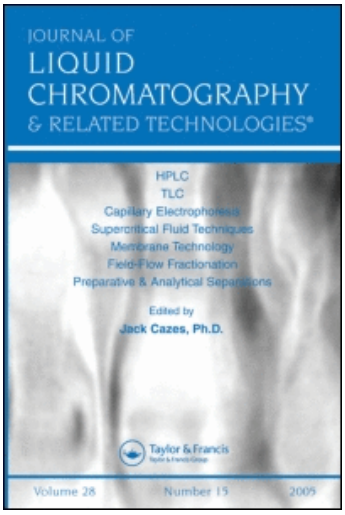

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CHARACTERIZATION OF PACKING COLUMNS FOR THE LIQUID CHROMATOGRAPHIC DETERMINATION OF CORTICOSTEROIDS IN HUMAN PLASMA

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ABSTRACT

Commercially available columns were characterized by examining the retentions of steroid hormones in liquid chromatography. The phase systems used were dichloromethane-ethanol for silica gel, acetonitrile-water for ODS and CN bonded columns. Solvent compositions were optimized so as to make the retention times of standard steroids less than sixty minutes. Retention data were obtained and reproducibility of the retention times was examined by repetitive injections of the standard samples. These chromatographic phase systems were applied to determine steroid hormones in human plasma following extraction by dichloromethane. CN columns showed the best reproducibility and resolution. Changes in the standing of corticosteroids in plasma under general anesthesia were investigated using CN column systems.

INTRODUCTION

The various constituents in biological fluids have often been determined by high performance liquid chromatography (HPLC). Many packings and columns have come to be commercially available, however, for a given

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sample mixture, an arbitrary selection is frequently made. To optimize analytical processes for steroid hormones as important constituents, three typical commercial columns, i.e., silica gel, chemically bonded ODS and CN columns, are examined in this report for characterization of their retentivity and reproducibility of retention. The standard samples used were clinically important steroids including progesterone, cortisone, corticosterone, 4-androstene-3,17-dione, testosterone and 17α-hydroxy-progesterone.

On the basis of plasma steroid hormone determinations, a detailed examination of endocrinological secretions is possible and the abnormal metabolism of steroid hormones can be detected in many cases. The clinical application of steroid hormone assays to determine endocrinological functions is also a possibility. The secretion of corticosteroids is considered to be associated with mental stress and physical load phenomenon.

Phase systems optimized by standard pure samples were examined to determine corticosteroids in human plasma following the pretreatment process involved in organic solvent extraction. The linear relation between the peak area of steroids in the chromatogram and sample amount injected into the columns was experimentally confirmed and the dynamic changes in the plasma corticosteroids of patients under general anethesia were investigated.

EXPERIMENTAL

Apparatus and Columns

A model LC-5A HPLC system (Shimadzu Co., Kyoto) was equipped with the variable wavelength detector model SPD-2A. UV signals were integrated on-line with the data system, Chromatopack CR-1B.

The columns used were silica gel (SIL), octadecyl and cyano-alkyl bonded (ODS and CN) Zorbax columns (Du Pont). The respective particles were 7 μ, 4.6 mm in ID, 250 mm in length. The eluents were organic solvents for SIL and aqueous solvents for the ODS and CN columns.

Samples and Reagents

Progesterone, cortisone, corticosterone, 4-androstene-3,17-dione, testosterone, 17α-hydroxy-progesterone and cortisol were purchased from Sigma. Solvents for the chromatography separation were of analytical grade (Wako Pure Chemicals Co., Osaka). For collection of blood samples, a Benedict blood collector containing EDTA-2K (Thermo Co., Tokyo) was used.

