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STUDIES OF MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

IV. APPLICATION OF THE MICRO PRE-COLUMN METHOD TO THE ANALYSIS OF CORTICOSTEROIDS IN SERUM

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SUMMARY

The micro pre-column method, which is a useful method for the pre-treatment of samples, was applied to the analysis of corticosteroids in serum. Corticosteroids were concentrated in a micro pre-column packed with porous styrene–divinylbenzene copolymer particles and separated in a micro separation column packed with porous silica particles. Concentrations of corticosterone, cortisone and cortisol in the 20–130 ppb range were determined individually by pre-treatment of only 0.2 ml of serum.

INTRODUCTION

Although there have been marked advances in high-performance liquid chromatography (HPLC) recently, sometimes pre-treatment of the sample is required in order to give a more suitable concentration or to separate the matrix or macro components from the sample.

For the determination of steroids, colorimetric and fluorimetric analysis have commonly been employed. However, in these methods, solvent extraction, column chromatography or thin-layer chromatography is needed in order to remove the matrix or macro components from the samples. Fluorimetric analysis has mainly been employed for the determination of corticosteroids in urea and blood, but difficult solvent extractions must be carried out and more complex pre-treatments are required for the separation of corticosterone, cortisone and cortisol, so that the amounts of sample required may be large.

Gas chromatography (GC) and HPLC have recently been employed for the analysis of steroids. However, it is often necessary to prepare suitable derivatives of the steroids before GC analysis, because of the high boiling points and instability towards heat or the presence of hydroxy groups in the parent compounds[1,2]. These disadvantages are avoided when HPLC is used[3–6], but some extraction and concen-

tration steps are necessary in the analysis of corticosteroids in blood and a sample of several millilitres must be treated.

We have previously adapted HPLC to the micro scale, using the abbreviation MHPLC[7]. In addition, we have developed a micro pre-column method as a pre-treatment method suitable for use with MHPLC, instead of conventional solvent extractions; the applicability of this method was demonstrated by the pre-treatment of aqueous phthalate ester solutions[8]. In this method, sample components are adsorbed by passing the sample solution through a packed micro pre-column. This pre-column is then connected to the top of a micro separation column and the adsorbed components are eluted from the former column with an appropriate mobile phase and developed on the latter column. The amounts of sample solution required are very small and the sample components can be pre-concentrated simply and rapidly. Therefore, this method may be very useful for the analysis of *in vivo* samples, especially when the amounts of sample supplied for analysis are very small or rapid analysis is required. In this paper, the application of the micro pre-column method to the analysis of corticosteroids in serum is described.

EXPERIMENTAL

Preparation of micro pre-columns and micro separation columns

These columns were prepared in PTFE tubing as described previously[7]. A micro pre-column is shown schematically in Fig. 1A. The pre-columns used for the pre-treatment of aqueous phthalate ester solutions in Part III[8] consisted of stainless-steel tubing (18 mm × 0.35 mm I.D.). However, use of such tubing tended to make the packing of particles difficult and the condition of the packing could not be readily observed. Therefore, semi-transparent PTFE tubing (0.5 mm I.D. and 2.0 mm O.D.) was used for the micro pre-columns in this work. The micro pre-columns were *ca.* 10–30 mm long and of 0.5 mm I.D. These micro pre-columns could be prepared more easily than the stainless-steel pre-columns.

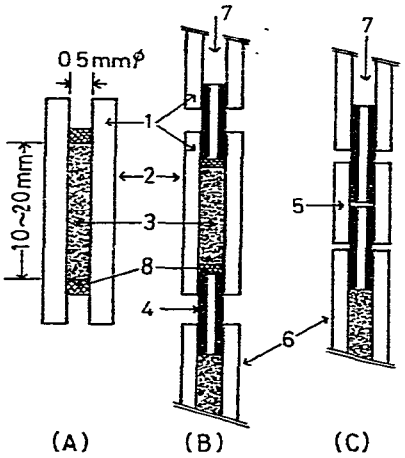


Fig. 1. Schematic diagrams of the micro pre-column and the connection to the micro separation column. 1 = PTFE tube; 2 = micro pre-column; 3 = packing material; 4 = stainless-steel tube; 5 = PTFE adapter tube; 6 = micro separation column; 7 = mobile phase; 8 = quartz-wool.

