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APPLICATION OF ULTRA-MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO TRACE ANALYSIS

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SUMMARY

A pre-column concentration technique in ultra-micro high-performance liquid chromatography has been developed and applied to trace analysis. The micro pre-columns are composed of PTFE tubing (3–12 × 0.1–0.2 mm I.D.) packed with commercially available materials. Components in water or samples are collected in a micro pre-column and then separated on ultra-micro packed columns (*ca.* 10 cm × 0.12 mm I.D.). Polynuclear aromatic hydrocarbons, phthalates and corticosteroids can be separated on these columns, in spite of the small dimensions. Phthalates in water and corticosteroids in serum have been determined by this technique.

INTRODUCTION

The use of micro-bore columns in high-performance liquid chromatography (HPLC) has several advantages: (1) consumption of the mobile phase, which is sometimes toxic, is low; (2) the amounts of packing materials can be reduced; (3) the amounts of sample required for analysis are small, which is especially favourable *in vivo*; (4) the low flow-rate of the mobile phase facilitates the direct combination of a liquid chromatograph with a mass spectrometer.

Ishii and co-workers have developed micro-HPLC[1] and examined various modes of chromatography with columns of 3–20 cm × 0.5 mm I.D.[2–7]. The internal volume of these columns was one-hundredth that of conventional HPLC columns. Since solute band broadening in a micro-HPLC column is small, solutes can be detected sensitively.

We have recently developed ultra-micro-HPLC and examined the parameters which affect the column efficiency[8]. Pyrex glass columns gave the highest efficiencies and satisfactory separations of aromatic hydrocarbons were obtained with columns of 3–10 cm × 0.12 mm I.D. The dimensions of columns employed in ultra-micro-HPLC are about one-tenth of those of micro-HPLC and one-thousandth of those of conventional HPLC. The sensitivity increased by a factor of ten compared with that in micro-HPLC and the consumption of the mobile phase could be markedly reduced.

In ultra-micro-HPLC the amounts of sample injected should be as small as

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possible (around 0.02 μl). Thus, the pre-treatment of a sample prior to injection is indispensable in the case of the analysis of constituents existing in dilute solutions.

The micro pre-column method was useful for the analysis of samples *in vivo*, as previously reported[4]. The pre-columns were composed of PTFE tubing (1–3 cm × 0.5 mm I.D.). The samples were first placed on the micro pre-column and then required solutes eluted by a mobile phase. The solutes were then loaded onto a separation column.

We now describe the use of the micro pre-column method in ultra-micro-HPLC, and the application of this technique to trace analysis.

EXPERIMENTAL

The apparatus employed was nearly the same as described previously[8]. Pyrex glass tubing was selected as the column material since a pyrex glass column gave the highest efficiency of all the materials examined.

The micro pre-column was composed of PTFE tubing (0.1–0.2 mm I.D.) packed with commercially available packings. After the concentration step, the pre-column was connected to a separation column by stainless-steel tubing (3–5 × 0.13 mm I.D.), as shown in Fig. 1. For the analysis of corticosteroids, the pre-column was freed from water using an Hitachi rotary oil vacuum pump (Hitachi, Tokyo, Japan).

Both separation and collection columns were packed with commercially available packing materials: silica ODS SC-01 (5 μm; Japan Spectroscopic, Hachioji-shi, Japan); Develosil ODS (3, 5 and 10 μm; Nomura Chemical, Setoshi, Japan); porous polymer Hitachi GEL No. 3011 (12 μm, Hitachi) and Nucleosil NH_2 (10 μm). The preparation of these micro-bore columns was as described previously[8].

The sample solution was passed into a pre-column by one of two methods: (1) manual feeding using a gas-tight syringe (1 ml) or (2) feeding via a glass pipe (*ca.* 0.3 mm I.D.) and nitrogen pressure (*ca.* 20 atm). The gas-tight syringe and glass pipe were carefully washed with organic solvents and distilled water prior to use, otherwise solutes were sometimes adsorbed on the vessel walls. For a micro pre-column (5 × 0.2 mm I.D.) packed with 10-μm materials, 5–20 min were required to pass 1 ml of an aqueous solution. The performance of the pre-columns was examined with poly-nuclear aromatic hydrocarbons and phthalates as samples.

