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DETERMINATION OF 17-HYDROXYCORTICOSTEROIDS IN URINE BY FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING Dns-HYDRAZINE AS A PRE-COLUMN LABELING REAGENT

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SUMMARY

A method is described for the determination of urinary 17-hydroxycorticosteroids using fluorescence high-performance liquid chromatography. After enzymatic hydrolysis, 17-hydroxycorticosteroids were extracted using an Extrelut column and then labeled with Dns-hydrazine in hydrochloric acid—ethanol solution. The labeled steroids were chromatographed on a microparticulate silica gel column, the mobile phase was dichloromethane—ethanol—water (900:60:40). The eluate was monitored on a fluorophotometer at 365 nm (excitation) and 505 nm (emission). Linearity of the fluorescence intensities (peak heights) of various 17-hydroxycorticosteroids were obtained between 60 pg and 20 ng. The assay was sensitive, precise and accurate. Comparison with the results obtained by radioimmunoassay gave correlation coefficients of 0.932 for tetrahydrocortisol and 0.930 for tetrahydrocortisone. The proposed method is clinically useful for the routine analysis of urinary 17-hydroxycorticosteroids.

INTRODUCTION

The most important steroid secreted by the human adrenal gland is cortisol. Cortisol in the blood is converted to water-soluble conjugates in the liver; they, along with other metabolites, are excreted as glucosiduronates of tetrahydrocortisol (THF) and tetrahydrocortisone (THE). Measurement of these, the so-called urinary 17-hydroxycorticosteroids (17-OHCS), has been used to screen for abnormalities in adrenocortical function [1]. The Porter—Silber reaction [2, 3], a non-specific color reaction for the estimation of 17,21-dihydroxy-20-oxocorticosteroids, has been widely used in clinical laboratories. However, there is interference by other steroids, non-steroid metabolites and certain drugs [4]. Specific radioimmunoassay (RIA) techniques [5] have been developed and used in routine clinical diagnosis. Although RIA is highly

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sensitive, its specificity is limited because steroid antibodies may cross-react to varying degrees with other steroids. High-performance liquid chromatography (HPLC) offers the desired selectivity and sensitivity for the analysis of steroids such as cortisol [6—13] and, because it does not require the use of radioisotope, HPLC represents a useful, safe alternative to RIA techniques in routine laboratory analysis. However, because tetrahydrocorticosteroids have no strong UV-absorbing group in their structure, no HPLC method for their detection has been reported; instead, urinary steroids have been assayed by gas chromatography [14—18].

We have reported the use of Dns-hydrazine as a fluorescent labeling reagent to increase the detection limit for 17-oxosteroids (17-OS) [19]. We now present a highly sensitive fluorescence HPLC method for the determination of urinary 17-OHCS.

EXPERIMENTAL

Materials

THF, tetrahydro-11-deoxycortisol (THS) and *allo*-tetrahydrocortisol (*allo*-THF) were kindly donated by Dr. D.K. Fukushima. THE, tetrahydrocorticosterone (THB) and Dns-hydrazine (grade II) were from Sigma (St. Louis, MO U.S.A.), and β-D-glucuronidase and β-D-glucuronidase/arylsulphatase from Boehringer Mannheim—Yamanouchi Co. (Tokyo, Japan). The Extrelut Refill Pack was from E. Merck (Darmstadt, G.F.R.). Other steroids, reagents and solvents were from commercial sources.

Instruments and chromatographic conditions

We used an Hitachi Model 635 high-performance liquid chromatograph equipped with a Kyowa Seimitus KHP-UI-130 injection valve, a stainless-steel column (250 × 4.6 mm I.D.) and a Jasco FP-110 fluorescence spectrophotometer equipped with a mercury lamp and a micro flow cell. A Zorbax SIL column (particle size 5—6 μm, 250 × 4.6 mm I.D.) was used. The mobile phase was the organic layer separated from a mixture of dichloromethane—ethanol—water (900:60:40) after 30 min shaking; the flow-rate was 1 ml/min. The detector wavelength was set at 365 nm and 505 nm for excitation and emission, respectively.

Reagent solutions

Dns-hydrazine solution. A 0.05% (w/v) solution was prepared by dissolving 5 mg of Dns-hydrazine in 10 ml of ethanol; it was stored in a refrigerator until use.

Hydrochloric acid—ethanol solution. This was prepared by mixing 2 ml of concentrated hydrochloric acid with 1000 ml of ethanol.

