Journal of Chromatography, 570 (1991) 1–11
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5980

Determination of serum cortisol by reversed-phase liquid chromatography using precolumn sulphuric acid–ethanol fluorescence derivatization and column switching

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(First received December 27th, 1990; revised manuscript received April 19th, 1991)

ABSTRACT

An assay method for serum cortisol, using precolumn sulphuric acid–ethanol fluorescence derivatization and reversed-phase liquid chromatography with a column-switching technique, has been developed. The crude precolumn fluorescence cortisol derivative was prepared by the addition of sulphuric acid to serum deproteinized with ethanol, and directly injected onto an octadecylsilane-bonded silica gel (ODS) precolumn for concentration and purification. After switching columns the samples were separated using an ODS analytical column and monitored fluorimetrically. When the pH of the mobile phase in the analytical separator decreased to 1.85, the emission wavelength of the cortisol derivative changed to 520 nm (excitation of 365 nm) and the fluorescence itensity increased. Among the sulphuric acid–ethanol derivatives of various steroids, cortisol, corticosterone and testosterone emitted fluorescence. However, their retention times differed from those of the cortisol derivatives (12.5 min). The detection limit of cortisol was 0.3 μg/dl (signal-to-noise ratio of 3). Use of the fully automated column-switching system contributed to good reproducibility and recovery.

INTRODUCTION

Because cortisol is an important physiological substance involved in stress and glucose metabolism, and is a marker of adrenocortical function [1–3], many assay methods for cortisol have been developed, including radioimmunoassay (RIA) [4], enzyme immunoassay (EIA) [5,6] and high-performance liquid chromatography (HPLC) with UV detection [7–9]. RIA and EIA for cortisol suffer from the problem of cross-reaction [10–12] with endogenous steroids, as well as synthetic steroidal drugs. However, the assays of serum cortisol by HPLC can be performed selectively.

Precolumn fluorescence labelling reagents, such as dansyl hydrazine [13] and

0378-4347/91/$03.50

9-anthroyl nitrile [14,15] have been used for sensitive detection of cortisol, but elimination of excess labelling reagents is required to prevent interference with the labelled cortisol peak.

Sulphuric acid–ethanol fluorescence derivatization [16–19] of cortisol has the advantages of not requiring the elimination of labelling reagents, because they are not used, and of yielding a relatively specific fluorescence derivative with a long wavelength of emission that is unique to intrinsic substances in human body fluids. However, the ordinary sulphuric acid–ethanol fluorescence derivatization methods for serum cortisol pose problems [20,21] such as interference by serum contaminants, despite cortisol extraction with dichloromethane, which increases the fluorescence background.

There have been some reports concerning the elimination of fluorescence from serum contaminants. One of those is based on the separation of the sulphuric acid–ethanol precolumn cortisol derivative by reversed-phase HPLC after cortisol extraction with dichloromethane [22,23]. The other method relies on the derivatization of cortisol with sulphuric acid after HPLC [24,25]. Although these methods eliminated the background fluorescence, preliminary extraction of cortisol from serum samples with dichloromethane and careful purification were still needed.

We observed that ethanol is effective both in deproteinizing serum and in the fluorescent derivatization of cortisol with sulphuric acid. There was no need for prior extraction of the fluorescent derivatized cortisol in the sulphuric acid–ethanol method.

We also found that the long maximum emission wavelength of 520 nm (excitation at 365 nm) for the cortisol derivative was obtained at pH 1.85 of the mobile phase in the analytical column, which enables more sensitive detection of cortisol than that by Gotelli *et al.* [22], and that the cortisol derivative was stable for *ca.* 15 h when it was kept at 5°C in the dark.

Therefore, we developed a new determination method for serum cortisol using precolumn sulphuric acid–ethanol fluorescence derivatization and fully automated reversed-phase and isocratic HPLC with column switching. This method is simple: only the addition of sulphuric acid to serum deproteinized with ethanol was needed, then the solution was allowed to stand in the dark at ambient temperature. The crude samples separated by reversed-phase HPLC with column switching were monitored with a fluorimeter using the emission wavelength of 520 nm (excitation at 365 nm).

