Journal of Chromatography, 305 (1984) 188—193
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1893

Note

New fluorimetric determination of 17-hydroxycorticosteroids after high-performance liquid chromatography using post-column derivatization with benzamidine

TOKUICHIRO SEKI* and YOSHIHISA YAMAGUCHI

College of Bio-Medical Technology, Osaka University, 1-1 Machikaneyamacho, Toyonaka, Osaka (Japan)

(First received April 16th, 1983, revised manuscript received August 18th, 1983)

Detection of steroids after high-performance liquid chromatography (HPLC) has usually been performed by ultraviolet (UV) absorption at 240 nm of the Δ^4-3-keto group of steroids or by derivatization with fluorescent compounds [1—3]. These methods, however, can not be applied to the analysis of urine in which most corticosteroids are present in tetrahydro form.

Recently, we developed a new fluorimetric method for a determination of urinary corticosteroids using a reaction with glycinamide [4, 5]. This method has the advantage of being highly selective and capable of measuring 17-hydroxycorticosteroids and most 17-deoxycorticosteroids.

Ohkura and Kai [6] have reported a method for the fluorimetric determination of guanidino compounds in which the reaction of guanidino compounds with benzoin, a ketol derivative, under alkaline conditions yields fluorescent diphenylimidazole derivatives. Based on their results we inferred that compounds having a ketolic group might give fluorescent compound(s) when heated with amidine derivative under alkaline conditions. Therefore, we tried a reaction of steroids carrying a ketolic group with benzamidine and found that 17-hydroxycorticosteroids yielded fluorescent compound(s) with excitation and emission maxima of 370 and 480 nm, respectively.

In this paper, application of the new method for the fluorimetric determination of 17-hydroxycorticosteroids separated by HPLC is described.

EXPERIMENTAL

Materials

Benzamidine hydrochloride was the product of Aldrich Chemical Company

0378-4347/84/$03 00 © 1984 Elsevier Science Publishers B.V.

and was purchased from Yashima (Osaka, Japan). Other reagents were of analytical grade and used without further purification.

Standard solution of steroids

Steroids were dissolved in aqueous methanol (methanol—water, 1:1, v/v) at a concentration of 10 μg/ml.

Reagent for fluorimetry

Reagent A is a sodium hydroxide solution (0.4 mol/l) and reagent B is a solution of benzamidine hydrochloride (0.5%, w/v) in a mixture of 2-propanol—water (1:1, v/v).

Mobile phase

Methanol (500 ml) was made up to 1000 ml with reagent-grade water and mixed well. Then air bubbles were removed using a suction pump under reduced pressure.

Apparatus

We used a TRiRotar high-performance liquid chromatograph equipped with a variable-loop injector, spectrofluorimeter equipped with a 30-μl flow cell (Model FP-550F), and a recorder (Model RC-125). The column, Finepak C_{18}, was octadecyl silica, 10 μm particle size, 4.6 mm diameter and 25 cm length. A reciprocal-type pump equipped with two pump heads pumping liquid alternatively (Model SP-2-24) was used to mix reagents A and B. All apparatus was from Japan Spectroscopic Co.

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 Fishman units/ml, from *Escherichia coli*), 0.2 ml of 0.2 *M* phosphate buffer (pH 6.5), and one drop of chloroform to the test tube and mix well. Incubate the mixture for 24 h at 37°C. Add 2 μg of β-methasone (20 μl of 100 μg/ml solution in methanol), as internal standard, and shake solution with 4 ml of methylene chloride for 3 min. Discard the urine layer and wash the organic layer with 0.5 ml of 0.1 *M* sodium hydroxide and 0.5 ml of water successively. After centrifugal separation, transfer 2 ml of the extract to another test tube and evaporate to dryness in a hot water bath at 80°C.

Chromatographic separation and fluorimetric determination of steroids

Add 100 μl of mobile phase to the dried residue of the extract and dissolve the extract, 10 μl of this solution were injected into the chromatograph. The column temperature was kept at 40°C and the flow-rate of the mobile phase was 0.8 ml/min. Effluent from the column is mixed with the mixture of reagents A and B. Reagents A and B were pumped at a flow-rate of 0.5 ml/min; they were mixed using a T-shaped connector. The mixed reagent was added to the effluent from the column via a T-shaped connector and heated at 95°C for 5 min in a PTFE tube (I.D. 0.5 mm, length 30 m) immersed in a water bath, and then cooled to room temperature by passing through a tube immersed in

a water cup. Fluorescence was measured at 480 nm, with excitation at 370 nm; the slit width for both excitation and emission was 20 nm.