The micro pre-column could be connected to a micro separation column by using stainless-steel connecting tubing (*ca.* 10 mm long), as shown in Fig. 1B. Such a connection resulted in a void space of *ca.* 1 μl, but it did not have an undesirable influence on the separations of interest.

Fig. 1C shows the connection for the situation when the sample solution is injected directly into the micro separation column.

Chromatography

A Familic-100 instrument (Japan Spectroscopic, Tokyo, Japan) was employed for feeding the mobile phase and direct injection of samples into the micro separation column. This instrument can deliver the mobile phase at a very slow flow-rate (2, 4, 8 or 16 μl/min). A micro amount of sample solution (0.1–10 μl) can also be injected with this instrument. The mechanisms of the delivery of the mobile phase and the injection of samples in the Familic-100 are similar to those described previously[7].

The pre-treatment with the micro pre-column and the development of the absorbed components on the micro separation column were carried out as described previously[8]; these procedures are outlined in Fig. 2.

A Uvidec-100 UV spectrophotometer (Japan Spectroscopic) was used as the detector and the micro flow-through cells consisted of a quartz tube of I.D. 0.3–0.4 mm with a volume of 0.05–0.4 μl. The wavelength used for the detection of corticosteroids was 240 nm.

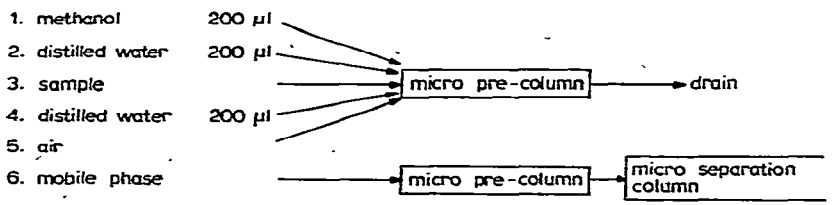


Fig. 2. Procedure for pre-treatment with the micro pre-column.

RESULTS AND DISCUSSION

Selection of packing materials for the separation of corticosteroids

The column efficiencies (capacity factor, HETP and resolution) of various packing materials were determined by using a standard solution of corticosterone, cortisone and cortisol. Zorbax ODS, Hitachi Gel No. 3010 (porous polymer particles, 18–22 μm), TSK-Gel LS-111 (porous polymer particles, 5 μm), TSK-Gel LS-310 (porous silica particles), Yanaco SIL S_A1 (spherical porous silica beads), Jasco SS-05 (porous silica beads, 5 μm) and Zorbax SIL were examined for use in micro separation columns. TSK-Gel LS-310, Hitachi Gel No. 3010 and Yanaco SIL S_A1 were inadequate as packings for the separation columns, as they gave poor column efficiencies and poor separations of corticosteroids. Zorbax ODS and TSK-Gel LS-111 gave satisfactory column efficiencies and separations. However, in the separation of corticosteroids in real serum, peaks due to macro components interfered.

Corticosteroids could be separated completely and rapidly by using a column packed with Zorbax SIL or Jasco SS-05, as shown in Fig. 3B, and macro components did not interfere because they had lower retention volumes than the corticosteroids. When a silica packing such as Jasco SS-05 was used in the separation columns, there was the risk of adverse effects on the separation column due to residual water in the micro pre-column. However, when drying of the pre-column in the procedure for pre-treatment shown in Fig. 2 was carried out adequately, a significant decrease in column efficiency and resolution in the separation column was not observed in many experiments.