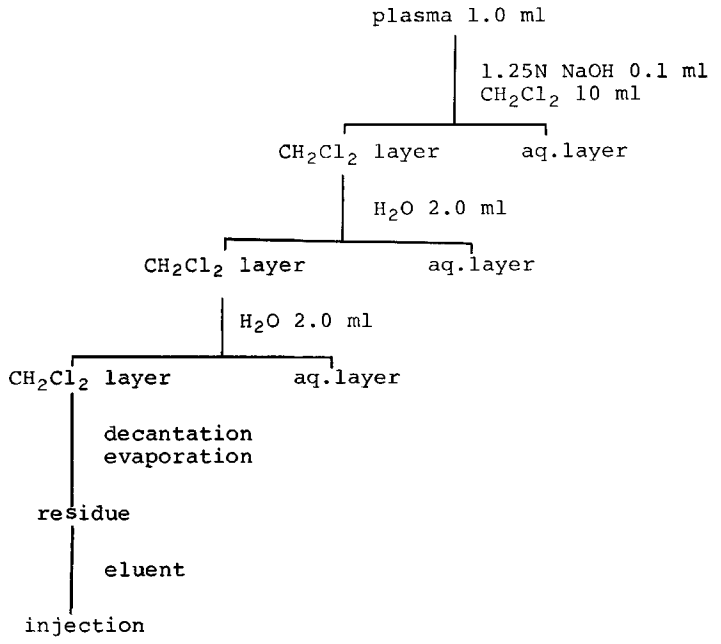

FIGURE 1. Extraction of Steroid Hormones from Plasma

Sample Preparation

The blood samples were collected from male and female normal adults and centrifuged (3000 rpm) for 10 minutes. The resultant plasma was decanted off and stored at -75°C until use for analysis. Frozen plasma was allowed to stand at room temperature and ca. 20°C and 1 ml of plasma was extracted by 10 ml of dichloromethane. The organic layer was washed twise with water and concentrated by evaporation. The flow chart of pre-treatment is shown in FIGURE 1. The extract was dissolved in the mobile phase solvent and injected into the HPLC columns.

Chromatography Procedure

Pure steroid samples were dissolved in the eluents (0.2 - 2 μg/ml). An aliquot (10 μl) of the result solutions was injected into each of the columns. An aliquot of plasma extracts dissolved in the eluents (20 μl) was used for the HPLC analysis. The flow rate of the eluents was 600 μl/min. A monitor detected UV absorption at 254 nm at a sensitivity of 0.005 Aufs. The chromatography was carried out at room temperature.

RESULTS AND DISCUSSION

1. Choice of Mobile Phase Solvents and Optimum Compositions.

To construct a practical separation system, eluents were selected so as to have retention times less than sixty minutes. Binary solvent systems consisting of dichloromethane-ethanol were applied onto the SIL columns. An examination of various compositions of the stronger solvents from 1 to 10 percent in the mobile phase solvents showed a composition of 2 percent to give the best results for given steroid samples. Organic solvent-water systems were applied onto the ODS and CN columns. Various compositions of the organic solvent in the eluents were examined. The optimized compositions of the acetonitrile-water system were 50 : 50 v/v for ODS and 35 : 65 v/v for CN columns. Using these phase systems, the following experiments were carried out.

The standard steroid samples in the eluents (2 - 20 ng/10μl) were injected into three commercial columns for measurement of peak area. The coefficient of variation of the linearity between peak area and steroid sample amount was found to exceed 0.99. This procedure was thus suitable for the quantitative determination of steroid hormones.

2. Reproducibility of Steroid Hormone Retention.

In our experiments, variation in retention time was often observed with the SIL and ODS column systems, although the controlled chromatographic systems and same experimental conditions were used. Consequently, the reproducibility of retention using six steroids for the three columns was examined. The samples were repeatedly injected into the columns under controlled HPLC conditions. The average retention time and coefficient of variation (C. V.) were obtained and are shown in TABLE 1. The C. V. values of the SIL columns were each approximately 1 percent and those of the ODS columns, 0.1 percent for cortisone which afforded the least retention time. However, for progesterone which afforded the large retention time, the C. V. value was 1.7 percent. It is thus apparent that the C. V. values increase with the corresponding retention times. A C. V. value of approximately 0.5 percent was obtained for all steroid samples on using the CN column system. A comparison of the reproducibility of the three column systems showed the CN column system to be the most suitable for our experiment.