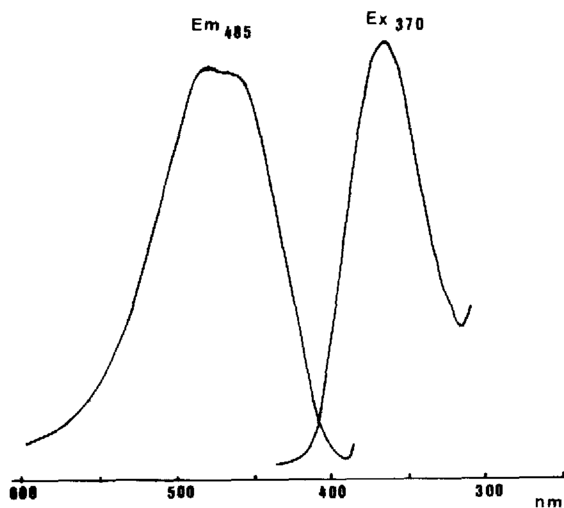

Fig 1. Excitation and emission spectra of fluorescent compounds Two millilitres of water, 0.5 ml of 0.4 *M* sodium hydroxide and 0.5 ml of 1% benzamidine were added to a test tube containing cortisol (50 μg per tube) and then the mixture was heated at 95°C for 5 min. The fluorescent compound formed has a maximum at 370 nm for excitation and at 480 nm for emission.

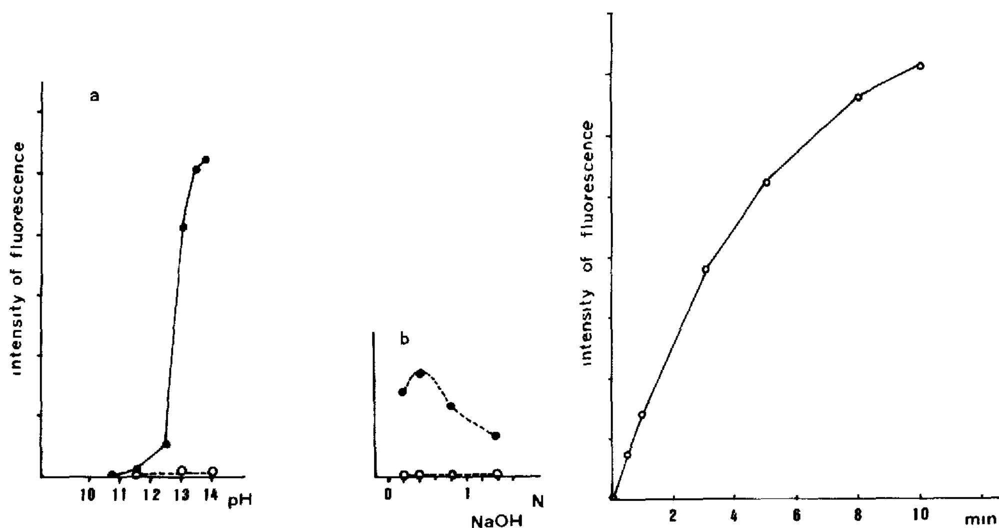

Fig. 2 Effect of pH on the intensity of fluorescence (a) in the test tube, (b) in HPLC analysis. (a) In the test tube, the intensity of fluorescence was highest between pH 13 and 14. (b) In HPLC analysis the concentration of sodium hydroxide in reagent A was examined and the highest fluorescence intensity was found around 0.4 *M*. (•), cortisol; (○), corticosterone.

Fig. 3. Time course of the reaction. Two millilitres of water, 0.5 ml of 0.4 *M* sodium hydroxide and 0.5 ml of 1% benzamidine were added to a test tube containing cortisol (50 μg per tube) and the mixture was heated at 95°C for the specified time, 5 min in the case of HPLC analysis.

RESULTS AND DISCUSSION

Excitation and emission spectra of the fluorescent compounds formed from the reaction of cortisol with benzamidine are shown in Fig. 1. Maximum fluorescence intensity was found at pH between 13 and 14 in the test tube and by the post-label method as shown in Fig. 2.

