Journal of Chromatography, 489 (1989) 364–370
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4634

Note

Measurement of 21-hydroxycorticosteroids in human and rat sera by high-performance liquid chromatography with fluorimetric detection

TAKASHI YOSHITAKE, SHUUJI HARA, MASATOSHI YAMAGUCHI* and MASARU NAKAMURA

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Johnan-ku, Fukuoka 814-01 (Japan)

YOSUKE OHKURA

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

and

SÁNDOR GORÒG

Chemical Works of Gedeon Richter Ltd., Pf 27, H-1475 Budapest 10 (Hungary)

(First received August 25th, 1988, revised manuscript received November 23rd, 1988)

The assay of serum 21-hydroxycorticosteroids is very useful for evaluation of adrenal and pituitary function, disorders of which cause such diseases as Cushing's syndrome and primary and secondary adrenal insufficiency.

Various methods, including enzyme immunoassay [1], radioimmunoassay [2], competitive protein binding assay [3] and fluorimetry [4] have been reported for the determination of individual 21-hydroxycorticosteroids in serum. Gas chromatographic–mass spectrometric [5] and high-performance liquid chromatographic (HPLC) [6–13] methods offer advantages over the above methods for simultaneous determination of the corticosteroids in serum.

Recently, we found that 1,2-diamino-4,5-methylenedioxybenzene (DMB) reacts with glyoxal compounds to form highly fluorescent quinoxaline derivatives [14]. Thus, we have developed a sensitive and simple HPLC method with fluorimetric detection for the determination of 21-hydroxycorticosteroids, based on the conversion of the corticosteroids into the corresponding glyoxal compounds followed by reaction with DMB. The method was applied to the assay of some 21-hydroxycorticosteroids of clinical importance in human and rat sera.

0378-4347/89/$03.50

EXPERIMENTAL

Reagents and solutions

Unless stated otherwise, all chemicals and solvents were of analytical-reagent grade. Aldosterone (ALD), hydrocortisone (F), prednisone (PRED), cortisone (E), 18-hydroxycorticosterone (18-OHB), dexamethasone (DXM), 18-hydroxydeoxycorticosterone (18-OHDOC), corticosterone (B), 11-deoxycortisol (S) and deoxycorticosterone (DOC) were purchased from Sigma (St. Louis, MO, U.S.A.). DMB·2HCl was prepared as described previously [15], although it is now commercially available (Dojindo Labs., Kumamoto, Japan).

DMB solution (7.0 m*M*) was prepared by dissolving DMB·2HCl in water containing 0.2 *M* β-mercaptoethanol and 0.25 *M* sodium hydrosulphite. The solution was stored at 4°C in the dark; under these conditions it was stable for at least two weeks. Cupric acetate solution (39 m*M*) was prepared by dissolving 0.7 g of cupric acetate in 10 ml of water and diluting the solution with methanol to 100 ml [15]. The internal standard (I.S.), 2.2 μM DXM prepared in methanol, could be used for at least one month.

Apparatus and HPLC conditions

Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorimeter (Tokyo, Japan) in 10×10 mm quartz cells; spectral bandwidths of 10 nm were used both for excitation and emission monochromators.

A Hitachi 655A high-performance liquid chromatograph was used. This was equipped with a Rheodyne 7125 syringe-loading sample injector valve (50-μl loop) and a Shimadzu RF-535 fluorescence spectromonitor (12-μl flow-cell). It was operated at an excitation wavelength of 350 nm and an emission wavelength of 390 nm. The column was TSK gel ODS-120T (250 mm×4.6 mm I.D.; particle size 5 μm; Tosoh, Tokyo, Japan). This column could be used for more than 1000 injections with only a small decrease in the theoretical plate number. The column temperature was ambient (ca. 20°C). For the separation of the DMB derivatives of the corticosteroids, stepwise gradient elution with methanol–acetonitrile–1.0 *M* ammonium acetate (48:15:37, 65:8:27 and 82:8:10, v/v; mobile phases A, B and C, respectively) was carried out with a Hitachi 833A solvent gradient device. Mobile phase A was used first for 27 min, mobile phase B for the following 22 min and mobile phase C for the next 16 min (Fig. 1A). The column was equilibrated with mobile phase A for 20 min before the start of the next sample. The flow-rate was kept constant at 1.0 ml/min. Peak areas were determined by a Water QA-1 data system.

Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with 20-μl flow-cell; the spectral bandwidths were 5 nm for both the excitation and emission monochromators.

Serum samples

Human sera were obtained from healthy volunteers (seven males and two females; 22–37 years old) in our laboratories. Rat serum was obtained from 13-week-old male Wistar rats weighing 210–230 g.

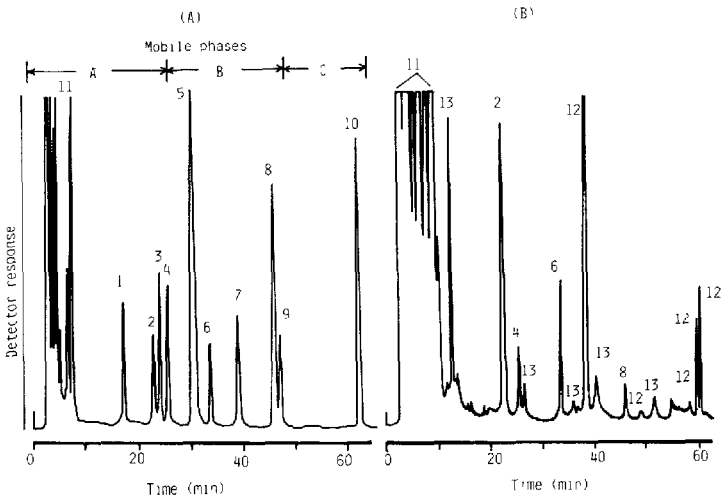


Fig. 1. Chromatograms obtained with (A) a standard solution and (B) a normal human serum. (A) A portion (100 μl) of a standard mixture of 21-hydroxycorticosteroids (concentrations. ALD, F, PRED, E, DXM, B, S and DOC, 1 0 nmol/ml each; 18-OHB and 18-OHDOC, 50 nmol/ml each) in methanol was treated as in the derivatization procedure. (B) A portion (500 μl) of a normal human serum was treated according to the procedure. Peaks: 1 = ALD; 2 = F; 3 = PRED; 4 = E; 5 = 18-OHB; 6 = DXM; 7 = 18 = OHDOC; 8 = B; 9 = S, 10 = DOC; 11 = blank components; 12 = endogenous α-dicarbonyl compounds; 13 = endogenous 20-keto-21-hydroxy steroids.

A 500-μl aliquot of serum was pipetted into a screw-capped culture tube together with 100 μl of the I.S. solution and 4 ml of diethyl ether–dichloromethane (60:40, v/v). The mixture was vortex-mixed for 2 min and centrifuged at 1000 *g* for 5 min. The organic layer (ca. 3 ml) was transferred into another screw-capped 1-ml vial, and was evaporated to dryness under a stream of nitrogen. The residue, dissolved in 100 μl of methanol, was used as a sample solution.

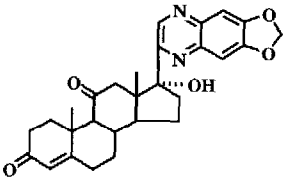
Derivatization procedure

A 100-μl portion of the sample solution, placed in a screw-capped 1-ml vial, was mixed with 20 μl of cupric acetate solution. The mixture was allowed to stand at room temperature for 1 h. Then, 100 μl of the DMB solution were added and heated at 60°C for 40 min. After cooling, the mixture was centrifuged at 1000 *g* for 5 min, and 50 μl of the supernatant were injected into the chromatograph.

The calibration graphs were prepared as in the procedure, except that 100 μl of the I.S. solution was replaced by the I.S. solution containing 1 pmol to 5 nmol each of the corticosteroids. The net peak-area ratios of the individual corticosteroids and DXM were plotted against the concentrations of the corticosteroids.

