Journal of Chromatography, 330 (1985) 113–119
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 779

AUTOMATED PRE-COLUMN SYSTEM FOR TRACE ENRICHMENT AND CLEAN-UP OF PLASMA SAMPLES IN NARROW-BORE LIQUID CHROMATOGRAPHY

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(Received April 4th, 1985)

SUMMARY

The design of a switching valve having an internal pre-column for automated sample handling and trace enrichment in narrow-bore liquid chromatography is described. The system combines low dispersion with the capability of direct plasma injection without any (off-line) clean-up procedure. Band broadening and enrichment factors have been studied for 100-μl trace enrichment *versus* 0.5-μl direct loop injection. As an example, the automated analysis of plasma samples containing clobazam and desmethylclobazam at the low-ppb level is reported.

INTRODUCTION

Narrow-bore liquid chromatography (LC), featuring separation columns of about 1 mm I.D., offers advantages over conventional LC, such as relatively low consumption of solvent and stationary phase and high mass sensitivity. However, owing to the limited injection volume of about 1 μl and the reduced path length in, *e.g.*, UV absorption detector flow cells, concentration sensitivity is often unsatisfactory.

Recently, we have developed a simple micro-pre-column that can be packed manually and used in on-line (automated) systems[1]. Critical parameters such as pre-column length, inlet capillary I.D. and the use of screens instead of frits were studied and the applicability to the direct analysis of plasma and serum samples, *i.e.*, without any off-line clean-up, was demonstrated. Unfortunately, extra-column band broadening caused by this pre-column was serious and resulted in a 3.5-fold increase in detection limit and a 12-fold decrease in plate number. Therefore, the actual enrichment factor was 60 instead of the expected 200 for a 100-μl trace enrichment experiment *versus* a 0.5-μl loop injection.

In this paper we describe a micro-pre-column inserted within the axis of a common six-port switching valve. In this way, extra-column band broadening could be reduced without losing the benefits of the previous design. The system has been applied to the determination of clobazam and its active metabolite desmethylclobazam in plasma samples without any off-line sample pre-treatment.

0021-9673/85/$03.30

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) Model 410 pump was used in combination with a Gilson (Villers le Bel, France) Model 302 pump and two modified Valco (Houston, TX, U.S.A.) six-port valves. For band broadening studies a home-made 0.5-μl injection valve modified from an early Jasco valve[2] was used. Valve switching was microprocessor-controlled using a Kipp & Zonen (Delft, The Netherlands) Model 5140 programmer. A Knauer (Bad Homburg, F.R.G.) fixed-wavelength (254 nm) photometer, equipped with a 1-μl flow-cell, was used for detection. Chromatograms were recorded on a Kipp & Zonen BD 40 recorder.

Chemicals

LC-grade water was obtained by purifying distilled water in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system. HPLC-grade acetonitrile was obtained from Promochem (Wesel, F.R.G.). Acetic acid was of analytical-reagent grade from Baker (Deventer, The Netherlands). The test mixture contained 3,5-dichlorophenol, 3,4,5-trichlorophenol and 2,3,4,6-tetrachlorophenol, which were purchased from Aldrich Europe (Beerse, Belgium).

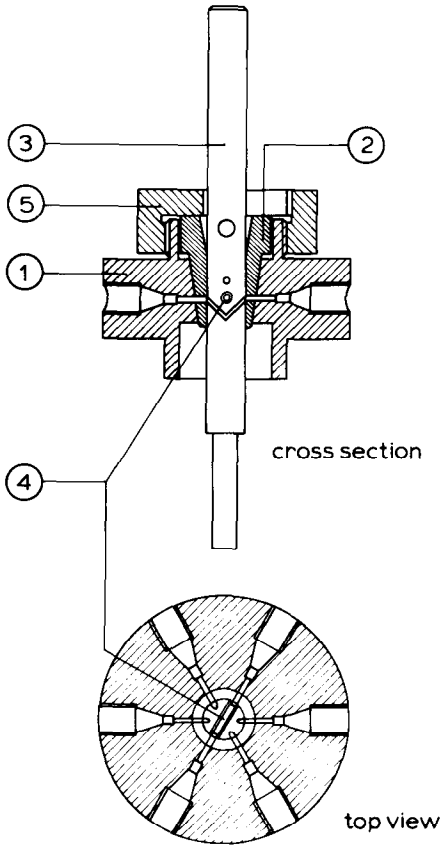

Fig. 1. Switching valve with internal micro-pre-column. For explanation, see text.

