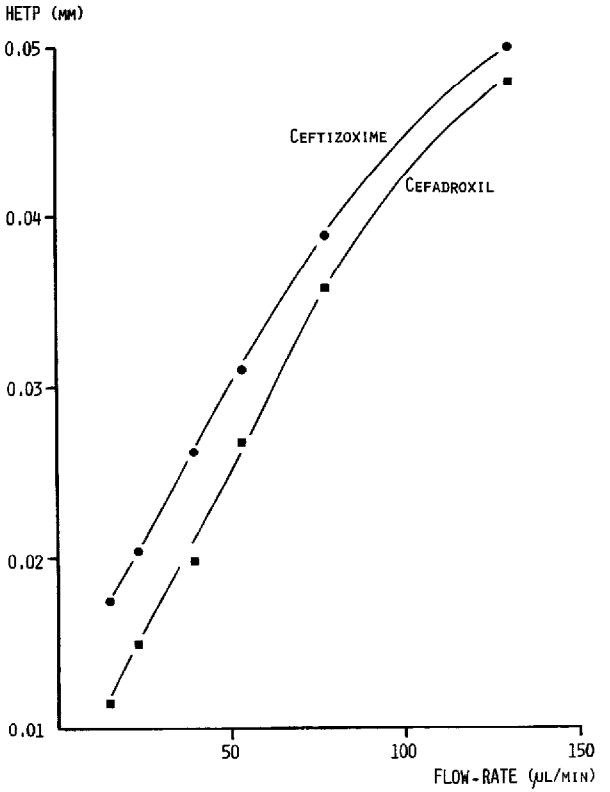

Fig. 2. HETP versus mobile phase flow-rate. Conditions: column, 15 cm × 1 mm I.D., Nucleosil C_{18} (5 μm); mobile phase, methanol–water–1.8 *M* H_2SO_4 (14:86:0.2, v/v/v); injector, WISP; volume injected, 5 μl; detection, 254 nm; flow cell, 0.5 μl; sample composition, water (200 μl), 10% (w/v) TCA (70 μl), 1000 μg/ml cefadroxil (20 μl), 1000 μg/ml ceftizoxime (20 μl).

TABLE I

INFLUENCE OF SAMPLE SIZE ON COLUMN PERFORMANCE FOR CADRALAZINE

Conditions: column, 15 cm × 1 mm I.D., Nucleosil C_{18} (5 μm); mobile phase, acetonitrile–0.1 *M* NaH_2PO_4–1 *M* NaOH (18:79:3, v/v/v); injector, Gilson; volume injected, 20 μl; flow-rate, 60 μl/min; detection, 254 nm; flow cell, 2.4 μl; sample concentration, 100 ml/ng cadralazine in 0.005 *M* sulphuric acid.

Parameter*	Injected volume (μl)			
	2	10	20	50
N	3170	3270	3270	3370
t_R (min)	6.7	6.8	6.8	7.4

*N = plate number calculated by using the bandwidth (b) at the peak half-height; t_R = retention time.

eluting solvents were injected with minimum influence on the efficiency [5–7] owing to the effect of the concentration of the solutes at the head of the column. Therefore, we investigated whether the injection of large sample volumes results in sufficient efficiency for cadralazine, cephalexin and cefroxadin.

Cadralazine. The influence of the injected sample size is shown in Table I for cadralazine. Cadralazine was dissolved in 0.005 *M* sulphuric acid and 2–50 μl were injected after adequate dilution in 0.005 *M* sulphuric acid so that the same amount of cadralazine was injected. No changes in peak bandwidth or retention time occurred over the range 2–20 μl and only slight increases were observed for a 50-μl injection.

Cephalosporins. Cephalexin and cefroxadin exhibited poor chromatographic performances when 5 μl of solution were injected on to a microbore column (Table II), although they were injected in a non-eluting solvent (water or dilute phosphoric acid). The apparent column efficiency was enhanced when a carboxylic acid (TCA) was added to the sample prior to injection and a further improvement was obtained when sodium alkylsulphonates were added. The retention time increased strongly whereas the peak bandwidth increased to a much lesser extent. The asymmetry factor decreased and became lower than 1 with sodium heptane- and octanesulphonates. The longer the hydrocarbon chain of the alkylsulphonate, the greater was the modification of the chromatographic parameters.

