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STUDIES OF MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

III. DEVELOPMENT OF A "MICRO-PRE-COLUMN METHOD" FOR PRE-TREATMENT OF SAMPLES

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SUMMARY

A sample pre-treatment method is described in which components are adsorbed on a micro pre-column, and an investigation of the method (with phthalic acid esters in water as sample components) is reported. The esters could be concentrated in the pre-column simply and rapidly, and were separated in a micro separation column. Concentrations of diethyl phthalate in the ppb-range were rapidly determined by pre-treatment of 10 ml or less of the aqueous solution. The method was suitable for use in micro high-performance liquid chromatography.

INTRODUCTION

Recently, there have been marked advances in high-performance liquid chromatography (HPLC), which is a useful method for the analysis of high-boiling compounds in biochemical, medical or environmental samples. However, some treatment of the sample is often required before analysis by HPLC, either because of the concentration of the sample or in order to separate matrix or macro components. Solvent extraction, followed by concentration of the extract, is the usual form of pre-treatment, and only part of the sample solution prepared by such a method is used for HPLC[1–7].

We have adapted HPLC to the micro-scale, calling the technique MHPLC[8]. If prior solvent extraction is used, much of the sample solution is not required, as the injection volume in MHPLC is much smaller than that in ordinary HPLC. Therefore, a pre-treatment is needed by which traces of components in a small amount of sample can be concentrated simply, effectively and without loss.

With this aim in mind, we developed a method in which components were adsorbed by passing the sample solution through a packed pre-column (*ca.* 15 mm long). This pre-column was then connected to the top of a micro separation column, and the adsorbed components were eluted from the pre-column and developed on

* In this paper, the American billion (10^9) is meant.

the separation column. In this procedure, the amounts of sample solution required for the pre-treatment were very small, and sample components were concentrated simply and rapidly. We used aqueous solutions of phthalic acid esters as test samples, because pollution of rivers by such esters constitutes a serious problem for which a rapid and simple analytical method is required. Investigation of this procedure showed that the "micro-pre-column method" was particularly suitable for pre-treating samples for analysis by MHPLC.

EXPERIMENTAL

Packing materials, instruments and preparation of micro separation column and micro pre-column

Hitachi gel 3010 (styrene-divinylbenzene porous polymer particles; size 20 μm) and TSK gel LS-111 (the same as Hitachi gel 3010, but of size 5 μm) were used as packings for the pre-column and the separation column, respectively, and the separation columns were prepared in PTFE tubing as described previously[8]. A UV spectrophotometer (UVIDEC-1) was used for detection, and the flow-through cell for the separation column had a volume of 0.05–0.4 μl and an I.D. of 0.3–0.4 mm. Micro pre-columns were prepared by the same method as the micro separation column; such a pre-column is shown at A in Fig. 1. The pre-column consists of stainless-steel tubing (18 mm × 0.35 mm I.D.) packed with Hitachi gel 3010 and having PTFE tubing (0.5 mm I.D.) for connections; it can be connected to the separation column without any void volume, as shown at B in Fig. 1.

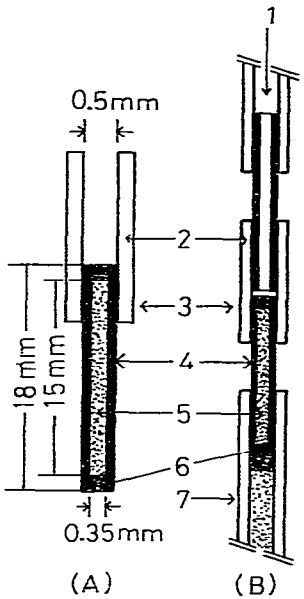

Fig. 1. Diagram of a micro pre-column (A) and its connection to the separation column (B). 1, Carrier liquid; 2, Teflon tube for connection; 3, micro pre-column; 4, stainless-steel tubing; 5, packing material; 6, glass wool; 7, micro separation column.

The chromatographic operation

The same methods as those used previously[8] were employed for supplying mobile phase and for direct injection of samples into the separation column. Fig. 2 shows the pre-treatment procedure for samples in the pre-column. Thus, 200 μl of methanol were caused to flow through the pre-column to remove any organic substances remaining in it, then 200 μl of distilled water were passed through it to remove methanol. After this washing procedure, organic components were adsorbed on the pre-column by passage of a suitable amount of aqueous sample solution at a flow-rate not exceeding 100 μl/min. The pre-column was then washed again with 200 μl of distilled water and was dried by passage of air. The pre-column, on which the organic sample components were concentrated, was connected to the separation column, as shown at B in Fig. 1, and sample components were eluted on to the separation column by supplying appropriate mobile phase.

