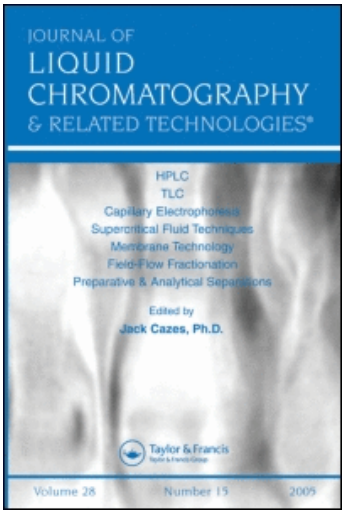

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NEW FLUORIMETRIC DETECTION METHOD OF CORTICOSTEROIDS AFTER HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING POST-COLUMN DERIVATIZATION WITH GLYCINAMIDE

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ABSTRACT

Some biological corticosteroids were detected by a new fluorimetric method using post-column derivatization with glycinamide .

Urinary corticosteroids which are hydrolyzed and extracted from sample fluid are detected by the reaction with glycinamide in the presence of hexacyanoferrate (III) in weakly alkaline media of borate buffer solution of pH 9.8 at 90 °C for 5 min.

This method is highly sensitive and specific for some corticosteroids and the reaction is performed under mild condition, compared with the fluorimetric method using the reaction with sulfuric acid, so that this method can be used for fractional assay of urinary corticosteroids of patients for a steroid abnormal metabolism.

INTRODUCTION

Urinary corticosteroids have been determined by colorimetric methods, such as Porter-Silber reaction

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and the Zimmermann reaction, gas-liquid mass spectrometric method and radioimmunoassay.

In an analysis of high-performance liquid chromatography (HPLC), a detection method of steroids has been performed by measuring UV absorbance at 254 nm based on Δ^4-3-keto group or by using derivatization reaction with fluorescent compounds (1-4).

Recently we have developed a new fluorimetric method for the determination of urinary 17-hydroxycorticosteroids using the reaction with glycinamide (5). The advantage of the method is in high specificity for some corticosteroids.

In this paper, we describe a modified method for the application of HPLC.

EXPERIMENTAL

Steroid standards were purchased from Sigma Chemical Co. and Steraloids Co. and used without further purification. All reagents were of analytical grade obtained from commercial sources and used without further purification. All reagent solution for HPLC was filtrated before use (millipore membrane, 0.4 μ).

Preparation of reagents

Methanol/water (1:1, by vol); Measure 500 ml of HPLC grade methanol, make up to 1000 ml with reagent grade water and mix, then air bubbles are removed using a suction apparatus.

Reagent for fluorimetric detection

In 300 ml of borate buffer solution (boric acid-KOH, 0.3 M, pH 9.8), 0.5 g of glycinamide and 30 mg of potassium ferricyanide are dissolved.

Stock standard solution; 20 μg/ml in methanol solution is prepared.

Working standard solution; Each steroid standard solution is prepared by diluting with water to a concentration of 10 μg/ml before use.

Liquid chromatography

HPLC used is a Model of Trirotar-III (Japan Spectroscopic Co. LTD) equipped with Finepak SIL C_{18} column (25.0 x 0.46 cm particle size 10 μ).

Fluorescence was measured at 385 nm, with excitation of 325 nm, by Spectrofluorometer Model FP-550F. The slit width of excitation and emission was set at 20 nm, and the scale of the sensitivity and selector were adjusted to x 10 and the variable was to x 0.1.

The flow rate of mobil phase was 0.8 ml/min.

Preparation of sample

Pipette 2 ml of urine sample into a 10 ml glass stoppered test tube and adjust to pH 6.5. Add 0.1 ml of β-glucuronidase (500 Fishmann units/ml, from E. coli EC 3.2.1.31), 0.2 ml of 0.2 M phosphate buffer (pH 6.5), and one drop of chloroform to the tube and mix well. Incubate the mixture for 24 h at 37 °C. Shake the solution with 4 ml of methylene chloride for 3 min . Discard urine layer and wash the organic layer with 0.5 ml of 0.1 N NaOH and 0.5 ml of water successively. After centrifugal separation, transfer 2 ml of the organic layer to another test tube and evaporated to dryness in a hot water bath at 80 °C.

To the dried residue of the extract, add 100 μl of methanol/water solution and dissolve the extract residue. This solution of 10 μl is injected into the HPLC in which column temperature is kept at 40 °C. Effluent of the chromatography is mixed with the fluorescent reagent at flow rate of 1.0 ml/min and mixed solution is heated at 90 °C for 5 min in a teflon tube dipped in a water bath (ID 0.5 mm, length

30 m of tube) and then cooled to room temperature by passing in a cold water bath. Fluorescence is measured at 385 nm, with excitation of 325 nm, and its intensity is recorded.