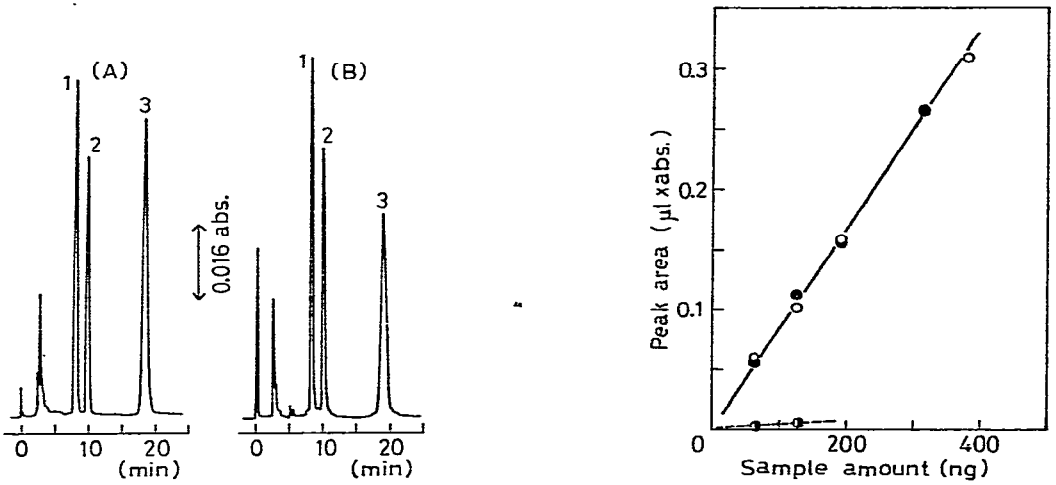


Fig. 3. Separation of corticosteroids. Peaks: 1 = corticosterone; 2 = cortisone; 3 = cortisol. Separation column: Jasco SS-05, 145 mm × 0.5 mm I.D. Mobile phase: dichloromethane–methanol (97:3); flow-rate, 8 μl/min. Wavelength of detection: 240 nm. (A) Separation of sample components in water pre-treated with micro pre-column. Sample concentrations: 1.2 ppm of corticosterone, 1.1 ppm of cortisone and 1.8 ppm of cortisol in water. Sample volume: 200 μl. Pre-column: Hitachi Gel No. 3010, 15 mm × 0.5 mm I.D. (B) Separation of sample components in dichloromethane injected directly into the separation column. Sample concentrations: $1.4 \cdot 10^{-2}$% of corticosterone, $1.8 \cdot 10^{-2}$% of cortisone and $2.0 \cdot 10^{-2}$% of cortisol in dichloromethane. Sample volume: 1 μl.

Fig. 4. Calibration graphs with pre-columns packed with different packing materials. Pre-column packings: ●, Hitachi Gel No. 3010; ○, Zorbax ODS; ◑, Permaphase ODS. Pre-column: 15 mm × 0.5 mm I.D. Sample concentration: 38 ppm of cortisol in water. Sample volume: 1–10 μl. Separation column: TSK-Gel LS-111, 150 mm × 0.5 mm I.D. Mobile phase: methanol; flow-rate, 4.2 μl/min. Wavelength of detection: 240 nm.

Finally, 14 cm × 0.5 mm I.D. PTFE columns packed with Jasco SS-05 were empioyed for the micro separation column for the analysis of corticosteroids in serum, and dichloromethane (water saturated)–methanol (97:3) was mainly used as the mobile phase.