TABLE 1. Retention Times and Coefficients of Variation in Steroid Hormones as Determined by the Three Packing Columns

Sample	SIL Column Retention Time (min)	C. V. (%)
Cortisone	37.78	2.1
Corticosterone	27.26	1.5
Testosterone	16.74	0.9
17α-Hydroxy-progesterone	11.51	1.2
4-Androstene-3,17-dione	8.98	1.2
Progesterone	8.36	0.9

ODS Column Retention Time (min)	C. V. (%)	CN Column Retention Time (min)	C. V. (%)
6.99	0.1	9.03	0.5
10.22	0.3	11.45	0.7
18.96	0.8	14.70	0.5
20.57	1.0	17.70	0.6
22.26	1.2	17.70	0.6
50.73	1.7	27.74	0.2

C. V.: coefficient of variation (n = 9)

3. HPLC Determination of Steroid Hormones in Serum.

Optimized phase systems for steroid hormone resolution were applied to determine the constituents in the biological fluid samples. Various phase systems involved in the three SIL, ODS and CN columns were characterized using plasma samples collected from normal male and female adults.

Seven steroids including cortisol were used as reference samples to identify the plasma constituents. Four peaks were identified in the chromatogram as those of cortisol, cortisone, corticosterone and testosterone by co-injection of the standard samples. Typical examples of the chromatogram are illustrated in FIGURE 2. These steroids were quantitatively determined by peak area. The results are shown in TABLE 2.

In this table, the amounts of testosterone in the male and female sera, obtained by the SIL columns, are much higher than those obtained by the other columns. The peaks were thus considered to be contaminated by obstructive unknown impurities. Testosterone values obtained from female blood were much higher than the experimental values in the literature, using the radio immunoassay (1). In contrast, the amounts of corticosterone obtained using the SIL columns were lower than those by the ODS and CN columns. The resolution of cortisol and cortisone could not be accomplished

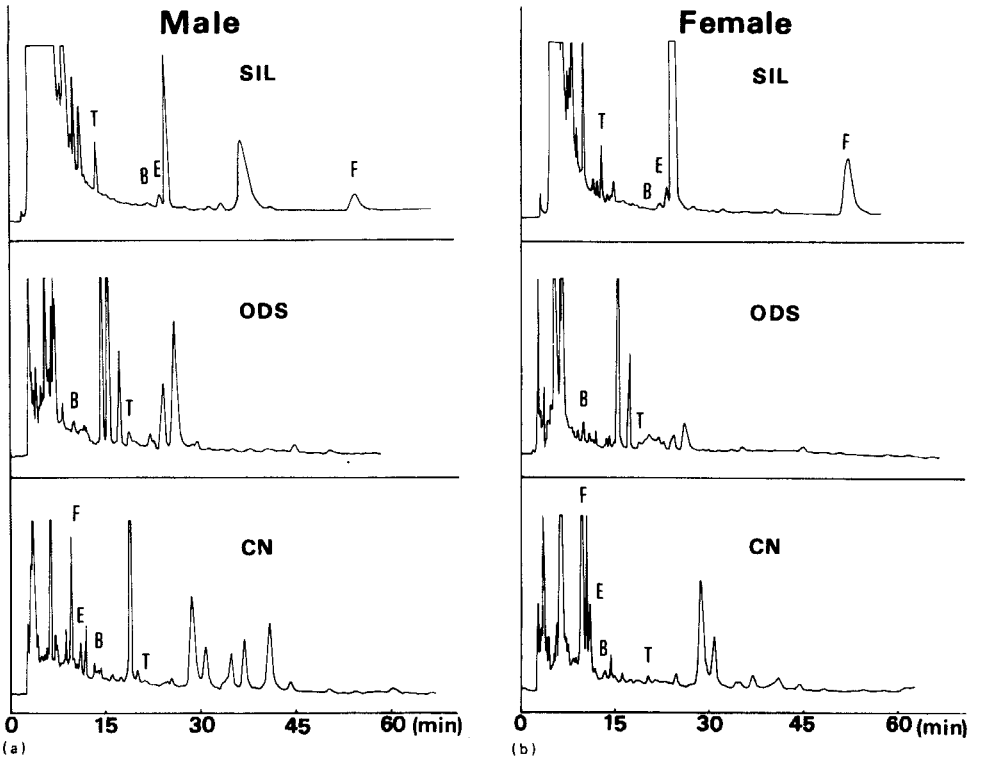

FIGURE 2. Chromatograms of Steroid Hormones from Male and Female Human Plasma by HPLC