Standard solution were also submitted to the whole procedure except the step of enzymatic hydrolysis.

RESULTS

Effect of final pH on fluorescence intensity for cortisol as standard of 20-keto-17,21-diol side chain carring steroid and for corticosterone as standard of 20-keto-21-ol steroid was shown in Figure 1. The maximum intensity for cortisol was around at pH 9.9 and for corticosterone at above 10.3. In this study, we have used buffer solution of pH 9.8 for urinary 17-hydrocorticosteroids fractionation.

Specifisity of the reaction by the present method was shown on Table 1 in which fluorimetric response

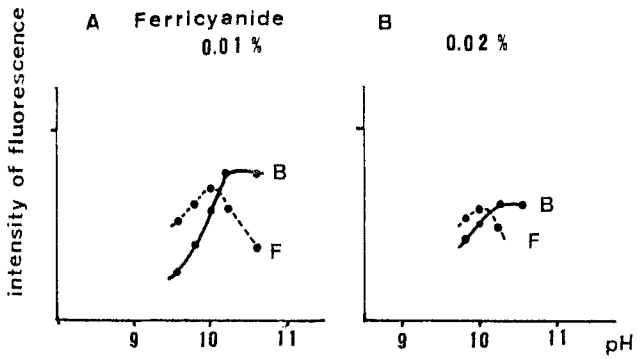

FIGURE 1. Effect of reaction pH on fluorescence intensity for cortisol and corticosterone.
Cortisol was used as a standard of 20-keto-17,21-diol steroid and corticosterone as a standard of 20-keto-21-ol steroids. F: cortisol, B: corticosterone.

TABLE 1. Specificity of the reaction

Compounds tested	Relative peak height (%)
Cortisol	100
Tetrahydrocotisol	85
Cortisone	90
Tetrahydrocortisone	80
11-Deoxycortisol	92
Tetrahydro-11-deoxycortisol	75
Tetrahydrocorticosterone	50
Corticosterone, Predonisolone	65
Tetrahydro-11-dehydrocorticosterone	50
Beta-methazone	10
Aldosterone	5
16-hydroxy-dehydroepiandrosterone	2
progesterone	0.2
pregnanediol	0.0
Androsterone	0.0
estradiol	0.0
Dehydroepiandrosterone	0.0

Cortisol was used as 100 %

of each steroids were shown by relative peak height at pH 9.8 using 10 μl of 0.3 mM of standard solution.

Linearity of each standard curve was observed , at least, up to 500 ng of steroids.

Recovery added to urine samples was 94 % (1 μg of tetrahydrocortisol was added to five urine samples).

Chromatograms of standard, normal subject and patient of Cushing's disease were shown in Figure 2.

DISCUSSION

Urinary corticosteroids are excreted mainly in tetrahydro form, so the detection by measuring UV absorbance can not be used for the analysis of these steroids. On the contrary, present method is based on the reaction at C_{17} side chain of corticosteroids so