Selection of packing for the micro pre-column

Non-polar packing materials such as porous polymer or chemically bonded ODS packings were examined for use in the micro pre-column. The efficiency of

adsorption of sample components was studied with three types of packings, *viz.*, Hitachi Gel No. 3010, Zorbax ODS and Permaphase ODS. An aqueous solution of cortisol was employed as the sample solution, as it was most difficult for cortisol to be adsorbed in the micro pre-column because of its polarity. Permaphase ODS was inadequate as a packing for the pre-column, as cortisol was adsorbed only slightly on it, as shown in Fig. 4, because of the inadequate surface area. Zorbax ODS and Hitachi Gel No. 3010 adsorbed cortisol quantitatively, as shown in Fig. 4, but Zorbax ODS was unsuitable because the small particle size (8–9 μm) made rapid passage of the aqueous solution of cortisol difficult. Use of such fine packings in the micro pre-column make it too time-consuming to pass large amounts of dilute sample solutions. Hitachi Gel No. 3010 had an appropriate particle diameter (*ca.* 18–22 μm) and sample solutions could be made to flow easily and rapidly through the micro pre-column. Hitachi Gel No. 3010 was therefore chosen as the packing for the micro pre-column.

Influence of pre-treatment with the micro pre-column on the separation of sample components

Fig. 3B shows a typical chromatogram obtained when 1 μl of a dichloromethane solution of corticosteroids was injected directly into the micro separation column. Fig. 3A shows a typical chromatogram obtained when an aqueous solution of corticosteroids was pre-concentrated by the micro pre-column method and was then separated in the micro separation column. The retention time, column efficiency and resolution in Fig. 3A are almost same as those in Fig. 3B. Hence the pre-treatment in the micro pre-column did not have an adverse effect on the separation of corticosteroids in the separation column.

The influence of the amount of sample on the separation of sample components was examined by pre-treating 10–1000 μl of an aqueous solution containing 1.2 ppm of corticosterone, 1.1 ppm of cortisone and 1.8 ppm of cortisol by the micro pre-column method. As shown in Table I, the retention times and column efficiencies are almost identical, regardless of the amount of sample. Hence the amount of sample used did not affect the separation of the sample components.

TABLE I

INFLUENCE OF SAMPLE SIZE ON SEPARATION

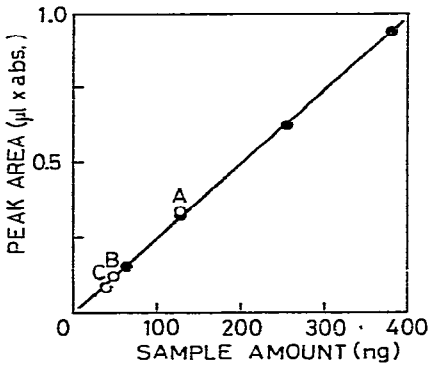
*Sample volume** (μl)	*Corticosterone*		*Cortisone*		*Cortisol*	
	R_t (min)	HETP (mm)	R_t (min)	HETP (mm)	R_t (min)	HETP (mm)
10	7.87	0.056	9.42	0.053	17.6	0.061
20	7.78	0.058	9.37	0.063	17.5	0.061
50	7.77	0.058	9.30	0.065	17.5	0.057
100	7.88	0.058	9.45	0.068	17.5	0.057
200	8.04	0.057	9.63	0.059	17.9	0.055
500	7.72	0.064	9.25	0.064	17.0	0.061
1000	8.00	0.060	9.60	0.061	17.4	0.065

* Concentrations of sample components: corticosterone, 1.2 ppm; cortisone, 1.1 ppm; cortisol, 1.8 ppm.

Efficiency of adsorption in the micro pre-column

A 1–10-μl volume of a standard aqueous solution containing 38 ppm of cortisol was pre-treated by the micro pre-column method in order to examine the efficiency of adsorption. The cortisol adsorbed was developed on the micro separation column and the area of the peak on the chromatogram was measured. Fig. 5 shows the calibration graph of amount of cortisol *versus* peak area. The rectilinear relationship indicates that the sample component was adsorbed and then eluted quantitatively for amounts of cortisol up to 400 ng. The points A, B and C in Fig. 5 correspond to the pre-treatment of increasingly dilute aqueous solutions. The fact that these points are situated on the calibration graph shows that very dilute solutions in the parts per billion (10^9) range could also be pre-treated quantitatively by the micro precolumn method.