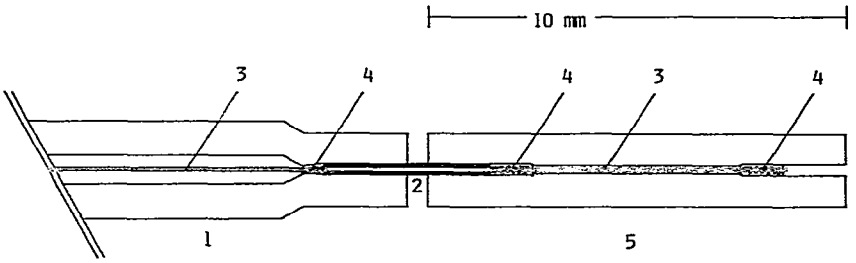


Fig. 1. Schematic diagram of a micro pre-column and the inlet of a separation column. 1 = Separation column; 2 = stainless-steel tubing (3–5 × 0.13 mm I.D.); 3 = packing; 4 = quartz wool; 5 = micro pre-column.

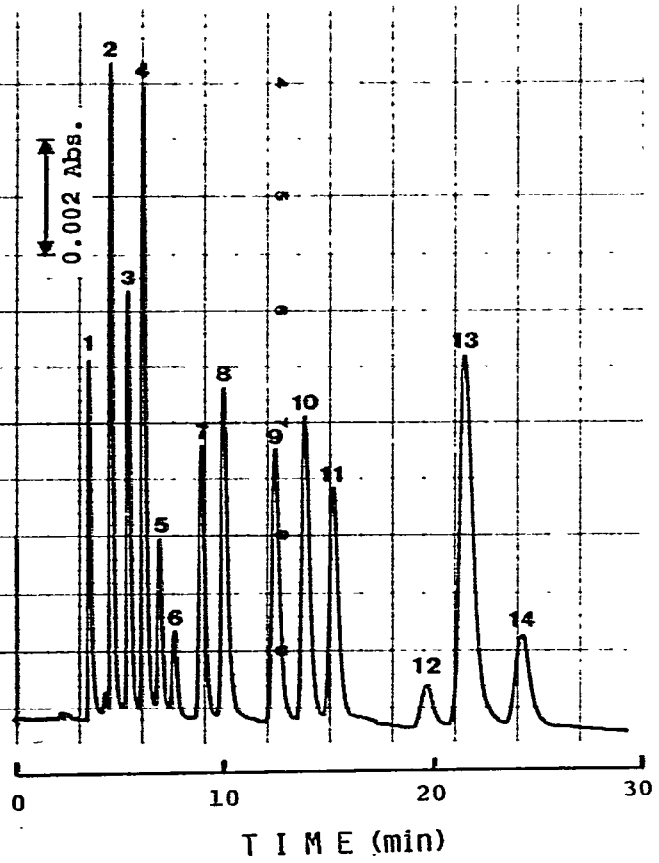


Fig. 2. Separation of aromatic hydrocarbons on an ultra-micro column (10.1 cm × 0.12 mm I.D. Pyrex glass capillary) packed with silica ODS SC-01. Mobile phase: acetonitrile–water (65:35); flow-rate 0.83 μl/min. Sample: 1 = 130 ng benzene; 2 = 11 ng naphthalene; 3 = 2.4 ng biphenyl; 4 = 3.4 ng fluorene; 5 = 0.6 ng phenanthrene; 6 = 0.4 ng anthracene; 7 = 3.4 ng fluoranthene; 8 = 3.4 ng pyrene; 9 = 3.4 ng *p*-terphenyl; 10 = 1.0 ng chrysene; 11 = 1.3 ng 9-phenylanthracene; 12 = 1.0 ng perylene; 13 = 4.1 ng 1,3,5-triphenylbenzene; 14 = 1.1 ng 3,4-benzopyrene. Wavelength of UV detection: 254 nm.

All reagents were purchased from Wako (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan). Horse serum was filtered through a membrane filter (0.45 μm) prior to use.