Steroid stock solutions. THF, THE, *allo*-THF, or THS (2 mg) were made up to 10 ml with ethanol and stored in a refrigerator until use.

Buffers. The acetate buffer was 2 *M* (pH 5.2); the phosphate buffer was 0.5 *M* (pH 6.2).

Extrelut column. A 10-ml syringe, closed at the bottom with a Fine Filter F (Ishikawa Manufactory, Tokyo, Japan) was used as a column. It was filled

with 2 g of Extrelut, a large-pore, granular Kieselguhr with high pore volume, and vibrated for 10 sec on a Vortex-type mixer; the surface of the packing was covered with a filter plate.

Procedure

Hydrolysis with β-D-glucuronidase/sulphatase. A mixture consisting of 0.5 ml of urine, 0.5 ml of acetate buffer and 40 μl of enzyme solution was incubated for 24 h at 37°C. Two drops of chloroform were added to avoid bacterial decomposition.

Hydrolysis with β-D-glucuronidase. After adding 0.5 ml of phosphate buffer, 20 μl of enzyme solution and two drops of chloroform to 0.5 ml of urine, the mixture was incubated overnight at 50°C.

Extraction on the Extrelut column. The hydrolysate was diluted to 3 ml with redistilled water, loaded onto a dry Extrelut column (2 g), and allowed to soak in for 10 min to distribute the aqueous phase on the column packing. 17-OHCS were eluted from the column with 6 ml of dichloromethane and the effluent was evaporated to dryness at 40°C under a stream of nitrogen gas. The resultant residue was assayed as described below.

Solvent extraction. After incubation, 6 ml of dichloromethane were added to the mixture; it was stirred vigorously for 1 min with a Vortex-type mixer and centrifuged for 3 min at 1000 *g*. The aqueous layer was removed with a Pasteur pipet. After drying by adding 1 g of anhydrous sodium sulphate, exactly 4 ml of the organic layer were evaporated to dryness at 40°C under a stream of nitrogen gas and the resultant residue was assayed by the following procedure.

Labeling reaction. The residue in a test-tube was dissolved by adding 0.2 ml of hydrochloric acid—ethanol solution, then admixed with 50 μl of Dns-hydrazine solution, left to stand for 10 min at 65°C, and evaporated to dryness under a stream of nitrogen gas. The labeled residue was dissolved in 500 μl of eluent and an aliquot was injected into the chromatograph described above.

RESULTS

Fluorescence spectrum

Fig. 1 shows the excitation and emission spectra of Dns-hydrazone of THF in chloroform. The hydrazone was stable in chloroform for at least one week at 4°C without any change of fluorescence intensity. As shown in Table I, the fluorescence intensity of Dns derivatives was affected by the polarity of the solvent. In polar solvents such as methanol and acetonitrile, it was much lower than in chloroform or dichloromethane. Therefore, normal-phase HPLC on a microparticulate silica gel column was used for the separation of 17-OHCS, using the organic layer of a dichloromethane—ethanol—water mixture as the mobile phase.

Concentration of hydrochloric acid

In acidic media, tetrahydrocorticosteroids reacted with Dns-hydrazine to form hydrazones. Fig. 2 shows the effect of the hydrochloric acid concentration on the fluorescence intensity peak height. The peak height on all three

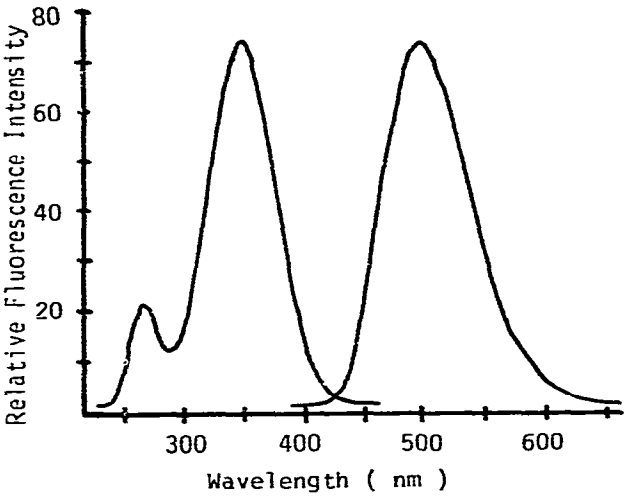

Fig. 1. Excitation and emission spectra of the fluorescent dansyl hydrazone of tetrahydrocortisol in chloroform. Excitation maximum, 350 nm; emission maximum, 505 nm.