High concentrations of alkylsulphonate resulted in band broadening, as shown in Table III for sodium pentanesulphonate.

The injection of cephalexin and cefroxadin in water before and after several injections of samples containing 0.1 *M* sodium heptanesulphonate resulted in similar retention times.

The addition of methanol to the mobile phase permitted the retention times to be decreased while the apparent column efficiency was maintained when cephalexin and cefroxadin were injected simultaneously with an ion-pairing reagent.

A likely explanation of the mode of retention involved when cephalosporins are injected simultaneously with the ion-pairing reagent is given below. The ion pair formed between the ionized amino group of the cephalosporin and TCA or

TABLE II

INFLUENCE OF THE INJECTED SAMPLE COMPOSITION ON CHROMATOGRAPHIC PERFORMANCE FOR CEPHALEXIN AND CEFROXADIN

Conditions. column, 15 cm × 1 mm I.D., Nucleosil C_{18} (5 μm); mobile phase, methanol–$2 \cdot 10^{-3}$ *M* phosphoric acid (19:81, v/v); injector, WISP; volume injected, 5 μl; detection, 254 nm; flow cell, 2.4 μl; flow-rate, 50 μl/min.

Sample composition*	Cephalexin**				Cefroxadin**			
	N	*b* (min)	t_R (min)	*As*	*N*	*b* (min)	t_R (min	*As*
400 μl water	1420	0.58	9.3	1.9	704	0.55	6.2	2.0
400 μl 0.3 *M* H_3PO_4	1400	0.63	10.0	1.5	1060	0.52	7.2	1.5
400 μl 0.1 *M* TCA	3850	0.52	13 7	1.4	2870	0.47	10.7	1.5
300 μl 0.1 *M* sodium pentanesulphonate + 100 μl 1.5 *M* H_3PO_4	4660	0.51	14.8	1.4	3810	0 45	11.8	1.4
300 μl 0.1 *M* sodium heptanesulphonate + 100 μl 1.5 *M* H_3PO_4	8120	0.87	33 3	0.8	7990	0.74	28.1	1 0
300 μl 0.1 *M* sodium octanesulphonate + 100 μl 1.5 *M* H_3PO_4	11000	1.3	58.0	0.65	9460	1.2	49.6	0.75

*30 μl of cephalexin (100 μg/ml) and 30 μl of cefroxadin (100 μg/ml) solutions were added.
**N, b, t_R as in Table I; As = asymmetry factor measured at 10% of peak height.

the alkylsulphonate was sorbed to the bonded phase at the head of the column until the injection was finished. With sodium heptane- and octanesulphonates, the front of the cephalosporin peak migrated more slowly than the rear, as the asymmetry factor was lower than 1. This might be the result of a higher concentration of the ion-pairing reagent at the front of the peak than at the rear. Gradient elution with decreasing concentrations of the ion-pairing reagent might be generated. Owing to its affinity for the sorbent, the injected ion-pairing reagent might be slowly eluted as a zone with a sharp front and continuously decreasing concentrations, as recently reported for the UV detection of *o*-xylenesulphonate sodium salt by Slais et al. [8]. At high concentrations of so-

TABLE III

INFLUENCE OF THE CONCENTRATION OF SODIUM PENTANESULPHONATE ADDED TO THE INJECTED SAMPLE ON CHROMATOGRAPHIC PERFORMANCE FOR CEPHALEXIN AND CEFROXADIN

Conditions as in Table II.

Concentration of sodium pentanesulphonate (*M*)	Cephalexin			Cefroxadin		
	N	*b* (min)	t_R (min)	*N*	*b* (min)	t_R (min)
0.01	1470	0.59	9.6	1020	0.50	6.8
0.05	3560	0.56	14.2	2830	0.50	11.3
0.1	4660	0.51	14.8	3810	0.45	11.8
0.2	1740	0.93	16.5	2050	0.68	13.1
0.5	1150	1.08	15.6	870	1.02	12.8

TABLE IV

INFLUENCE OF DETECTOR CELL VOLUME ON COLUMN PERFORMANCE WITH CADRALAZINE AS TEST SUBSTANCE

Conditions as in Table I.