The time course of the reaction of formation of fluorescent compounds in the test tube is shown in Fig. 3. The selectivity of the reaction utilized in the post-label method is shown in Table I. Compared with the method using glycinamide, which can detect both 17-hydroxy-20-oxo-21-hydroxycorticosteroids and 20-oxo-21-hydroxycorticosteroids, this method is selective for the compounds carrying a dihydroxyacetone side-chain or group.

Chromatograms of a standard mixture and of samples from a normal subject and a subject with Cushing's disease are shown in Fig. 4. This method is based on the reaction of benzamidine with the dihydroxyacetone side-chain of corticosteroids at C-17 so that both Δ^4-3-keto-17-hydroxycorticosteroids and tetrahydro-17-hydroxycorticosteroids could be measured, and at the highest sensitivity of the fluorimeter cortisol in serum could be measured at a level of 5—50 ng per injection as shown in Fig. 5.

The within-assay coefficient of variation (C.V.) calculated for a normal urine sample (five repeated assays) was 2.4% for tetrahydrocortisol and 3.2% for tetrahydrocortisone. The between-assay C.V. was 5.2% for tetrahydrocortisol and 7.8% for tetrahydrocortisone.

TABLE I

SELECTIVITY OF THE REACTION

To determine the selectivity of the reaction using benzamidine, 2 μl of 0.1 m*M* standard solution were injected and the relative peak heights were compared. β-Methasone was used as an index of 100%. For selectivity of the reaction using glycinamide [5], as has been described previously, the reactivity of the reaction is dependent on pH. In this table, reactivity at pH 9.8 is shown and cortisol used as an index of 100%.

Steroids	Reactivity (%) with	
	Benzamidine	Glycinamide
β-Methasone	100	10
Cortisol, cortisone	95	100
11-Deoxycortisol	90	92
Prednisolone	70	65
Tetrahydrocortisone, tetrahydrocortisol	85	80
Tetrahyro-11-deoxycortisol	70	75
Androsterone	0	0
Dehydroepiandrosterone	0	0
Progesterone	0	0.2
Corticosterone	0	65
11-Deoxycorticosterone	0	50
Aldosterone	0	5
16-Hydroxydehydroepiandrosterone	0	2

Fig. 4. Chromatograms of standard mixture and patient samples. (A) Chromatogram of 10 μl of standard solution, containing 50 ng each of F, IS, THE and THS, and 100 ng of THF. (B, C) Chromatograms of a urine sample from a normal subject. (D) Chromatogram of a urine sample of a patient with Cushing's disease. F = cortisol, THE = tetrahydrocortisone, THF = tetrahydrocortisol, THS = tetrahydro-11-deoxycortisol, IS = β-methasone (internal standard).

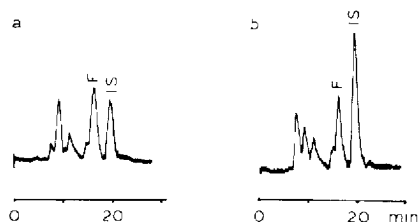

Fig. 5. Chromatograms of serum cortisol (F) assay. To 1 ml of serum sample, internal standard (β-methasone solution of 100 ng or 200 ng, 10 μl or 20 μl of 10 μg/ml) was added and mixed with methylene chloride. After extraction, the organic layer was washed with alkaline solution and water, and then evaporated to dryness. The residue was dissolved in 100 μl of 50% methanol and 30 μl of the solution were injected for HPLC. (a) 100 ng of internal standard were added to 1 ml of serum. (b) 200 ng of internal standard were added to 1 ml of serum

The present method is more selective than the UV absorption method so an analysis of corticosteroids in biological fluids could be performed by the present fluorimetric method.

REFERENCES

1 J.Q Rose and J W. Jusko, J. Chromatogr., 162 (1979) 273
2 A.M Caldarella, G.E. Reardon and E.E. Canalis, Clin. Chem., 28 (1982) 538.
3 R. Horikawa, T. Tanimura and Z. Tamura, J. Chromatogr., 168 (1979) 526.
4 T Seki and Y. Yamaguchi, Anal Lett., 15 (B13) (1982) 1111.
5 T Seki and Y. Yamaguchi, J. Liquid Chromatogr., 6 (1983) 1131.
6 Y. Ohkura and M. Kai, Anal Chim. Acta, 106 (1979) 89