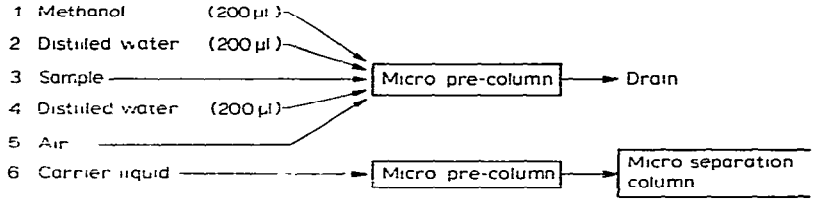

Fig. 2. Procedures involved in adsorption of sample components on the pre-column and transfer to the separation column.

The esters used were dimethyl, diethyl, dibutyl, diheptyl, dioctyl, didecyl and dilauryl phthalates (DMP, DEP, DBP, DHP, DOP, DDP and DLP, respectively), and methanol–dichloromethane (5:1) was used as mobile phase. A typical separation is shown in Fig. 3.

RESULTS AND DISCUSSION

Selection of packings for pre-columns and separation columns

When methanol–water mixture is used as mobile phase in reversed-phase liquid partition chromatography, the retention volumes of organic sample components can be increased by increasing the water content of the mobile phase and can be made infinitely large by using only water as mobile phase. Thus, organic sample components that are slightly soluble in water are adsorbed on non-polar packing and can be concentrated in a pre-column containing such a packing by passage of the aqueous sample solution through it. The components so concentrated in the pre-column can be eluted and developed on the separation column by passage of a mobile phase less polar than water (such as methanol).

From this point of view, such non-polar materials as porous polymers or chemically bonded ODS packings were examined for use in the micro pre-column. The efficiency of adsorption of sample components was studied with four kinds of packings, *viz.*, Hitachi gel 3010, TSK gel LS-111, Zorbax ODS (a porous silica, with chemically bonded octadecyl groups) and Permaphase ODS (superficially porous silica, with chemically bonded octadecyl groups). Permaphase ODS proved to be

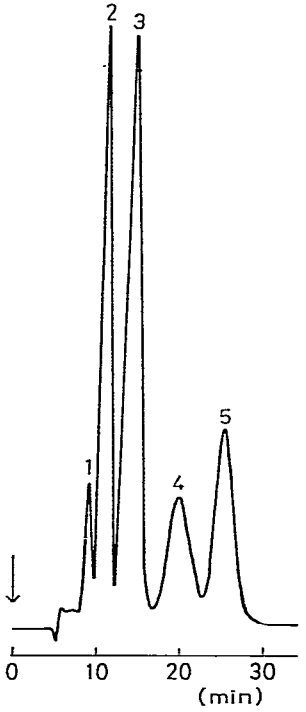

Fig. 3. Typical separation of phthalate esters on a micro separation column (170 mm × 0.5 mm I.D.) packed with TSK gel LS-111. Peaks: 1 = DEP; 2 = DBP; 3 = DHP; 4 = impurity in DDP; 5 = DDP. Sample concentrations (in methanol): DEP, 0.43%; DBP, 2.8%; DHP, 5.8%; DDP, 6.9%. Sample size, 0.06 μl; mobile phase, methanol–dichloromethane (5:1); flow-rate, 6.25 μl/min; temperature, ambient; wavelength of detection, 240 nm.

nadequate for packing the pre-column, as its surface area (0.8 to 1.0 m^2/g) was nsufficient for complete adsorption of sample components. The other three materials adsorbed the sample components completely, but LS-111 and Zorbax ODS were unsuitable because their fineness (5 μm and 8–9 μm, respectively) made rapid passage of aqueous sample solutions difficult. Hitachi gel 3010 had an appropriate particle diameter (*ca.* 20 μm), and aqueous solutions could be made to flow easily and rapidly through the pre-column.

Initially, the silica gel packings were used in the micro separation columns, but we thought that small amounts of residual water in the pre-column might adversely affect the column efficiency and separation of sample components. Thus, porous polymer packings, which were hardly affected by residual water, were examined as packings for the separation column; of the polymers tested, LS-111 was most suitable for the separation of phthalic acid esters.