EXPERIMENTAL

Materials

Sulphuric acid, ethanol, acetonitrile and tetrahydrofuran were all reagent grade and purchased from Kantoh (Tokyo, Japan). Potassium biphthalate and trifluoroacetic acid were from Tokyo Kasei Kogyo (Tokyo, Japan). Cortisol

(11β,17α,21-trihydroxypregn-4-ene-3,20-dione), allotetrahydrocortisol (5α-pregnane-3α,11β,17α,21-tetrol-20-one), prednisolone (1,4-pregnadiene-11β,17α,21-triol-3,20-dione), cortolone (5β-pregnane-3α,17α,20α,21-tetrol-11-one), cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione), prednisone (17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione), dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), betamethasone (9α-fluoro-16β-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), cortol (5β-pregnane-3α,11β,17α,20β,21-pentol), tetrahydrocorticosterone (5β-pregnane-3α,11β,21-triol-20-one), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), androstenediol (3β,17β-dihydroxy-5-androstene), androstenedione (4-androstene-3,17-dione), testosterone (4-androsten-17β-ol-3-one), dehydroepiandrosterone (5-androsten-3β-ol-17-one), β-estradiol (1,3,5[10]-estratriene-3,17β-diol), estrone (1,3,5[10]-estratrien-3-ol-17-one) and progesterone (4-pregnene-3,20-dione) were from Sigma (St. Louis, MO, USA). These reagents were all used without further purification. Distilled water was further purified by passing through an ion-exchange column (Milli-Q, Millipore, Bedford, MA, USA).

Preparation of samples

A stock solution of cortisol was prepared at 80 μg/ml in ethanol and stored in the dark at 4°C for up to one week. Various working solutions of cortisol were prepared by diluting the stock solution with water on the day of use.

Patients' serum samples were collected by venipuncture. After measurement of serum cortisol with the "GammaCoat Cortisol" RIA kit (Baxter Healthcare, Cambridge, MA, USA) in our laboratory, the samples were stored at −20°C. Frozen serum samples were thawed only once immediately prior to the analysis.

Methods

Derivatization of serum cortisol with sulphuric acid and ethanol. Ethanol (250 μl) was added to 250 μl of serum in a capped brown polypropylene sample cup. The solution was vortex-mixed and centrifuged at 12 100 *g* for 10 min (CM-60RN, Tomy Seiko, Tokyo, Japan). A 320-μl aliquot of the supernatant was transferred to another sample cup. After addition of 480 μl of sulphuric acid, the solution was vortex-mixed and allowed to stand for 10 min in the dark at ambient temperature, then cooled with crushed ice for 15 min in the dark. After adding 700 μl of the precolumn mobile phase solution, the solution was again vortex-mixed and cooled for 15 min in the dark with crushed ice. The samples were kept at 4–5°C in an autosampler until injection into the precolumn. Analysis of all samples was completed within 15 h after sample preparation.

Column-switching HPLC system. Fig. 1 shows a block diagram of the column-switching HPLC system. S1 and S2 are mobile phase solutions. Both P1 and P2 are HPLC pumps (CCPM, TSK, Tokyo, Japan) used in the single plunger mode. V is a high-pressure column-switching valve unit (PT-8000, TSK) and AS is an

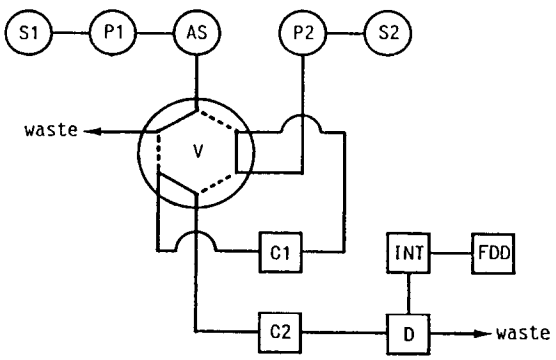

Fig. 1. Block diagram of the column-switching HPLC system. S1, S2, P1, P2, V, AS, C1, C2, D, INT and FDD are described in *Methods*.

autosampler (AS-8000, TSK). C1 is a precolumn and C2 an analytical column. D is a fluorescence detector (FS-8000, TSK), INT is an integrator (CP-8000, TSK) and FDD is a floppy disk drive (FD-8021, TSK).