Parameter*	Detector flow-cell volume (μl)		
	8 (10 mm)**	2.4 (6 mm)**	0.5 (1 mm)**
N	3030	3690	4580
As	2.1	1.6	1.3
h (cm)	78	64	12

*N = plate number calculated by using the bandwidth at the peak half-height; As = asymmetry factor measured at 10% of peak height; h = peak height.
**Optical path length.

dium pentanesulphonate, the decrease in column efficiency was probably due to the formation of micelles of the ion-pairing reagent, as found by Riley et al. [9] when the ion-pairing reagent was used in the mobile phase at a constant concentration.

Detector flow cell

A small detector cell is generally used with microbore columns to minimize band broadening. However, a reduction in the detector cell volume involves a reduction in the optical path length and therefore a decrease in sensitivity as the detector response decreases in proportion to the optical path length.

The influence of the cell volume was investigated with cadralazine ($k'=4$). When using the 8-μl cell with a microbore column, the plate number and asymmetry factor were severely affected whereas the peak heights were only about 20% higher than with the 2.4-μl cell (Table IV). The 2.4-μl cell also provided poor chromatographic performances compared with the 0.5-μl cell. Peaks obtained with the 2.4-μl cell were 5.3 times higher than those with the 0.5-μl cell instead of 6 times as predicted by the optical path-length ratio. The 2.4-μl cell was selected to obtain a higher sensitivity, and the plate number was sacrificed.

Determination of cephalexin in plasma

A method previously described for the determination of cephalexin in plasma with a conventional system and using cefroxadin as an internal standard [2] was adapted for a 1 mm I.D. column. The modifications to this procedure were as follows: addition of 70 μl of perchloric acid (1.2 *M*) to 200 μl of plasma instead of TCA to precipitate plasma proteins; addition of 70 μl of 0.5 *M* heptanesulphonate to the mixture before agitation and centrifugation; reduction of the injected volume of the supernatant from 20 to 5 μl; replacement of the packing material LiChrosorb RP-8 (5 μm) with Nucleosil C_{18} (5 μm); a slight decrease in the methanol content in the mobile phase from 28 to 26%; reduction of the mobile phase flow-rate from 1 ml/min to 50 μl/min; and reduction of the volume of the detector flow cell from 8 to 2.4 μl.

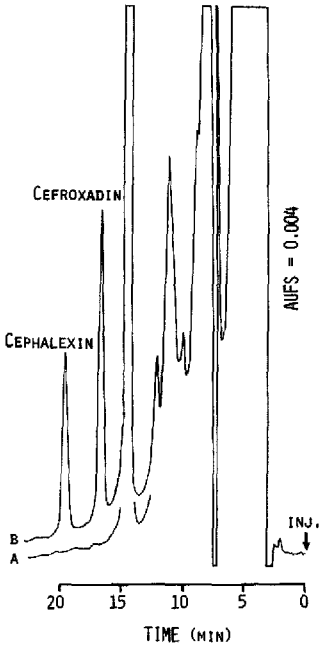

Fig. 3. Chromatograms of (A) blank plasma and (B) plasma containing 1 μg/ml cephalexin and 2 μg/ml cefroxadin. Conditions: pre-column, 4 cm × 1 mm I.D., Co:Pell ODS (30–38 μm); column, 15 cm × 1 mm I.D., Nucleosil C_{18} (5 μm); mobile phase, methanol–$2 \cdot 10^{-3}$ *M* phosphoric acid (26.74, v/v); injector, WISP; volume injected, 5 μl; detection, 254 nm; flow cell, 2,4 μl; flow-rate, 50 μl/min.

The ion-pairing reagent was added to enhance the apparent microbore column efficiency (about 8000 theoretical plates were obtained for both cephalosporins). Nucleosil C_{18} was used instead of LiChrosorb RP-8 because the microbore column exhibited a higher efficiency when filled with this packing material.

Examples of chromatograms and the characteristics of the procedure are exhibited in Fig. 3.