RESULTS AND DISCUSSION

Performance of ultra-micro columns

Despite the fact that the internal volume of an ultra-micro column (10 cm × 0.12 mm I.D.) is only about 1 μl, sufficient separability is observed. Fig. 2 illustrates the separation of polynuclear aromatic hydrocarbons. Solute amounts less than 1 ng can be detected since there is little spreading through the column; solute band broadening is only 0.3–1.5 μl.

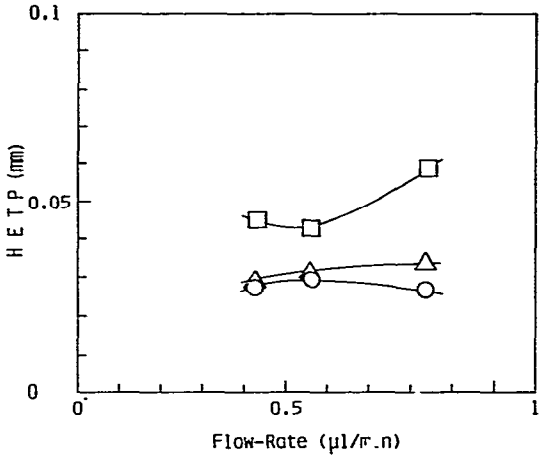


Fig. 3. Influence of particle diameter of packing materials on column efficiency. Columns: ○, 4.9 cm × 0.12 mm I.D., packed with Develosil ODS, 3 μm; △, 10.1 cm × 0.12 mm I.D., packed with Develosil ODS, 5 μm; □, 10.1 cm × 0.12 mm I.D., packed with Develosil ODS, 10 μm. Mobile phases: ○, □, acetonitrile–water (60:40 v/v); △, acetonitrile–water (65:35 v/v). Sample: pyrene.

The influence of the particle diameter of the packing materials on column efficiency is shown in Fig. 3. A column packed with smaller particles gave a lower value of the height equivalent to a theoretical plate (HETP). However, the pressure drop over the column, which was packed with 3-μm particles, was larger than usual. Thus, columns packed with 5–10 μm packings were effective in ultra-micro-HPLC.

Examination of the micro pre-column method

The effect of pre-column length on height equivalent to an effective theoretical plate (HEETP) was examined using SC-01 as the packing for both the separation and collection columns. Fig. 4 shows that a slightly lower HEETP value was observed for

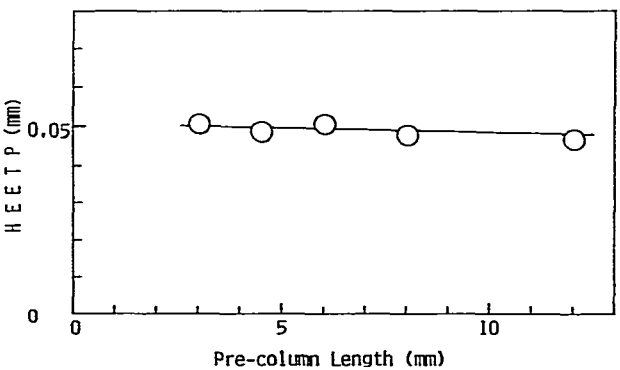


Fig. 4. Effect of pre-column length on column efficiency. Column: 10.1 cm × 0.12 mm I.D. packed with SC-01. Pre-column: 0.15 mm I.D., packed with SC-01. Mobile phase: acetonitrile–water (65:35); flow-rate 0.83 μl/min. Sample: 58 ppb anthracene in distilled water; volume 10 μl. Wavelength of UV detection: 254 nm.