Samples, injected by an autosampler every 20 min, passed through V, and were trapped and purified in the C1 column. Simultaneously, solvent S2 flowed into the C2 column by the same channel (dotted line). After 5 min the valve was switched so that waste solvent S1 was eliminated from V, and S2 was allowed to pass through columns C1, C2 and finally D before elimination as waste. Electric signals from D were collected by the INT and recorded with the FDD.

Chromatographic conditions. The precolumn (C1) was ODS-80Tm, (10 mm × 4.6 mm I.D., 5 μm) (TSK) and the analytical column (C2) was ODS-80Tm (150 mm × 4.6 mm I.D. 5 μm) (TSK). Column temperatures were ambient. The constitution of S1 for C1 was 10 m*M* potassium biphthlate (adjusted to pH 1.85 with trifluoroacetic acid), and that of S2 for C2 was acetonitrile–tetrahydrofuran 19 m*M* potassium biphthalate (40:6:54, v/v/v, adjusted to pH 1.85 with trifluoroacetic acid). Flow-rates of S1 and S2 were both 1.0 ml/min. Sample volume per injection was 1.0 ml. Eluates from the analytical column were monitored fluorimetrically at 365 nm (excitation) and 520 nm (emission).

RESULTS

Precolumn fluorescence derivatization of cortisol with sulphuric acid and ethanol

Conditions of fluorescence derivatization. The concentration of sulphuric acid was optimized. Sulphuric acid, ethanol and water were added to 1.0 μg of cortisol to a total reaction volume of 4.0 ml. The fluorescence emitted from the derivatized cortisol was measured with a Model 204 spectrofluorimeter (Hitachi, Tokyo, Japan). Fig. 2 shows the relative fluorescence of the derivative. Sulphuric acid (60%) and 17.5% ethanol in the reaction mixture resulted in the highest

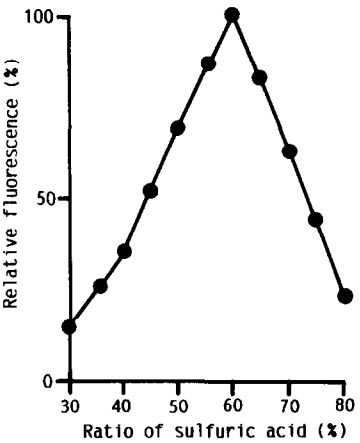

Fig. 2. Optimal concentration of sulphuric acid for fluorescence derivatization of cortisol in 4.0 ml of sulphuric acid–ethanol reaction mixture determined for derivatization of 1.0 μg of cortisol; 60% sulphuric acid (17.5% ethanol) emitted the strongest fluorescence with excitation at 365 nm and emission at 520 nm.

fluorescence intensity at 365 nm (excitation) and 520 nm (emission). Therefore, the optimal volumes of sulphuric acid and deproteinized serum were 480 and 320 μl, respectively. Deproteinized serum was obtained by mixing equal volumes of ethanol and serum.

The fluorescence intensity of derivatized cortisol was stable for 5–60 min of reaction time at ambient temperature. The optimal time for the H_2SO_4–ethanol–serum reaction was 10 min.

Influence of dilution on fluorescence emission. Since the aliquot of the sulphuric acid–ethanol–serum reaction mixture was too viscous to be injected onto the precolumn with an autosampler, the mobile phase (S1) for the precolumn was added to the reaction mixture to decrease the viscosity. The resulting fluorescence emission was highest when the mixture was 46.7% with respect to S1. Fig. 3 shows the relationship between the volume of S1 and the relative fluorescence of the cortisol derivative.

The fluorescence intensity of the derivative with the mobile phase S1 was stable for *ca.* 15 h in the dark at 4°C in an autosampler.