Stationary phases and columns

The analytical column was a 20 cm × 1 mm I.D. glass-lined stainless-steel column home-packed with 3-μm Spherisorb ODS-2 (Phase Separations, Queensferry, U.K.). The pre-column was packed with 40-μm Octyl (C_8) (Baker). Screens (36 μm) were obtained from Dinxperlo (Dinxperlo, The Netherlands).

Design of the switching valve with internal pre-column

The switching valve with its internal pre-column (Fig. 1) consists of three main parts, as follows.

(a) The valve body (1) is constructed from a standard Valco six-port switching valve. All ports were drilled to 1 mm. The analytical column fits directly into one of these ports without any connective tubing in between.

(b) The seal (2) is home-made and fits in the valve body. Note that this seal is fixed, whereas the seal in the standard switching valve is not.

(c) The axis of the valve (3) was drilled through, to give a 4.5 × 1 mm I.D. hole that can be filled with packing material to obtain the actual micro-pre-column (4). The axis contains one permanent screen; the other screen is held by a PTFE ring. The axis also contains two dummy channels for the other flow-lines of the six-port valve. These channels are drilled in such a way as not to touch each other, nor the micro-pre-column. The valve is leak-tight up to 250 bar when the wheel (5) has been tightened. The valve rotates extremely easily, thanks to the very small contact area between the rotating and the fixed part, and is easily pneumatically activated.

The geometrical volume between pre-column and separation column is about 2.5 μl, which is relatively high for narrow-bore LC. However, because of the absence of changes in inner diameter (all parts are 1 mm I.D.) the final band broadening caused by this dead volume is extremely small.

Pre-column packing procedure

For packing or emptying the pre-column, the axis is taken out of the switching valve and placed in a syringe adapter (Fig. 2). A syringe filled with a thin slurry of the packing material in methanol is fitted into the adapter and the pre-column is packed manually. Excess of packing material at the pre-column inlet is removed after the axis has been taken out again, and a screen and PTFE ring are inserted to retain the packing material. Then the axis is placed in the switching valve again and the wheel is tightened; the pre-column is now ready for use. The precolumn is emptied in a similar way by using a syringe filled with methanol, fitting the syringe into the adapter from the opposite side.

The entire packing procedure takes less than 5 min. It was not necessary to repack the pre-column frequently because of the routinely used on-line regeneration. Arbitrarily, we repacked the pre-column once a week.

RESULTS AND DISCUSSION

Band broadening due to the micro-pre-column/switching valve

In order to determine the contribution of the micro-pre-column/switching valve to the total band broadening of the LC system, a comparison was made between 0.5-μl loop injections of the chlorophenol test mixture (1 mg/ml dissolved in the

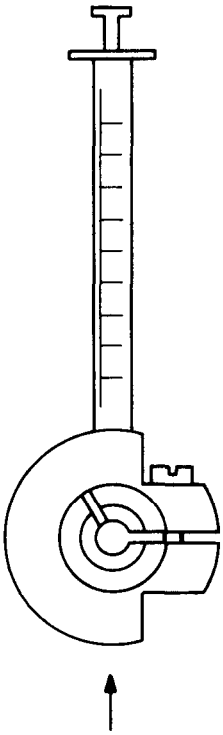

Fig. 2. Syringe adapter for manual packing and emptying (arrow position) of the micro-pre-column.

mobile phase) and 100-μl trace enrichment on the micro-pre-column after 200-fold dilution of the test mixture with 0.05% acetic acid.

Desorption after trace enrichment was carried out in both the forward- and the back-flush mode and all experiments were carried out in triplicate. From the results given in Table I, it can be seen that there is only a 30% (1.3-fold) increase in detection limit and a 40% (1.7-fold) decrease in plate number caused by extra-column

TABLE I

INFLUENCE OF MICRO-PRE-COLUMN BAND BROADENING ON SYSTEM EFFICIENCY AND DETECTION LIMITS

Conditions: 20 cm × 1 mm I.D. 3-μm Spherisorb ODS-2 column, acetonitrile–0.11% acetic acid (55:45) at 50 μl/min; N = 10 000 for test mixture; 100-μl (5 μg/ml) trace enrichment (flush volume 800 μl of 0.05% acetic acid) *vs.* 0.5-μl loop injection (1 mg/ml).