TABLE I

RELATIVE FLUORESCENCE INTENSITY (RFI) AND FLUORESCENCE MAXIMUM IN VARIOUS SOLVENTS

Solvent	RFI	Fluorescence maximum (nm)	
		Excitation	Emission
Chloroform	100.0	350	500
Dichloromethane	116.7	350	500
Eluent*	112.0	350	505
Acetonitrile	55.6	350	525
Methanol	44.4	350	525

*Organic layer of dichloromethane—ethanol—water (900:60:40).

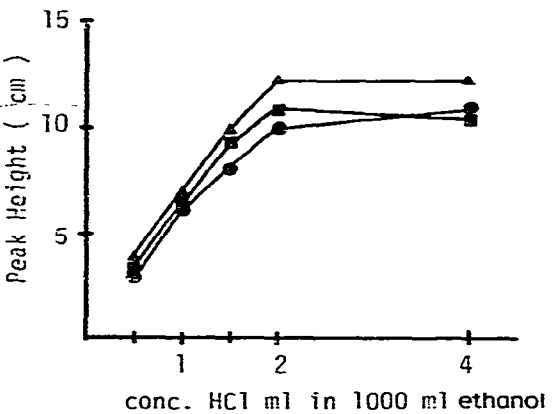

Fig. 2. Effect of hydrochloric acid concentration on fluorescence intensity peak height. (▲) Tetrahydrocortisone, (●) tetrahydrocortisol, (■) tetrahydro-11-deoxycortisol; 20 ng of each steroid were injected.

steroids increases with increasing hydrochloric acid concentration in ethanol up to 2 ml/l; it reached a constant value at 4 ml/l.

Therefore, hydrochloric acid—ethanol solution containing 2 ml of concentrated hydrochloric acid in 1000 ml of ethanol was used for the labeling reaction solvent.

Reaction temperature

As shown in Fig. 3, maximum peak heights were obtained at 65°C; they decreased at higher reaction temperatures. Therefore, the reaction temperature was set at 65°C for the labeling reaction. The reaction time used was 10 min because maximum peak heights were obtained at more than 5 min.

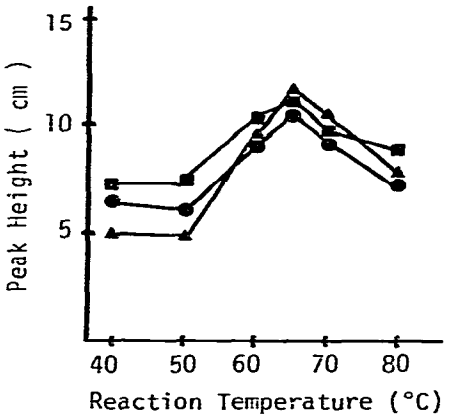

Fig. 3. Effect of reaction temperature on peak height. (▲) Tetrahydrocortisone, (●) tetrahydrocortisol, (■) tetrahydro-11-deoxycortisol; 20 ng of each steroid were injected.

Selection of eluent

Many solvent systems and columns were examined to obtain the complete separation of 17-OHCS. An organic layer consisting of a mixture of dichloromethane—ethanol—water (900:60:40) was found to be suitable when used on a Zorbax SIL column. The chromatograms presented in Fig. 4 show good separation of standard tetrahydrocorticosteroid mixtures including THF, THE, *allo*-THF, THS, and THB. The testing of other silica gel columns showed that urinary 17-OHCS were efficiently separated on various columns (Table II).

Working curves and sensitivities

Linearity of fluorescence (peak heights) intensity with the injected amounts of 17-OHCS (THF, THE, *allo*-THF, THS) were obtained in the range 60 pg—20 ng. When a 0.5-ml urine sample was used for the assay, the detection limit for 17-OHCS was about 3 ng/ml of urine.

Recovery of steroids from Extrelut column

Recovery of radiolabeled steroids upon Extrelut column extraction was 96.8 ± 4.4% (n = 5) and 90.0 ± 5.4% (n = 5) for [1,2-^{3}H]THE and [1,2-^{3}H]-THF, respectively.