Influence of pH of the mobile phase S2 on fluorescence intensity of the cortisol derivative. Fig. 4 shows the changes in emission of the cortisol derivative as a function of mobile phase (S2) pH ranging from 5.0 to 1.85. The cortisol derivatives were removed after HPLC separation under various mobile phase pH conditions, and their emission wavelengths were scanned with a Model 204 spectrofluorimeter at an excitation wavelength of 365 nm. The maximum emission wavelength was 480 nm with a mobile phase S2 pH ranging from 5.0 to 2.3, 480 and 520 nm at pH 2.2, and 520 nm at pH ranging from 2.1 to 1.85. The strongest fluorescence of the cortisol derivative was observed with emission at 520 nm (excitation at 365 nm) and a pH of 1.85 of the mobile phase. The optimal condi-

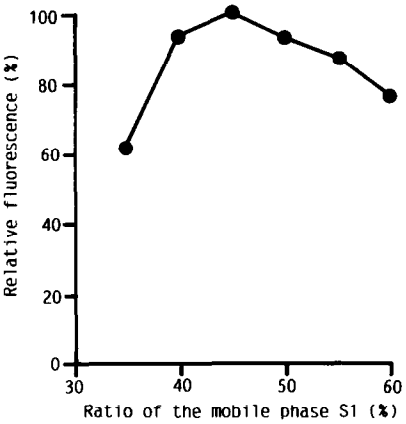

Fig. 3. Influence of dilution of the sulphuric acid–ethanol reaction mixture with mobile phase S1 on fluorescence of the cortisol derivative. Mobile phase S1 was added to the reaction mixture in various amounts to reduce the viscosity and to enable sample solutions to enter the autosampler. The strongest fluorescence of the cortisol derivative was observed when 700 μl of S1 were added to 800 μl of the sulphuric acid–ethanol reaction mixture. The resulting mixture was 46.7% with respect to S1.

tions for excitation and emission wavelengths and the mobile phase (S2) pH were 365 nm, 520 nm and 1.85, respectively.

Calibration

The study of the correlation between the concentrations of authentic cortisol (0, 0.10, 0.30, 0.50, 1.0, 3.0, 5.0, 10, 30, 50, 100, 150 and 200 μg/dl) and the corresponding peak areas of the cortisol derivative resulted in the following regression line: $y = 6.94\ x - 1.48$ (y-axis is peak area, mV), and a coefficient of correlation $r = 1.000$ ($n = 11$). The detection limit for cortisol was 0.3 μg/dl at a signal-to-noise ratio of 3.

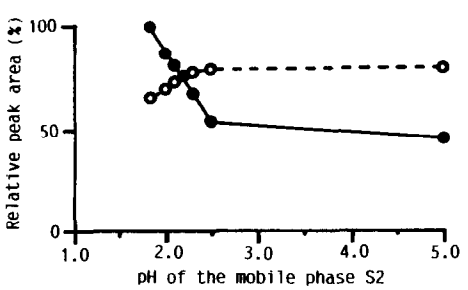

Fig. 4. Influence of the pH of mobile phase S2 on the fluorescent excitation and emission wavelengths of cortisol derivatized with sulphuric acid and ethanol. The solid and broken lines showed the peak areas of the cortisol derivative monitored at 520 and 480 nm emission wavelengths, respectively. Changes of pH of the mobile phase S2 influenced the fluorescence of the derivatized cortisol. The maximum emission wavelength of the cortisol derivative changed from 480 to 520 nm when the pH of S2 changed from 5.0 to 1.85 with excitation of 365 nm. However, the maximum excitation wavelength of the cortisol derivative was not affected at 365 nm following a change in the pH of S2.

Chromatograms

Fig. 5a and b shows the chromatograms of authentic cortisol containing 10.8 ng of cortisol and a patient's serum sample containing 13.3 ng of cortisol per injection, respectively. The retention time of the sulphuric acid–ethanol fluorescent derivative of cortisol was 12.5 min.