Compound	*k'*	*Increase in detection limit*		*Decrease in plate number*	
		*FF**	*BF**	*FF*	*BF*
3,5-Dichlorophenol	3	1.3	1.2	1.7	1.4
2,4,5-Trichlorophenol	4	1.3	1.3	1.7	1.7
2,3,4,6-Tetrachlorophenol	6	1.3	1.2	1.7	1.4

* FF, forward-flush desorption; BF, back-flush desorption.

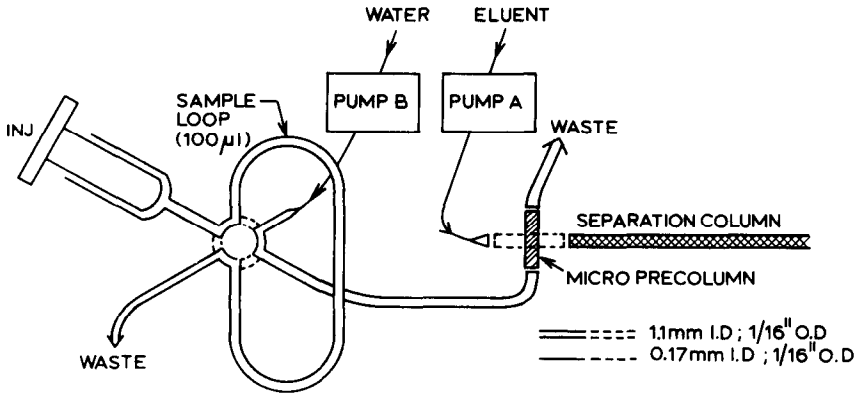

Fig. 3. Set-up for the automated sample handling and trace enrichment of plasma samples. Conditions: 20 cm × 1 mm I.D. 3-µm Spherisorb ODS-2 column, acetonitrile–water (50:50) at 50 µl/min; detection at 254 nm, 0.02 a.u.f.s. Micro-pre-column, 4.5 × 1 mm I.D. 40-µm Octyl (C_8). Sampling at 200 µl/min.

band broadening. Hence, the actual increase in sensitivity as a result of trace enrichment will be 150 rather than the expected value of 200.

One should note the small difference between back-flush and forward-flush desorption. This difference indicates that retention on the pre-column during the elution step is not negligible; that is, the extra-column band broadening must be attributed to the kind of packing material rather than to the design itself. When untreated plasma samples have to be analysed, we prefer the forward-flush mode in order to maintain the protective filter aspect of the pre-column; in this way no blocking of the analytical column will occur.

Automated handling of plasma samples

As an example, the micro-pre-column was used for the automated handling of untreated plasma samples spiked with the tranquillizer clobazam and its active metabolite desmethylclobazam; the set-up used is shown in Fig. 3.

After filling the loop, the switching programme summarized in Table II was

TABLE II

SWITCHING PROGRAMME FOR THE AUTOMATED ANALYSIS OF CLOBAZAM AND DESMETHYLCLOBAZAM IN PLASMA, USING THE SET-UP IN FIG. 3

Conditions: 20 cm × 1 mm I.D. 3-µm Spherisorb ODS-2 column, acetonitrile–water (50:50) at 50 µl/min; detection at 254 nm, 0.02 a.u.f.s. Micro-pre-column, 4.5 × 1 mm I.D. 40-µm Octyl (C_8). Sampling at 200 µl/min.

Time (sec)	*Event*
0	Sample loop (100 µl) filled
1	Inject on to micro-pre-column and flush with 3000 µl of water
900	Forward-flush desorption with 50 µl of eluent
960	Reset pre-column valve; flush pre-column with 1800 µl of water
1497	Reset sampling valve
1500	End