Preparation of the fluorescent product (Fig. 2) from cortisone

E (110 mg, 0.3 mmol) was dissolved in 10 ml of methanol. To the solution were added 50 ml of the cupric acetate solution, and the mixture was allowed to stand at room temperature for 1 h. To the resulting solution were added DMB·2HCl (ca. 110 mg, 0.5 mmol), 4 ml of β-mercaptoethanol and ca. 300 mg of sodium



(I)

Fig. 2. Structure of compound I, the fluorescent product from cortisone.

hydrosulphite, and the mixture was heated at 80°C for 20 min and cooled. The reaction mixture was filtered, and the filtrate was evaporated to dryness in vacuo. The residue was dissolved in 5 ml of chloroform–acetone (19:1, v/v) and chromatographed on a silica gel 60 column (ca. 75 g, 70–230 mesh; Japan Merck, column size, 25 cm×2 cm I.D.) with the same solvent. The main fraction was evaporated to dryness, and the residue was recrystallized from acetone to form colourless needles (dec. 275°C; 64 mg).

RESULTS AND DISCUSSION

HPLC conditions

The best separation of the DMB derivatives of ten 21-hydroxycorticosteroids was achieved on a TSK gel ODS-120T column by stepwise elution with mixtures of methanol, acetonitrile and 1.0 *M* ammonium acetate, as described in Experimental. The change in methanol and acetonitrile concentrations actually had no effect on the fluorescence excitation (maximum, 350 nm) and emission (maximum, 390 nm) spectra or the intensities of the derivatives of all the corticosteroids. A typical chromatogram obtained with a standard mixture is shown in Fig. 1A. The individual corticosteroids gave unique peaks. All the peaks were well separated within 63 min.

Derivatization conditions

21-Hydroxycorticosteroids are easily oxidized by cupric acetate to form the corresponding glyoxal compounds. The oxidation conditions were as described previously [16].

DMB·2HCl reacted with glyoxal compounds and gave the most intense peaks with reagent solution at concentrations greater than 4.0 m*M*; 7.0 m*M* was used in practice. β-Mercaptoethanol and sodium hydrosulphite most effectively stabilized DMB during the reaction at concentrations of 0.2 *M* and 0.25 *M*, respectively.

The derivatization reaction occurred at low temperatures (0–37°C), but higher temperatures allowed it to proceed more rapidly. However, at 80°C, the peak areas decreased after prolonged heating (45 min). At 60°C, the peak areas for all the corticosteroids approached maxima after heating for 30 min, hence heating at 60°C for 40 min is the recommended procedure. The DMB derivatives of all the corticosteroids were stable for at least 6 h in daylight at room temperature (20–25°C).

The within-day precision was estimated from eight replicate analyses of a standard mixture of corticosteroids (ALD, B, E, DOC, S, F and DXM, 50 pmol per 100 μl; 18-OHB and 18-OHDOC, 5 nmol per 100 μl). The relative standard deviations did not exceed 5% for all the corticosteroids. The limits of detection (pmol) were 0.51 (ALD), 0.71 (F), 0.42 (PRED), 0.45 (E), 9.2 (18-OHB), 0.77 (DXM), 29.4 (18-OHDOC), 0.14 (B), 0.69 (S) and 0.21 (DOC) in a 50-μl injection volume at a signal-to-noise ratio of 3. The relatively low sensitivities for 18-OHB and 18-OHDOC may be due to the following reason: after the cupric acetate oxidation of the 21-hydroxy group in these compounds, the 21-aldehyde formed undergoes an intramolecular reaction with the 18-hydroxy group to form a cyclic ketal derivative, which allows only a few percent of the glyoxal to react with DMB.

Fluorescent product in the determination of cortisone

In order to investigate the structure of the fluorescent products, E was employed as a model compound. Since the products in the reaction of α-dicarbonyl compounds with DMB have been shown to be the 6,7-methylenedioxyquinoxaline derivatives [14], the reaction product from E should be a quinoxaline derivative (Fig. 2). This structure was confirmed by ^1H nuclear magnetic resonance, infrared and electron-impact mass spectra. The fluorescence excitation (maximum, 350 nm) and emission (maximum, 390 nm) spectra of the product in methanol–acetonitrile–1.0 *M* ammonium acetate (48:15:37, v/v) were almost identical with those of the eluates for the other corticosteroids. These results indicate that the fluorescent products of the corticosteroids tested are the corresponding quinoxaline derivatives.