The identity of the peak of the cortisol derivative in patient's serum was confirmed as follows: the coincidence of the retention time with that of the authentic cortisol derivative, the increase in peak area at the same retention time on addition of cortisol standard to serum samples without the change in the peak shape, and no or small peaks at 12.5 min in the chromatograms of the blank serum specimens assayed with the GammaCoat Cortisol kit.

Characteristics of the fluorescent emissions of sulphuric acid–ethanol derivatized steroids

Table I shows the retention times and relative fluorescence intensities of 5 μmol each of various steroids derivatized with sulphuric acid and ethanol in comparison with that of derivatized cortisol. The derivatives of cortisol, corticosterone and testosterone emitted strong fluorescence of 100, 218 and 304, and the derivatives of allotetrahydrocortisol and β-estradiol emitted very weak fluorescence of 2.0 and 2.7. Other derivatives emitted virtually no fluorescence. Synthetic steroidal drug derivatives did not emit any fluorescence.

Retention times of the derivatives of corticosterone, testosterone, allotetra-

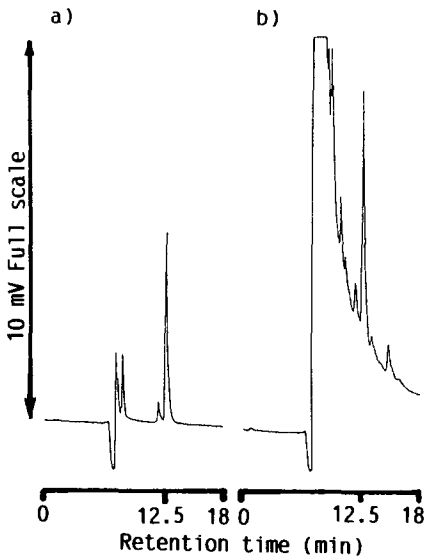

Fig. 5. (a) The peak of derivatized authentic cortisol (10.8 ng); the retention time was 12.5 min. (b) A peak corresponding to serum cortisol of 13.3 ng.

TABLE I

RETENTION TIMES AND RELATIVE FLUORESCENCE INTENSITIES OF THE STEROIDS DERIVATIZED WITH SULPHURIC ACID AND ETHANOL

Retention times and relative fluorescence of 5 μmol each of various steroids in comparison with that of cortisol. Strong fluorescence was observed only for the cortisol, corticosterone and testosterone derivatives.

Steroid	Retention time (min)	Relative fluorescence intensity
Cortisol	12.5	100
All-tetrahydrocortisol	12.3	2.0
Prednisolone	12.5	0.4
Cortolone	13.2	0.2
Prednisone	–[a]	
Dexamethasone	–[a]	
Betamethasone	–[a]	
Cortol	–[a]	
Tetrahydrocortisone	–[a]	
Corticosterone	23.0	218
Androstenediol	9.75	0.5
Androstenedione	11.5	0.6
Testosterone	76.8	304
Dehydroepiandrosterone	–[a]	
β-Estradiol	11.8	2.7
Estriol	12.9	0.7
Estrone	–[a]	
Progesterone	–[a]	

[a] No peak observed.

hydrocortisol and β-estradiol were 23.0, 76.8, 12.3 and 11.8 min, respectively. These peaks did not interfere with the cortisol derivative peak.

Precision

Intra-assay reproducibility. Intra-assay reproducibility was studied with three kinds of serum specimen. Mean concentrations of cortisol were 8.64, 60.1 and 113 μg/dl (n = 10). The corresponding coefficients of variation (C.V.) were 1.24 of 0.559 and 0.619%, respectively.

Inter-assay reproducibility. Inter-assay reproducibility was determined with serum samples at three different cortisol concentrations: 8.65, 59.5 and 106 μg/dl (n = 10). The C.V. of cortisol values were 1.31, 1.03 and 1.30%, respectively.

Recovery. Authentic cortisol was added to a serum specimen containing 8.78 μg/dl cortisol. The expected cortisol value of 13.8 μg/dl was found to be 13.4 μg/dl. The recovery of cortisol was 92.4%. Likewise, authentic cortisol was added to serum samples containing 58.1 and 107 μg/dl cortisol. The recoveries were 99.0 and 110%, respectively.