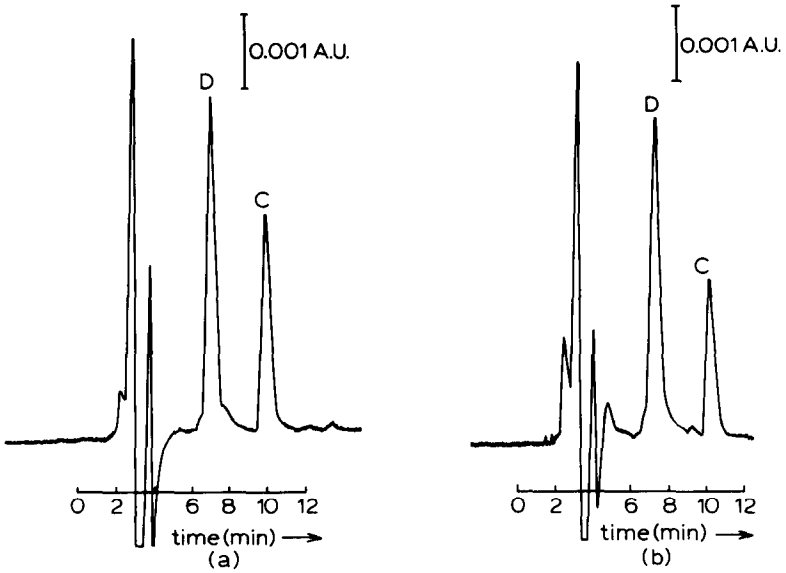

Fig. 4. Chromatograms of a 100 ng/ml standard solution of desmethylclobazam (D) and clobazam (C) diluted 1:1 with water (a) and with blank plasma (b). Conditions as in Table II and Fig. 3.

started. A 100 ng/ml standard solution of clobazam and desmethylclobazam in water was diluted with an equal volume of water or with fresh human plasma and concentrated on the micro-pre-column and analysed (Fig. 4a and b). During each analytical separation the pre-column was flushed on-line with, arbitrarily, about 2 ml of water to remove the remaining water-soluble protein fragments and then re-used for the next sample. From the chromatogram it can be seen that the sorption–desorption procedure on the pre-column provides excellent clean-up; even with the non-selective UV detection at 254 nm, no interferences are observed.

Relevant analytical data are given in Table III. The detection limits are 2.5 and 5 ng/ml in real samples for desmethylclobazam and clobazam, respectively, and are comparable to those in conventional LC with post-column photochemical reaction and fluorescence detection[3,4].

TABLE III

ANALYTICAL DATA FOR THE AUTOMATED ANALYSIS OF CLOBAZAM AND DESMETHYLCLOBAZAM IN FRESH PLASMA

Conditions: 100 µl samples, analysed following the switching programme in Table II.

Compound	*Repeatability (%) for*		*Recovery (%) from plasma (n = 6)*	*Detection limit in plasma samples*	
	Standards (n = 10)	*Plasma (n = 6)*		*ng*	*ng/ml*
Desmethylclobazam	±0.7	±1.7	91	0.25	2.5
Clobazam	±2.2	±1.1	71	0.5	5

CONCLUSION

The sample capacity in narrow-bore LC can be successfully improved by on-line trace enrichment on a micro-pre-column. An increase in sensitivity of more than two orders of magnitude can easily be obtained (100-μl trace enrichment *versus* 0.5-μl loop injection). The present switching valve with its internal pre-column allows automated sample handling and trace enrichment without excessive additional band broadening ($\sigma_{v,\text{pre-column}} = 3.5$ μl; $k' = 3$). Even with a high-performance analytical system ($N = 10\,000$), 60% of the separation power is maintained, which contrasts favourably with earlier results[1] where over 90% of the separation power was lost. The additional band broadening apparently is mainly caused by the nature of the packing material in the pre- and analytical columns and not by the pre-column design; in other words, a further reduction in band broadening should be attainable.

The present design, which features wide-pore screens, relatively large packing material (d_p 40 μm) and wide-bore valve ports, allows the repeated injection of fresh plasma samples without clogging of the system, as has been demonstrated for clobazam and desmethylclobazam with good repeatability and detection limits at the low ng/ml level.

The on-line combination of several pre-columns packed with different types of packing materials for an increase in selectivity in the handling of more complex samples, using automated narrow-bore LC systems, is currently under investigation.

ACKNOWLEDGEMENTS

The Free University mechanical workshop, and especially R. Boegschoten, is acknowledged for improving the design of the first micro-pre-column prototype. J. H. Wolf is thanked for providing the plasma samples and Kipp & Zonen for the loan of their Programmer.

This work was supported by the Dutch Foundation for Technical Sciences, grant no. VCH 44.0616.

REFERENCES

1 M. W. F. Nielen, E. Sol, R. W. Frei and U. A. Th. Brinkman, *J. Liq. Chromatogr.*, (1985) in press.
2 T. Takeuchi and D. Ishii, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 469.
3 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *Anal. Chim. Acta*, 114 (1980) 137.
4 M. Uihlein and E. Schwab, *Chromatographia*, 15 (1982) 140.