Influence of micro pre-column on separation of sample components in micro separation column

We thought that connection of the pre-column to the separation column might affect the efficiency and separating power of the latter column because of differences

in the packings of the two columns, and that the pre-treatment procedures used with the pre-column might cause undesirable effects in the separation column because of residual water or spread adsorption of sample components in the pre-column. The following experiments were performed in order to investigate these factors.

First, 0.06 μl of a methanol solution of phthalic acid esters was injected into a micro pre-column connected to a micro separation column; the resulting chromatogram is shown in Fig. 4, from which it can be seen that separation of the esters was almost the same as that shown in Fig. 3, although the retention volumes were larger. Thus, connecting the pre-column to the separation column does not undesirably affect the efficiency of separation of the sample components.

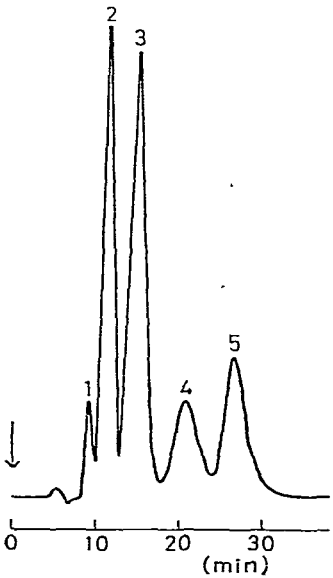

Fig. 4. Typical chromatogram of phthalate esters obtained by injecting into a micro pre-column (15 mm × 0.35 mm I.D.) packed with Hitachi gel 3010 to a micro separation column as described in Fig. 3. Peaks and chromatographic conditions as in Fig. 3.

Next, the capacity factor, the HETP and the resolution were measured in both instances, in order to establish the effects of pre-treatment. In the first instance (A in Table I), 0.06 μl of a methanol solution of phthalic acid esters was directly injected into the pre-column connected to the separation column; in the second instance (B in Table I), esters concentrated in the pre-column by passage of the aqueous test solution were developed on the separation column after connection of the pre-column. The separation column (170 mm × 0.5 mm I.D.) was packed with LS-111 gel and the pre-column (15 mm × 0.35 mm) with Hitachi gel 3010; the flow-rate of mobile phase was 4.17 μl/min. Table I shows the results obtained, and, as the values are the same in both instances, pre-treatment on the pre-column obviously has no adverse effect on the separation.

Efficiency of adsorption of sample components in the pre-column

The efficiency of adsorption of components in the pre-column was investigated

TABLE I

EFFECT OF PRE-TREATMENT WITH THE MICRO PRE-COLUMN ON CHROMATOGRAPHIC DATA

Test ester	*Conditions**	*Sample concn. in methanol, %*	*Injection volume, µl*	*Sample concn. in water, ppm*	*Volume of pre-treatment, µl*	*Capacity factor*	*HETP, mm*	*Resolution***
DEP	A	0.43	0.06	—	—	0.87	0.30	1.35
	B	—	—	17	67	0.88	0.32	1.35
DBP	A	2.76	0.06	—	—	1.33	0.29	—
	B	—	—	12	100	1.35	0.32	—
DOP	A	1.98	0.06	—	—	2.55	0.37	2.44
	B	—	—	2	5000	2.56	0.34	2.46

* A: Injection of methanol solution into the pre-column connected to the separation column. B: Pre-treatment of the aqueous solution on the pre-column.

** Between DBP and the other esters.

by varying the amounts of sample components, their concentrations and the amounts of the aqueous solution. The more dilute the aqueous solution, the larger the amount that must be applied to the pre-column to attain the same sensitivity. Thus, a close relationship exists between the concentration of the sample solution and the amount pre-treated on the micro pre-column.

Graphs of the retention of DEP and DBP against variation in the amount of aqueous solution passed through the pre-column are shown in Fig. 5; the volumes of aqueous solution applied to the pre-column (10–100 µl) corresponded to 100–1000 ng of sample component. The rectilinearity of the graphs shows that the esters are quantitatively retained by the pre-column.