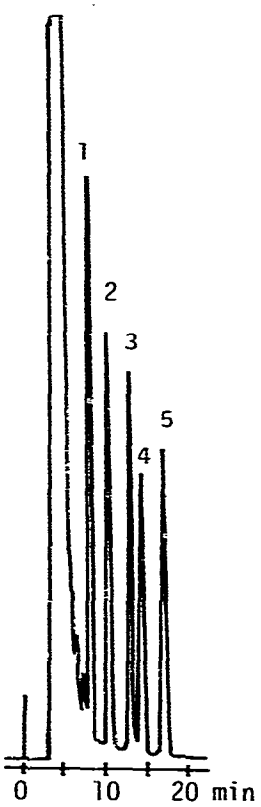

Fig. 4. Separation of fluorescent derivatives of tetrahydrocorticosteroid standard mixtures. Peaks: 1 = tetrahydrocorticosterone, 2 = tetrahydro-11-deoxycortisol, 3 = tetrahydrocortisone, 4 = allo-tetrahydrocortisol, 5 = tetrahydrocortisol. Column: Zorbax SIL column (250 × 4.5 mm I.D.). Mobile phase: dichloromethane—ethanol—water (900:60:40); flow-rate, 1 ml/min.

TABLE II

COMPARISON OF CAPACITY FACTORS (k') OF TETRAHYDROCORTICOSTEROIDS ON DIFFERENT SILICA GEL COLUMNS

Column packing	Producer	Particle size (μm)	Dimensions (length × I.D., mm)	Eluent*	Capacity factor (k')			
					THS	THE	*allo*-THF	THF
Hitachi gel 3042	Hitachi	5	250 × 4.0	I	3.46	5.20	6.06	7.40
Hitachi gel 3043	Hitachi	10	250 × 4.0	II	2.58	3.70	4.15	5.27
LiChrosorb Si 100	Merck	10	250 × 4.0	II	2.47	3.80	4.33	5.28
Zorbax SIL	DuPont	5—6	250 × 4.6	I	2.35	3.15	4.71	5.49
Finepak SIL	Jasco	5	250 × 4.6	I	2.47	3.29	3.81	4.61

*Eluent I: organic layer of dichloromethane—ethanol—water (900:60:40). Eluent II: organic layer of dichloromethane—ethanol—water (920:50:30). Flow-rate: 1 ml/min.

Recovery and reproducibility

An aqueous standard solution (2 ml) containing 0.5 μg each of the three 17-OHCS was added to a urine hydrolysate, and Extrelut column extraction, labeling reaction and HPLC were performed. As shown in Table III, 17-OHCS were recovered in the range 90.8—102.2% with a coefficient of variation (C.V.) range of 3.2—4.3%.

TABLE III

RECOVERY OF TETRAHYDROCORTICOSTEROIDS ADDED TO URINE PRIOR TO EXTRACTION

Urine (0.5 ml) to which 0.5 μg of each of the three 17-OHCS was used.

Steroid	Recovery (%)	*n*	C.V. (%)
THE	102.2	5	3.2
allo-THF	92.5	5	4.3
THF	90.8	5	4.1

Typical chromatograms of urine samples

Fig. 5 shows typical chromatograms of normal human and patient urine samples; urinary 17-OHCS were clearly separated and identified by comparison with authentic samples.

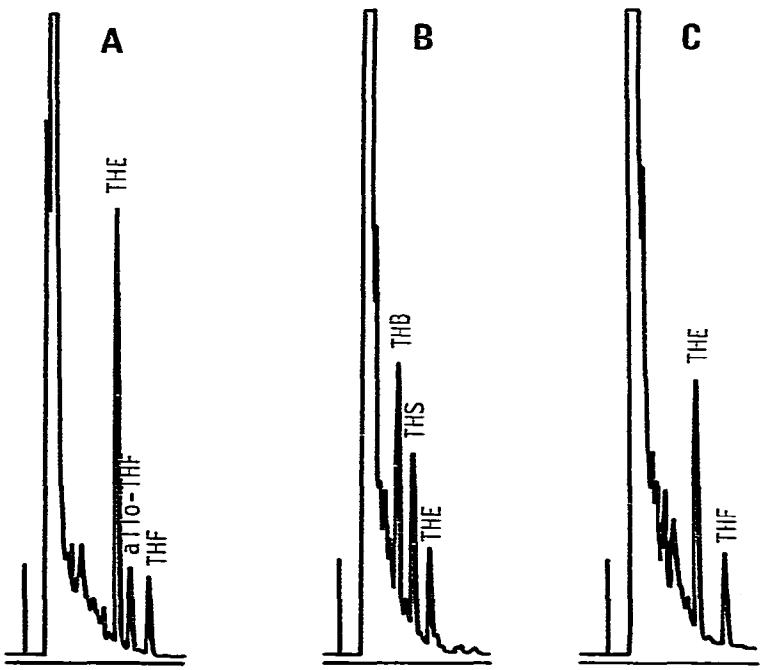

Fig. 5. Typical chromatograms of urine samples. (A) Normal human; (B) pituitary tumor patient; (C) normal human after oral administration of carbamazepine.