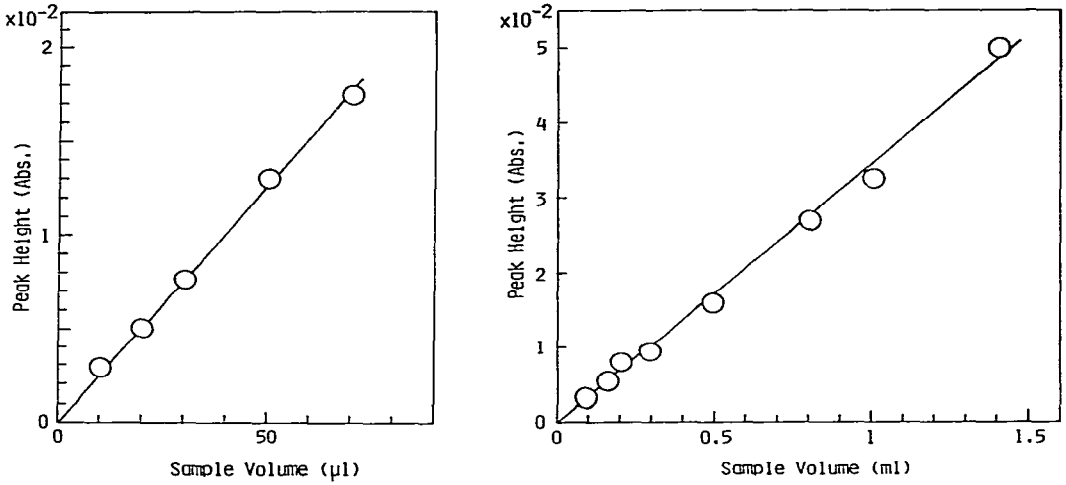


Fig. 5. Relationship between sample volume and peak height. Pre-column: 4 × 0.15 mm I.D. Sample: 42 ppb phenanthrene in distilled water. Other operating conditions as in Fig. 4.

Fig. 6. Relationship between sample volume and peak height. Column: 10.1 cm × 0.12 mm I.D., packed with SC-01. Pre-column: 4 × 0.15 mm I.D., packed with SC-01. Mobile phase: acetonitrile–water (65:35); flow-rate 0.83 µl/min. Sample: 20 ppb diisopropyl phthalate in distilled water. Wavelength of UV detection: 230 nm.

the longer pre-column. However, too much time was required to pass an aqueous solution through this column. The peak heights of solutes were similar for the different lengths examined, indicating that solutes were collected completely in a micro pre-column. Thus, pre-columns with dimensions of 4–6 × 0.1–0.2 mm I.D. were selected for the following studies; the internal volume was 0.03–0.19 µl.

Fig. 5 shows the relationship between sample volume and peak height, which is linear in the range 10–70 µl. A linear relation was also observed in the range 0.1–1.4 ml, Fig. 6. In the former case 0.42–2.9 ng phenanthrene and in the latter case 2–28 ng diisopropyl phthalate were collected, respectively. In addition, similar HEETP values were obtained, independent of sample volume.

Fig. 7 shows the relationship between sample concentration and peak height, which is linear in the range 0.02–0.88 ppm. The samples were prepared in acetonitrile–water (20:80). The effect of sample concentration on the column efficiency was negligible, as illustrated in Fig. 7. A linear relationship between sample concentration and peak height was obtained at low concentrations (1–20 ppb*), Fig. 8.

For dilute sample solutions, the problem of adsorption of solutes on the vessel walls is serious. Ogan *et al.*[9] found that adsorption could be reduced by treating glass walls with dimethyldichlorosilane. In contrast, we found that addition of 1 ppm of ethylene glycol to a sample solution was sufficient to reduce adsorption of phthalates.

The non-zero intercept in Fig. 8 indicates that di-*n*-butyl phthalate is present as an impurity in distilled water and/or ethylene glycol. Unfortunately, di-*n*-butyl

* Throughout this article, the American billion (10^9) is meant.

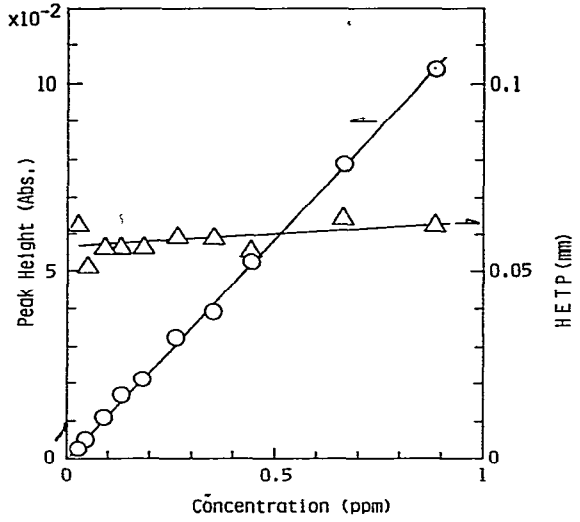


Fig. 7. Dependence of peak height and column efficiency on sample concentration. Sample: phenanthrene in acetonitrile–water (20:80); volume 15 μl. Other operating conditions as in Fig. 4.

phthalate and diisobutyl phthalate have the same retention in the reversed-phase system employed. Ishida *et al.*[10] reported that di-*n*-butyl phthalate was present as an impurity in water and various organic solvents. The content of di-*n*-butyl phthalate was determined, assuming the absence of diisobutyl phthalate, to be *ca.* 3 ppb in distilled water containing 1 ppm ethylene glycol.