The efficiency of adsorption from more dilute aqueous solutions was also investigated, with DEP (relatively difficult to adsorb because of its polarity) as test compound. Thus, 1, 6 and 10 ml of aqueous DEP solution (330, 82 and 7.7 ppb,

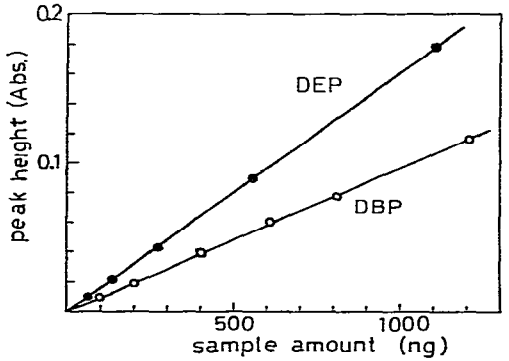

Fig. 5. Calibration graphs for DEP (17 ppm) and DBP (12 ppm) in water. Micro pre-column and micro separation column as Fig. 4; mobile phase, methanol–dichloromethane (5:1); flow-rate, 4.17 µl/min.

respectively) were passed through the pre-column, and the adsorbed DEP was developed on the separation column with methanol–dichloromethane. Sample amounts were determined, by using the calibration graph in Fig. 5, from the peak heights on the chromatograms and were compared with the amounts taken in the experiments; the results are listed in Table II. The values determined from the calibration graph agreed relatively well with those calculated from the concentration and amount of sample solution used. From these results, we concluded that DEP at the ppb level in 10 ml of test solution could be quantitatively retained on the micro pre-column. The estimated limits of detection (signal-to-noise ratio = 2) for DEP and DBP were 25 and 40 ng, respectively.

TABLE II

EFFICIENCY OF RETENTION OF DEP ON MICRO PRE-COLUMN FROM DILUTE AQUEOUS SOLUTION

DEP concn. in water, ppb	*Sample vol. for pre-treatment, ml*	*Amount of DEP calculated from sample concn. and vol., ng*	*Amount of DEP determined from calibration graph, ng*
330	1	330	330
82	6	492	469
7.7	10	77	83

Analysis of a micro amount of phthalic esters in water by using the "micro pre-column method"

Fig. 6 shows a typical chromatogram for phthalic acid esters obtained by pre-treating the dilute aqueous test solution on the pre-column, followed by separation on the micro separation column. The first peak (t_R = 6 min) may be due to an

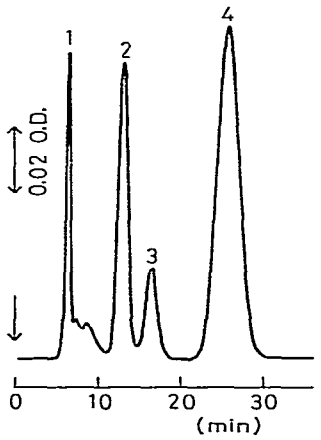

Fig. 6. Typical separation of phthalate esters in aqueous solution by the "micro pre-column method". Sample concentrations: DEP (peak 2), 130 ppb; DBP (peak 3), 60 ppb; DOP (peak 4), 2 ppm. Micro pre-column, micro separation column, mobile phase and flow-rate as in Fig. 5. Temperature, ambient; wavelength of detection, 240 nm. For peak 1 see text.

impurity in the water. The concentration of the test solution could be determined, by using the calibration graphs in Fig. 5, from the peak height for each compound. The estimated values for DEP and DBP (113 and 58 ppb, respectively) agreed well with the actual concentrations in the sample (130 and 60 ppb).

CONCLUSIONS

A method for pre-treatment of samples (the "micro pre-column method") was developed to facilitate application of MHPLC to the analysis of natural samples. By using phthalic acid esters in water as test compounds, an investigation of the method was carried out; this showed that sample pre-treatment could be carried out simply and rapidly. The amounts of sample required were small. The use of various combinations of pre-column and separation column should make possible extensive application of this method to biochemical, medical or environmental samples.

REFERENCES

1 M. Novotny, M. L. Lee and K. D. Bartle, *J. Chromatogr. Sci.*, 12 (1974) 606.
2 P. R. Musty and G. Nickless, *J. Chromatogr.*, 89 (1974) 185.
3 P. W. Albro and B Moore, *J Chromatogr.*, 94 (1974) 209.
4 J. A. Schmit, R. A. Henry, R. C. Williams and J. F. Dreckman, *J. Chromatogr. Sci.*, 9 (1971) 645.
5 R. J. Dolphin, *J. Chromatogr.*, 83 (1973) 421.
6 S. J. Mulé, *J. Chromatogr.*, 39 (1969) 302.
7 J. E. Evans, *Anal. Chem.*, 45 (1973) 2428.
8 D. Ishii, K. Asai, K. Hibi, T. Jonokuchi and M. Nagaya, *J. Chromatogr.*, 144 (1977) 157.