Comparison with results obtained by RIA and the colorimetric method

The reliability of the newly devised HPLC method for the determination of urinary 17-OHCS (THF and THE) was assessed by comparing the results with those obtained by RIA. RIA was as described by Kambegawa and Honma [20]; it involves Sephadex LH-20 chromatography for the separation of THF and THE. As illustrated in Fig. 6, the values obtained by both methods were in good agreement; the correlation coefficients were 0.932 and 0.930 for THF and THE, respectively.

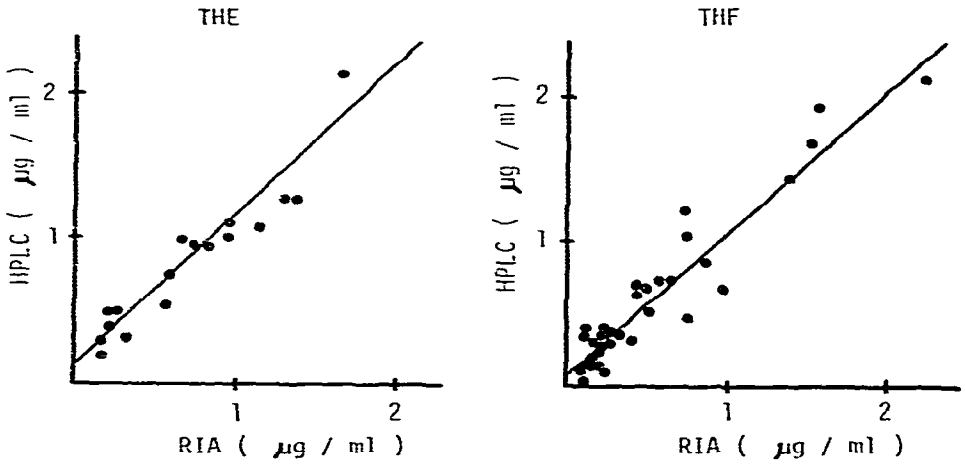

Fig. 6. Correlation between fluorescence HPLC and RIA values of urinary tetrahydrocortisone (THE) and tetrahydrocortisol (THF). THE: $y = 0.98x + 0.14$; $r = 0.930$; $n = 17$. THF: $y = 0.96x + 0.10$; $r = 0.932$; $n = 32$.

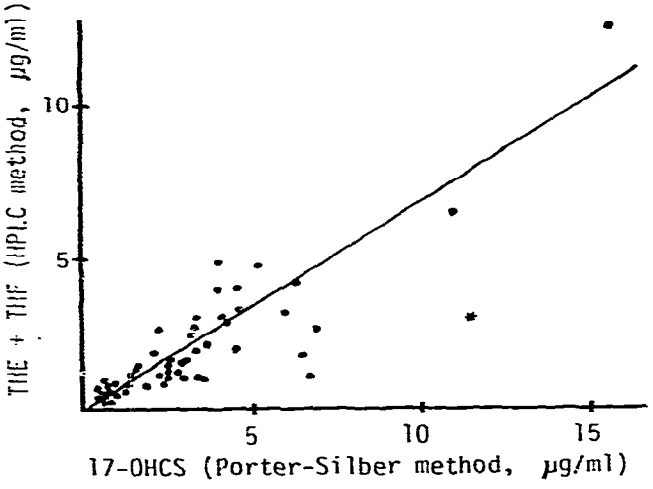

Fig. 7. Comparison of the results obtained with fluorescence HPLC and the colorimetric method for the determination of urinary 17-OHCS. $y = 0.67x - 0.05$; $r = 0.915$; $n = 53$. *, After oral administration of carbamazepine.

Furthermore, the total urinary THF and THE values obtained by the HPLC method were compared with the 17-OHCS values obtained by an ordinary colorimetric method, Porter—Silber reaction, used in routine assay. As shown in Fig. 7, the correlation coefficient was 0.915 and the regression line, $y = 0.67\ x - 0.05$, where x equals the values determined by the colorimetric method.