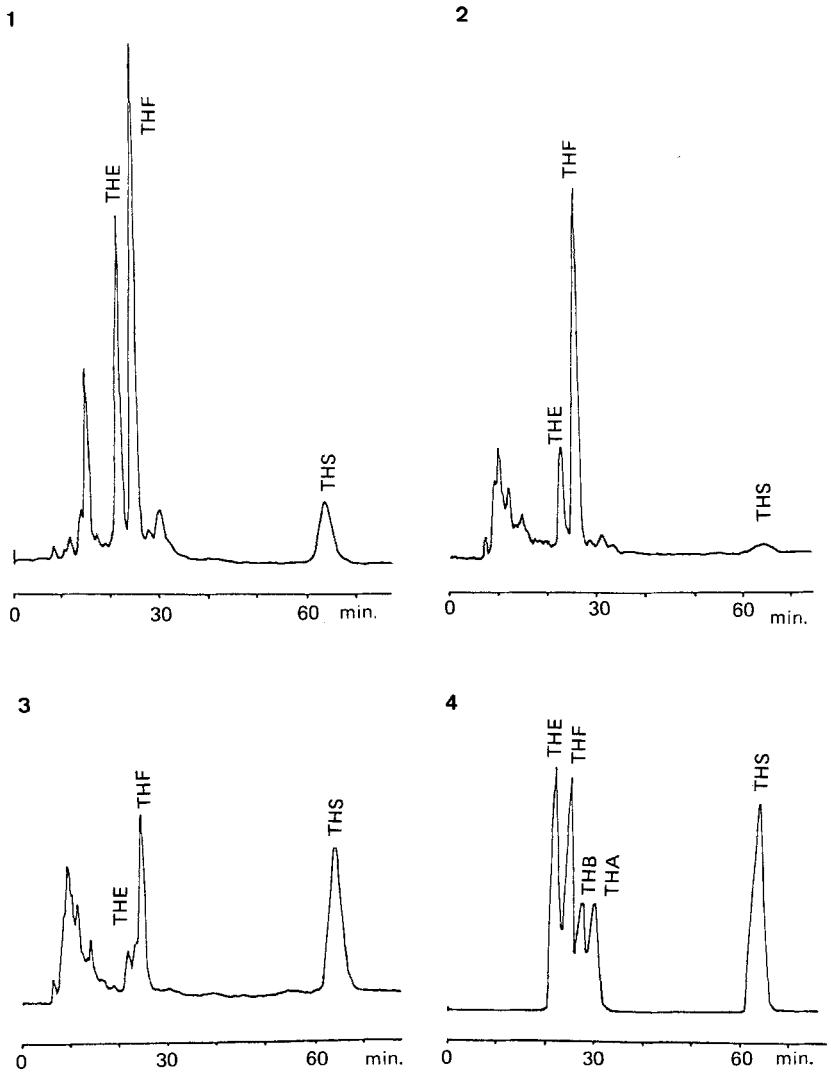

FIGURE 2. Chromatograms of samples with Cusing's disease, normal subject, Su-4885 test, and standards.
(1) Cushing's disease, (2) normal subject
(3) Su-4885 test, which is a 11β-hydroxylase inhibitor of steroid metabolism enzyme. (4), standards

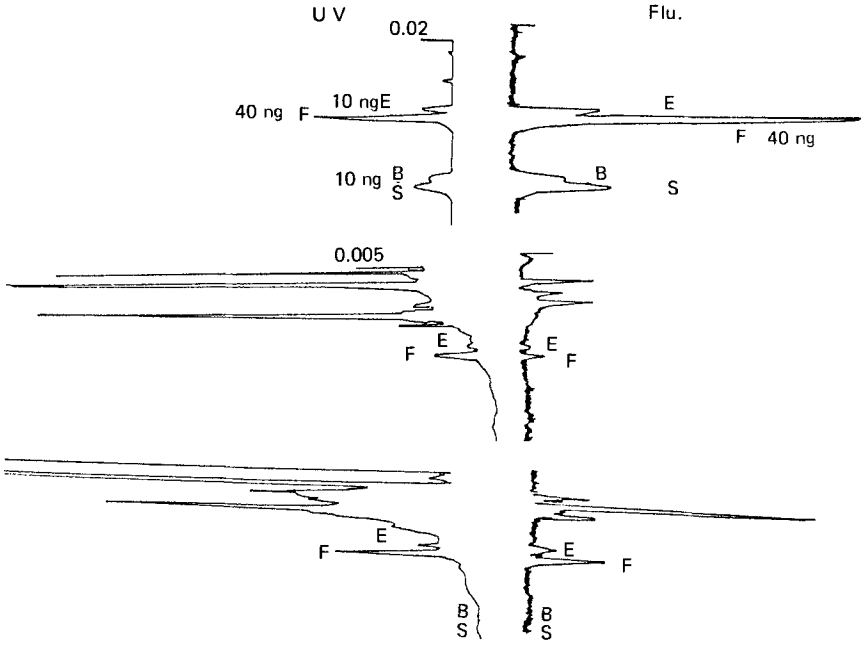

FIGURE 3. Chromatograms of serum Δ^4-3-keto steroids by UV detection and fluorometric detection. Procedure of serum analysis was performed by the method described by Caldarella et al (3). The flow rate of mobile phase (methanol/water, 3:4 by vol) was 1.0 ml/min. UV absorption was detected at 254 nm and 0.02 and 0.005 of UV means absorbance units range of UV detector. Fluorescence was measured at 385 nm, with excitation 325 nm.

that both Δ^4-3-keto corticosteroids and tetrahydro-corticosteroids could be measured.

By increasing a sensitivity of fluorometer, cortisol in serum was also detected at a level of 5 to 50 ng steroid/ injection as shown in Figure 3.

There are many fluorimetric method for steroid analysis using sulfuric acid but these methods have diffical-

ty for the application to HPLC analysis because of the use of strong acid. On the contrary, our method is performed under mild reaction condition so the method could be easily applied to the chromatographic method.

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