DISCUSSION

Sulphuric acid fluorescence derivatization of serum cortisol

Ethanol effectively deproteinized serum and derivatized cortisol fluorescently with sulphuric acid. We tried to develop a new rapid and simple determination method for serum cortisol using only the addition of sulphuric acid to deproteinized serum specimens and storing samples in the dark at ambient temperature. This method did not need prior cortisol extraction from serum with dichloromethane as in previously reported methods [16–25].

Other serum substances besides cortisol that were derivatized at the same time were removed by the precolumn, and further separated from the cortisol derivative on the analytical column. The problem of high background fluorescence in ordinary sulphuric acid–ethanol fluorimetry for serum cortisol was solved by this method. There can be no interference from the excess of fluorescence labelling reagents because they are not used.

Derivatization of cortisol was influenced by the amounts of water, ethanol and sulphuric acid in the reaction mixture. Precolumn derivatization enables the amounts of these reactants to be adjusted in order to maximize the yield of derivatized product.

This precolumn derivatization method for serum cortisol also contributed to the reproducibility and accuracy of the method, because loss of cortisol through prior extraction was eliminated.

Stability of the fluorescent derivative of cortisol

There have been some reports [19,20,26] that indicated that the fluorescence intensity of sulphuric acid–ethanol derivatized cortisol decreased steadily over time. However, under our conditions the fluorescence intensity remained stable for up to 15 h at 4–5°C in the dark in an autosampler after derivatization. Cooling the derivatized samples to 4–5°C was very effective in preventing any further reactions.

Influence of pH

The emission wavelength of the cortisol derivative changed from 480 to 520 nm with excitation at 365 nm following a change in the pH from 5.0 to 1.8 in the mobile phase S2 in the analytical column. The fluorescence intensity of the cortisol derivative also increased when emission at 520 nm and excitation at 365 nm were used compared with the fluorescence with emission at 488 nm and excitation at 366 nm employed by Gotelli *et al.* [22]. It contributed to decrease in the detection limit of cortisol to 0.3 μg/dl, which is lower than that of the GammaCoat Cortisol RIA kit.

Specificity of fluorescence of cortisol derivative with sulphuric acid and ethanol

Among the sulphuric acid–ethanol derivatized steroids studied, only those of

cortisol, corticosterone and testosterone were highly fluorescent, whereas those of other steroids, including synthetic steroidal drugs, did not emit fluorescence. This specificity is very useful for the selective determination of serum cortisol.

The HPLC retention times of sulphuric acid–ethanol derivatized cortisol, corticosterone and testosterone were different, which increased the specificity of the assay.

The separation of cortisol and prednisolone in the serum specimens has been quite difficult by isocratic reversed-phase HPLC–UV methods [9] in clinical work. However, our HPLC method enabled the determination of cortisol in serum even though the serum contained both cortisol and prednisolone, as the fluorescence intensity of the prednisolone derivative was very weak in comparison with that of the cortisol derivative.

Column lifetime

Although the samples injected into the precolumn were strongly acidic, and the sample volume per injection was 1.0 ml, ODS-80Tm gel precolumns could be used for 100–200 injections. This is probably because of rapid dilution of samples with the mobile phase S1 (pH 1.83) in the precolumn after injection, and end-capping of the silica of ODS-80Tm gel.

The use of an ODS-80Tm gel cartridge for precolumns (10 mm × 4.6 mm I.D.) (TSK) may facilitate precolumn maintenance.

The ODS-80Tm gel analytical column also withstood the mobile phase of pH 1.85 for the reasons mentioned above.

Fully automated reversed-phase HPLC system with column switching

Good reproducibility and recovery were obtained using the fully automated reversed-phase-HPLC system with column switching. This automatically purified the crude samples, separated on-line and enabled the fluorescence to be monitored after column switching.

CONCLUSION

This method eliminates the need for prior extraction of cortisol in serum specimens. It is rapid, and the stable derivative of cortisol can be sensitively detected.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Hiroko Kawamoto of Tottori University College of Medical Care Technology (Yonago, Japan) for her advice during this study.

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