Linear calibration graphs, established over the range 0.2–20 μg/ml cephalexin (0.2 μg/ml being the limit of quantitation), were obtained with correlation coefficients of 0.999 by plotting peak-height ratios of cephalexin and cefroxadin versus cephalexin concentrations. The reproducibility and accuracy were checked by analysing replicate plasma samples containing 0.5 and 1 μg/ml cephalexin and 2 μg/ml cefroxadin. The mean values ± standard deviations ($n = 6$) were 0.53 ± 0.02 μg/ml (coefficient of variation = 3.9%) and 1.02 ± 0.04 μg/ml (coefficient of variation = 3.9%), respectively.

The peak heights obtained with the microbore system were about twice those obtained by using a conventional system with a 4.6 mm I.D. column, although the amount of sample injected on to the microbore column was four times lower. The analysis times were similar. The incorporation of a pre-column did not affect the microbore column efficiency but decreased the peak symmetry (the asymmetry factor measured at 10% of the peak height without pre-column was 0.7 and with

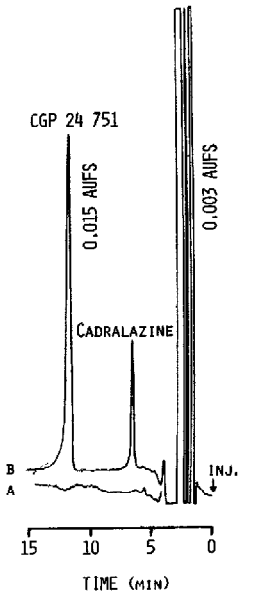

Fig. 4. Chromatograms of extracts of (A) blank plasma and (B) plasma containing 25 ng/ml cadralazine and 1 μg/ml CGP 24 751 (internal standard). Conditions: column, 15 cm × 1 mm I.D., Nucleosil C_{18} (5 μm); mobile phase, acetonitrile–0.1 *M* NaH_2PO_4–1 *M* NaOH (18:79:3, v/v/v); injector, Gilson; volume injected, 20 μl; flow-rate, 60 μl/min; detection, 254 nm; flow cell, 2.4 μl.

a pre-column 0.5). The pre-column should be replaced after about 80 injections of biological samples.

Determination of cadralazine in plasma

The method previously reported by Hauffe and Dubois [3] for the determination of cadralazine in plasma by conventional HPLC was adapted for a 1 mm I.D. column with the following minor modifications: reduction of all volumes by a factor of 5 for the sample preparation step [200 μl of plasma were extracted with 1.2 ml of chloroform–ethanol (95:5, v/v) and back-extracted into 100 μl of pH 2 buffer (0.09 *M* potassium chloride in 1 l of 0.01 *M* hydrochloric acid)]; reduction of the injected volume of the supernatant pH 2 buffer from 100 to 20 μl; replacement of the packing material LiChrosorb RP-8 (10μm) with Nucleosil C_{18} (5 μm); increase in the acetonitrile content in the mobile phase from 15 to 18%; reduction of the mobile phase flow-rate from 2.7 ml/min to 60 μl/min; and reduction of the volume of the detector flow cell from 8 to 2.4 μl.

Typical chromatograms and the characteristics of the procedure are shown in Fig. 4.

Linear calibration graphs were established over the range 5–500 ng/ml plasma (5 ng/ml being the limit of quantitation) by plotting the peak-height ratios of cadralazine and the internal standard versus cadralazine concentrations. The correlation coefficients were 0.999. The reproducibility and precision were evaluated by analysing replicate spiked plasma samples. For a series of six plasma samples containing 10 and 250 ng/ml cadralazine, the mean values ± standard

deviations found were 10.1 ± 0.5 ng/ml (coefficient of variation = 4.9%) and 255 ± 7 ng/ml (coefficient of variation = 2.7%), respectively.

The peak heights obtained by the present method were about 50% higher than those obtained with a conventional system, although the volume of plasma used for the microbore column was five times lower. The retention times were similar. The microbore column was routinely used by injecting 20-μl plasma extracts with up to 200 injections.

CONCLUSION

It would be of interest to use microbore systems for the routine determination of drugs in plasma. In this study, the size of the blood sample required for analysis with microbore columns was smaller than that with conventional columns and the solvent consumption was reduced. The analysis time was similar. The limit of quantitation was also similar, provided that the volume of sample injected on to the microbore column was larger than usual.

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