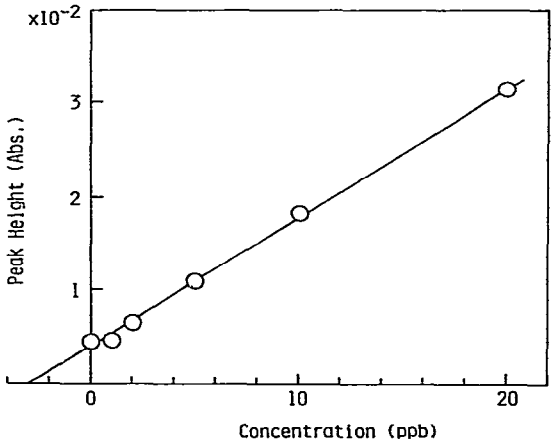


Fig. 8. Relationship between sample concentration and peak height. Column: 9.9 cm × 0.12 mm I.D., packed with SC-01. Pre-column: 5–6 × 0.15 mm I.D., packed with SC-01. Mobile phase: acetonitrile–water (65:35); flow-rate 0.83 μl/min. Sample: di-*n*-butyl phthalate in distilled water containing 1 ppm ethylene glycol; volume 1 ml. Wavelength of UV detection, 230 nm.

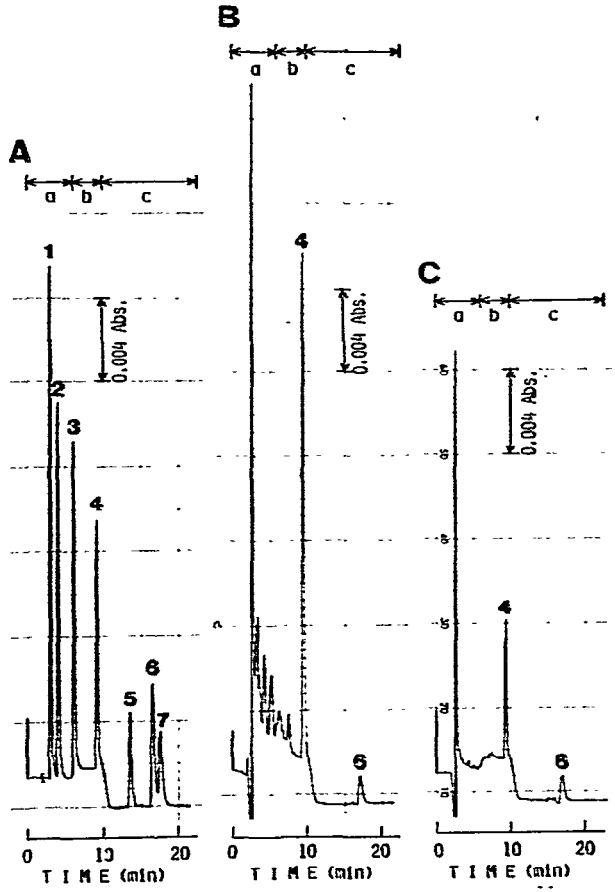


Fig. 9. Analysis of phthalates in water. Column: 10.2 cm × 0.12 mm I.D., packed with SC-01. Pre-column: 5 × 0.19 mm I.D., packed with Develosil ODS (10 μm). Mobile phases: acetonitrile–water (55:45) (a); (65:35) (b); (90:10) (c); flow-rate 0.83 μl/min. Samples: A, 1 = 9.9 ng dimethyl; 2 = 9.8 ng diethyl; 3 = 9.8 ng diisopropyl; 4 = 9.8 ng di-*n*-butyl; 5 = 6.6 ng diheptyl; 6 = 7.3 ng di-2-ethylhexyl; 7 = 8.3 ng dinonyl phthalate; B, deionized water, 200 μl; C, tap-water, 400 μl. Wavelength of UV detection: 235 nm.