DISCUSSION

In clinical pathology, fluorometric and colorimetric methods such as the Porter—Silber reaction [3], the tetrazolium reaction [21] and ketogenic steroid

estimation [22], have been routinely used for the determination of 17-OHCS. As these methods are of limited sensitivity and do not have highly specific reactions, there may be some interference by other steroids, non-steroid metabolites and drugs. Sensitive RIA methods for THF [20, 23, 24] and THE [20, 24] have been reported, however, they require chromatographic separation such as Sephadex LH-20 column [20] or paper chromatography [24]. HPLC methods for the determination of steroids in biological fluids have been devised [18, 25]. In our preceding paper [19] we reported the determination of 17-OS in biological fluids by fluorescence HPLC using Dns-hydrazine as the fluorescence labeling reagent. In the present report, we describe a fluorescence HPLC method for the determination of urinary 17-OHCS, THF, THE, THS, *allo*-THF and THB. The Porter—Silber reaction involves the acid-catalyzed rearrangement of the adrenocortical side-chain to a 20,21-glyoxal grouping, followed by the formation of 21-phenylhydrazone [26] which showed a strong band at 1660 cm^{-1} arising from α,β-unsaturated β-hydrazinoketo group. On the other hand, the fluorescent product of THF obtained by the derivatization with Dns-hydrazine showed no band near 1660 cm^{-1}. It seems that the carbonyl group at C-20 position of THF reacted with Dns-hydrazine to yield the Dns-hydrazone of THF. THB, 17-deoxycorticosteroid, reacted with Dns-hydrazine under the same reaction conditions shown in Fig. 4.

We chose chromatographic conditions which, in the shortest possible analysis time, gave acceptable resolution between the Dns-hydrazone of tetrahydrocorticosteroids and the fluorescent coexisting substance in urine samples. As shown in Fig. 4 and Table II, good separation can be achieved on various silica gel columns, using the organic layer of a dichloromethane—ethanol—water mixture as the eluent. The sensitivity of this method is superior to that of other HPLC methods which employ a UV detector. Tetrahydrocorticosteroids have no strong UV-absorbing group in their molecules; therefore, when an refractive index detector is used, their detection limits exceeded 1 μg. When we hydrolyzed urine with β-D-glucuronidase and/or β-D-glucuronidase/sulphatase [27], we obtained equally good results (Table IV). The use of β-D-

TABLE IV

ANALYSIS OF URINARY STEROIDS USING DIFFERENT HYDROLYSIS AND EXTRACTION METHODS

Enzyme	Extraction	Steroid	Peak height (mm)	n	σ_{n-1}	C.V. (%)
Helicase	Mixing with dichloromethane	THE	137.5	4	0.64	4.6
		allo-THF	19.5	4	0.17	5.8
		THF	39.0	4	0.18	4.6
Helicase	Extrelut column	THE	128.5	4	0.36	2.8
		allo-THF	16.2	4	0.06	3.5
		THF	34.5	4	0.17	5.0
β-Glucuronidase	Mixing with dichloromethane	THE	130.0	4	0.45	3.5
		allo-THF	15.8	4	0.10	6.0
		THF	25.0	4	0.07	2.8

glucuronidase/sulphatase makes it possible to obtain simultaneously a hydrolysate for 17-OHCS and 17-OS detection. Although dichloromethane can be used for the extraction of 17-OHCS, this method sometimes produces an emulsion. The Extrelut column extraction method permits quantitation and it is as good as other methods commonly used for recovery. Ende et al. [28] observed contamination when they used the Extrelut column for steroid determination. However, we noted no interference peaks. There was a good correlation between the urinary THF and THE values obtained by our newly devised method and the RIA method, and the total THF and THE values obtained by our method correlated well with those of 17-OHCS obtained by the colorimetric method. A high 17-OHCS value (11.5 μg/ml) was recorded upon application of the Porter—Silber method to a urine sample that was obtained after the oral administration of carbamazepine; our method, used in the same sample, gave a normal (3 μg/ml) summation value for THF and THE. This indicates that urinary 17-OHCS can be determined by our method without interference by a metabolite of carbamazepine [29].

Although the estimation of total urinary neutral 17-OHCS is used in screening for adrenal disease, specific changes will go unnoticed. For example, the THF/THE ratio was increased in patients suffering from a wide range of diseases [30] and a highly significant correlation was obtained between THF-glucuronide and the cortisol secretion rate [24]. THS-glucuronide values were also highly elevated after metyrapone administration to humans [31]. Therefore, to obtain meaningful and accurate information, the determination of individual components of this group of steroids is very important. Our newly developed method may be clinically useful in the routine assay of urinary 17-OHCS.

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