Fig. 6A shows a chromatogram obtained when the micro pre-column was washed with 5 ml of water after adsorption of the sample components, instead of washing with the usual volume of 200 μl of water. The peak height, retention time, column efficiency and separation were almost identical with those in Fig. 6B (representing the usual washing). In addition, the peak area obtained after washing with 8 ml of water coincided with the calibration graph in Fig. 5. Hence, it was considered that the amount of aqueous sample solution pre-treated in the micro pre-column could be increased up to 8 ml.



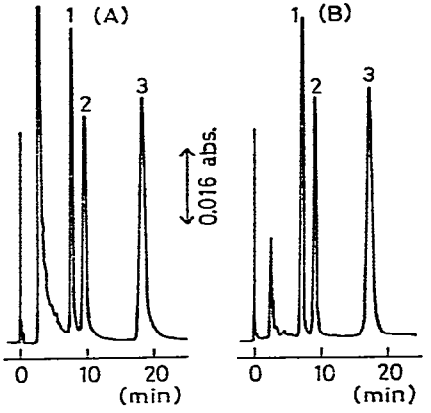


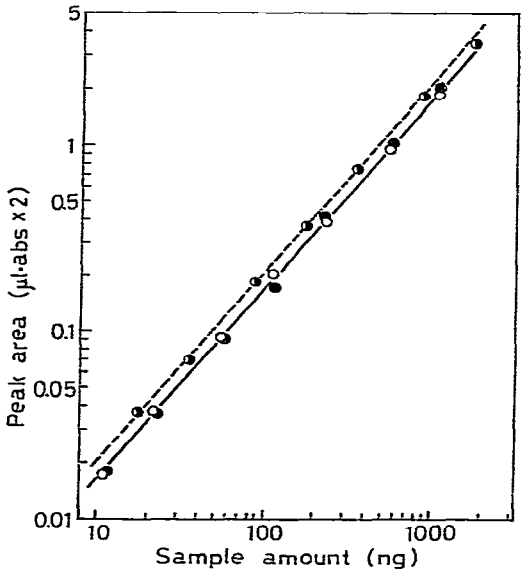
Fig. 5. Calibration graph obtained with a micro pre-column packed with Hitachi Gel No. 3010. Sample: 38 ppm of cortisol in water (1–10 μl); point A, 3.8 ppm of cortisol in water (33.3 μl); point B, 380 ppb of cortisol in water (133 μl); point C, 38 ppb of cortisol in water (1000 μl). Pre-column: 15 mm × 0.5 mm I.D. Separation column: TSK-Gel LS-111, 130 mm × 0.5 mm I.D. Mobile phase: methanol; flow-rate, 4.2 μl/min. Wavelength of detection, 240 nm.

Fig. 6. Influence of washing volume on adsorption and separation. Washing volume: (A) 5 ml; (B) 200 μl. Peaks: 1 = corticosterone; 2 = cortisone; 3 = cortisol. Sample concentrations: 1.2 ppm of corticosterone, 1.1 ppm of cortisone and 1.8 ppm of cortisol in water. Sample volume: 200 μl. Micro pre-column: Hitachi Gel No. 3010, 15 mm × 0.5 mm I.D. Micro separation column: Jasco SS-05, 130 mm × 0.5 mm I.D. Mobile phase: dichloromethane–methanol (97:3); flow-rate, 8 μl/min. Wavelength of detection: 240 nm.

Analysis of corticosteroids in rabbit serum and horse serum

Fig. 7 shows the calibration graphs for corticosterone, cortisone and cortisol over a wide range of concentrations, which were obtained by pre-treating a standard aqueous solution containing 1.2 ppm of corticosterone, 1.1 ppm of cortisone and 1.8 ppm of cortisol by the micro pre-column method. The linearity of the calibration graphs shows that adsorption in the micro pre-column and development on the micro separation column occur quantitatively, so that these calibration graphs can be used for the determinations of corticosteroids. However, in real serum, the various materials present may prevent the quantitative recovery of corticosteroids in the micro pre-column. Therefore, the serum samples were diluted 10-fold with distilled water and 2 ml of this dilute solution was pre-treated on the micro pre-column.