Application to trace analysis

The pre-column method was applied to the analysis of phthalates in water. Fig. 9A shows the stepwise gradient separation of standard mixtures of phthalates. Gradient elution was performed as follows. Prior to a chromatographic run, different proportions of mobile phases were stored in a fused silica glass capillary tubing (0.25 mm I.D.) in the order as indicated in Fig. 9 and then forwarded onto the separation column via the injection part by feeding the third solution (c) from a pump. A sample solution was sucked in the top of the mobile phase manually.

Fig. 9B and C shows the analysis of phthalates in deionized and tap-water using the pre-column method, respectively. Solutes were identified from their retention times and the amounts of di-*n*-butyl and di-2-ethylhexyl phthalate were determined from standard samples. The results are given in Table I.

Fig. 10 shows the analysis of drained water from a sewage treatment plant. A

TABLE I

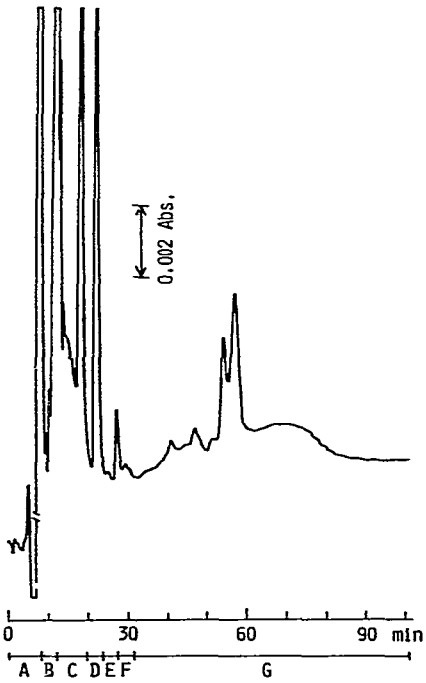
ANALYTICAL RESULTS FOR PHTHALATES IN WATER

DBP = di-*n*-butyl phthalate; DEHP = di-2-ethylhexyl phthalate.

Sample	*Concentration (ppb)*	
	DBP	*DEHP*
Deionized water	101	8.4
Tap-water	14	4.0
Distilled water	1.5	2.8

number of peaks were detected. Unfortunately the solutes could not be identified, but use of a mass spectrometer may solve this problem.

The analysis of corticosteroids can be performed with the pre-column method, as previously reported[4], using either reversed- or normal-phase systems. In this work, the latter mode was adopted. However, trace amounts of water remaining in the



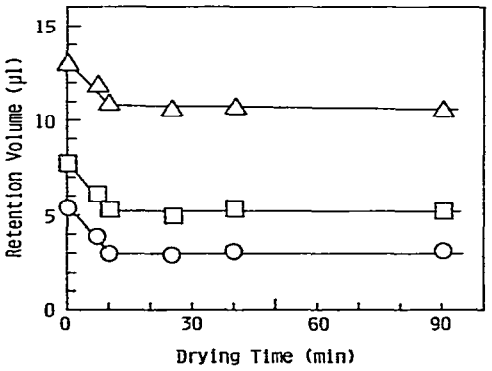


Fig. 10. Analysis of drained water from a sewage treatment plant. Column: 10.0 cm × 0.12 mm I.D., packed with SC-01. Pre-column: 4 × 0.2 mm I.D., packed with SC-01. Mobile phases: acetonitrile–water (3:7) (A); (4:6) (B); (5:5) (C); (6:4) (D); (7:3) (E); (8:2) (F); (9:1) (G). Sample: drained water, 100 µl. Flow-rate: 0.42 µl/min. Wavelength of UV detection: 220 nm.

Fig. 11. Influence of drying time on solute retention. Column: 10.1 cm × 0.12 mm I.D., packed with Nucleosil NH_2. Pre-column: 4–6 × 0.19 mm I.D., packed with Hitachi GEL No. 3011. Mobile phase: dichloromethane saturated with water–methanol (98.5:1.5); flow-rate 0.83 µl/min. Samples: ○, corticosterone; □, cortisone; △, cortisol.