Male age: 31 Female age: 22
T: Testosterone B: Corticosterone E: Cortisone F: Cortisol
Mobile phase: SIL, dichloromethane-ethanol (98:2 v/v)
ODS, acetonitrile-water (50:50 v/v)
CN, acetonitrile-water (35:65 v/v)
Flow rate: 0.6 ml/min Detection: UV at 254 nm (0.005 AUFS)

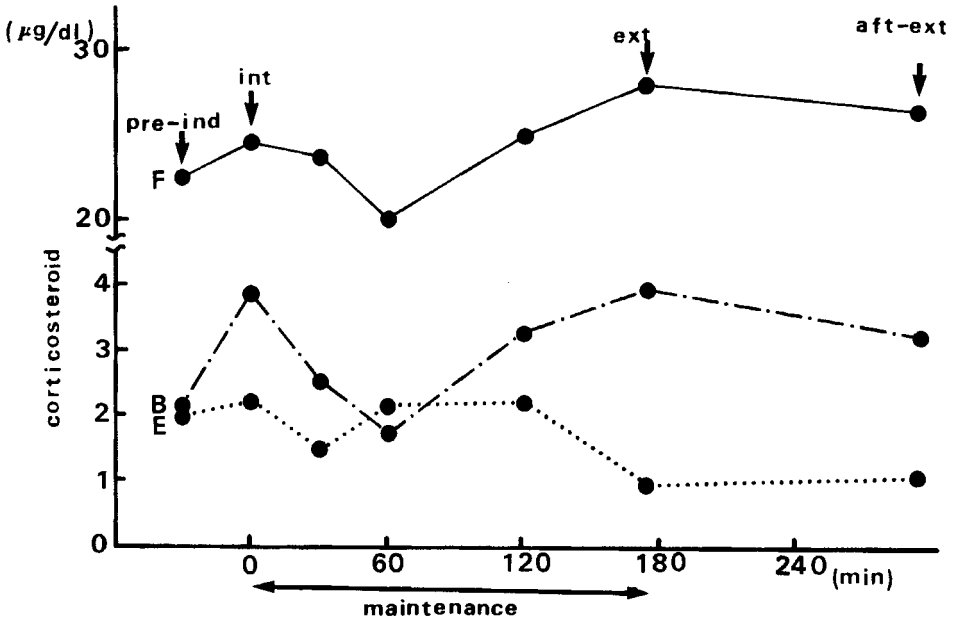

FIGURE 3. Changes in the Standing of Corticosteroids in Human Plasma under General Anesthesia

B, E, F: as in FIGURE 2. pre-ind: pre-induction int: intubation ext: extubation aft-ext: 180 min following extubation

TABLE 2. Determination of Corticosteroids in Male and Female Plasma by Using the Three Packing Columns

	Column	Cortisol	Cortisone	Corticosterone	Testosterone
Male	SIL	4.93	2.75	0.25	2.50
	ODS	--	--	0.79	0.56
	CN	4.78	2.41	0.81	0.56
Female	SIL	17.03	2.76	0.71	2.01
	ODS	--	--	0.90	0.24
	CN	12.87	4.67	0.92	0.23

μg/dl

by the ODS columns. Based on the above results, the authors concluded that the CN column system is best among the three commercial column systems examined for determining plasma steroid hormones.

4. Quantitative Determination of Corticosteroids in Human Plasma from Patients under Anesthesia.

In view of the results presented above, the quantitative determination of corticosteroids in human plasma was examined for the feasibility of its clinical application. The dynamic change in corticosteroids has been noted to be closely related to mental and physical stress. Thus measurement of corticosteroids may provide useful information for estimating mental stress even under general anesthesia.

Although various phase systems were developed to determine corticosteroids (2-4), the CN column system was found the most reliable. One of the results obtained by HPLC determination of corticosteroids in plasma using the CN column system is illustrated in FIGURE 3. Blood samples were collected from twenty year-old male under general anesthesia for dental treatment. The results show that the amounts of corticosteroids increased immediately following endotracheal intubation. It seems reasonable to consider variation in the amount of corticosteroid in the plasma to be an index for the mental and physical stress load of a patient.

Using the CN column system for HPLC analysis, it was possible to determine quantitatively small amounts of corticosteroids in the plasma. This phase system may thus be used for solving clinical problems. Its applications will be presented in a future paper.

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