Fig. 8B shows a typical result for rabbit serum and Fig. 8A shows a typical separation of a standard corticosteroid sample. Chromatogram B was identified by comparison with the standard chromatogram A, and it was found that corticosterone was present in rabbit serum. Fig. 8C shows the typical chromatogram for the mixed sample A + B. By comparing these three chromatograms, the recoveries of corticosterone, cortisone and cortisol in serum obtained with the micro pre-column method



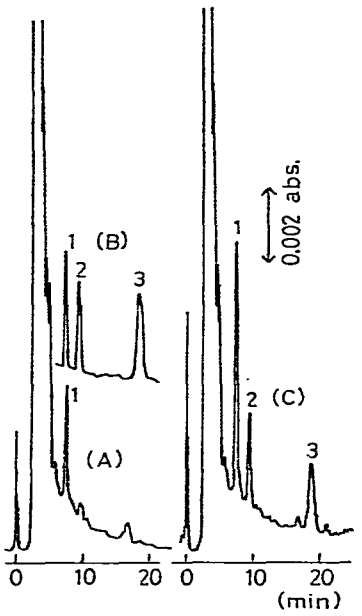


Fig. 7. Calibration graphs for corticosterone, cortisone and cortisol. Sample concentrations: 1.2 ppm of corticosterone, 1.1 ppm of cortisone and 1.8 ppm of cortisol in water. Sample volume: 10–1000 µl. Micro pre-column: Hitachi Gel No. 3010, 15 mm × 0.5 mm I.D. Micro separation column: Jasco SS-05, 145 mm × 0.5 mm I.D. Mobile phase: dichloromethane–methanol (97:3); flow-rate, 8 µl/min. Wavelength of detection: 240 nm. ●, Corticosterone; ○, cortisone; ◑, cortisol.

Fig. 8. Separation of rabbit serum. Sample: (A) 0.2 ml of rabbit serum in 1.8 ml of water; (B) 1.2 ppm of corticosterone, 1.1 ppm of cortisone and 1.8 ppm of cortisol in water (10 µl); (C) sample A + sample B. Pre-column: Hitachi Gel No. 3010, 30 mm × 0.5 mm I.D. Separation column: Jasco SS-05, 130 mm × 0.5 mm I.D. Mobile phase: dichloromethane–methanol (97:3); flow-rate, 8 µl/min. Wavelength of detection: 240 nm.

were measured by the addition method. The recoveries were *ca.* 100% for corticosterone and cortisone and *ca.* 90% for cortisol. The accuracy was about 15%.

By a similar method, corticosteroids in horse serum were analysed; corticosterone, cortisone and cortisol were found, although corticosterone was not determined because of a shoulder in the peak due to interfering substances.

The overall analytical results are given in Table II, and indicate that 10 ng of corticosterone in 0.2 ml of rabbit serum and 13 ng of cortisone and 4 ng of cortisol in 0.2 ml of horse serum were determined.

TABLE II

ANALYTICAL RESULTS

*Sample**	*Corticosterone (ng)*	*Cortisone (ng)*	*Cortisol (ng)*
Rabbit serum	10	—	—
Horse serum	—**	13	4

* Amounts of serum used, 0.2 ml.

** Corticosterone was not determined because of interfering components.

CONCLUSION

The micro pre-column method appears to be applicable to the analysis of medical or biochemical samples, such as corticosteroids in real serum, because the amounts of sample required are small and the analyses can be carried out simply and rapidly. The use of various combinations of micro pre-column and micro separation column should make possible more extensive applications of this method.

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