TABLE II

EFFECT OF THE INJECTION METHOD ON COLUMN EFFICIENCY

Operating conditions as in Fig. 11.

Injection method	*Corticosterone*		*Cortisone*		*Cortisol*	
	HETP (mm)	*Retention volume (μl)*	*HETP (mm)*	*Retention volume (μl)*	*HETP (mm)*	*Retention volume (μl)*
On-column injection	0.086	3.0	0.11	5.0	0.12	10.5
Pre-column injection	0.074	3.1	0.077	5.3	0.090	10.6

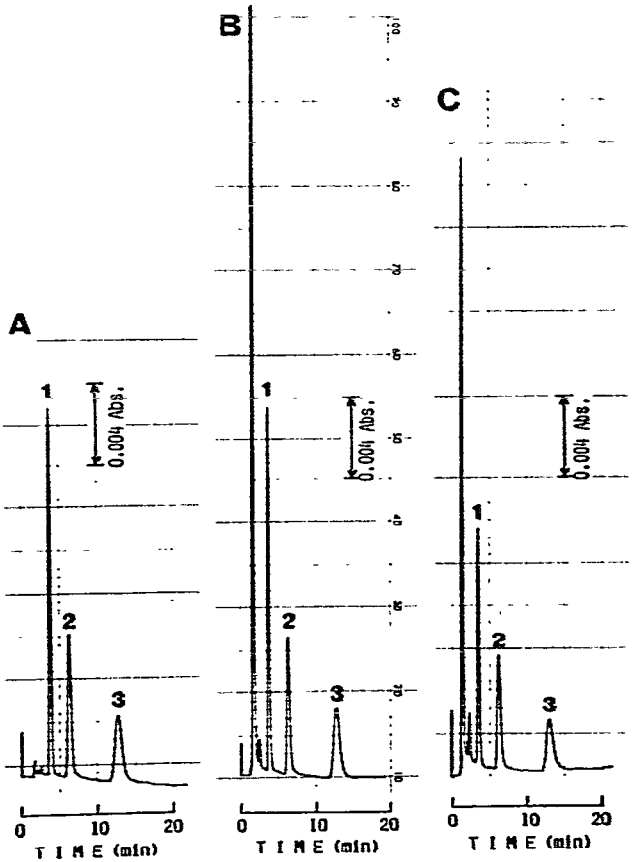


Fig. 12. Analysis of corticosteroids. Conditions as in Fig. 11. Samples: A, 0.024 μl dichloromethane solution containing 0.019% (w/w) corticosterone (1), 0.017% cortisone (2) and 0.016% cortisol (3); B, 500 μl of aqueous solution containing 12 ppb corticosterone, 11 ppb cortisone and 9.9 ppb cortisol; C, 16 μl of horse serum in 144 μl distilled water. Wavelength of UV detection, 240 nm.

collection column affect the separation of solutes. Thus, after a concentration step, the micro pre-column was dried *in vacuo* by using a rotary oil pump.

Fig. 11 shows the influence of drying time on the retention of corticosteroids. Drying for longer than 10 min gave constant solute retention.

Table II shows the effect of the injection method on column efficiency. Despite the fact that the extra-column dead volume for the pre-column injection is larger than for the on-column injection, lower HETP values are observed with the former method. This indicates that collected solutes are removed from the collection column in a narrower band by the mobile phase and loaded onto the separation column quantitatively.

Separations of standard mixtures of corticosteroids are shown in Fig. 12A and B, with on-column injection and pre-column injection, respectively. Fig. 12C shows the analysis of corticosteroids in horse serum, which was diluted in distilled water ten times after filtration. In previous work[4], certain components interfered with the determination of corticosterone; here this problem was absent. This difference seems to be due to the difference in packing materials: silica gel (used previously) and silica-NH_2. In 1 ml of horse serum 0.24 μg corticosterone, 0.28 μg cortisone and 0.23 μg cortisol were present.

CONCLUSION

The pre-column method developed in this work is useful for the analysis of constituents in natural water and *in vivo*. In combination with mass spectrometry, this technique has great potential.

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