Determination of 21-hydroxycorticosteroids of clinical importance (hydrocortisone, cortisone and corticosterone) in normal human and rat sera

21-Hydroxycorticosteroids in serum were extracted with diethyl ether–dichloromethane (6:4, v/v) in the usual manner [9–12].

Fig. 1B shows a typical chromatogram obtained using normal human serum. Peaks 2, 4 and 8 were identified as F, E and B, respectively, on the basis of their retention times and fluorescence excitation and emission spectra. This was achieved by comparison of the spectra with those of the standard compounds and also by co-chromatography of the standards and the serum samples with aqueous 50–100% methanol or acetonitrile as mobile phase. Peak 12 may be due to the endogenous α-dicarbonyl compounds in human serum. This was suggested by the following results. When serum sample was replaced with water, no peaks except for the blank components (peak 11) were detected in the chromatogram. Moreover, each elution from peak 12 exhibited fluorescence excitation and emission maxima et ca. 350 and 390 nm, almost identical with those of the DMB derivatives of the corticosteroids. Even when the oxidation step with cupric acetate was omitted, peak 12 was observed in the chromatogram. On the other hand, peak 13 appeared when the oxidation with cupric acetate was carried out. This indicates that these peaks may be ascribed to other endogenous 20-keto-21-hy-

droxysteroids not included in the standards investigated in this study. Those peaks did not interfere with the determination of E, F and B in serum.

DMB reacts with biogenic α-dicarbonyl compounds, such as glyoxal, methylglyoxal and α-keto acids (α-ketoglutaric, pyruvic, α-ketoisovaleric, α-ketoisocaproic and α-keto-β-methylvaleric acids), to produce fluorescent derivatives [14, 15]. However, all the DMB derivatives of those compounds were eluted at retention times of 5–12 min. Thus, further clean-up procedure was not necessary.

For calibration graphs, linear relationships were observed between the ratios of the peak areas of the corticosteroids to that of DXM and the amounts of the corticosteroids added in the range from 2 pmol to 2 nmol (corresponding to ca. 230 fmol to 230 pmol per injection volume) each to 1 ml of serum sample.

The recoveries (%, mean $\pm$S.D., $n=8$) of the corticosteroids (10 nmol per 500 μl) added to a pooled normal human serum were 85.3 ± 6.8 (ALDO), 99.9 ± 5.9 (F), 92.2 ± 3.2 (PRED), 103.4 ± 2.8 (E), 96.2 ± 4.0 (18-OHB), 83.4 ± 3.8 (DXM), 90.5 ± 1.9 (18-OHDOC), 99.0 ± 3.6 (B), 100.0 ± 7.5 (S) and 94.2 ± 0.7 (DOC).

The precision was established by repeated assays ($n=10$) of normal human serum. The relative standard deviations were 7.0% for F, 3.5% for E and 5.0% for B.

The amounts of F, E and B in normal human sera (mean $\pm$ S.D.) were 359.5 ± 107.6, 58.5 ± 125.9 and 11.2 ± 6.1 pmol/ml, respectively. The serum concentration of B in rat sera (mean $\pm$ S.D.) was 311.2 ± 166.9 pmol/ml. The mean values are similar to those obtained by other workers [1–3, 6–9]. The other corticosteroids could not be detected in human or rat sera.

CONCLUSION

The study has provided the first HPLC method with fluorimetric detection for the simultaneous determination of F, E and B in human serum. The method is sensitive enough to measure the corticosteroids in 500 μl of normal serum. The method is readily performed and could therefore be useful for diagnosis for pituitary and adrenal function.

REFERENCES

1 N. Monji, N.O Gomez, H. Kawashima, H. Alei and A. Castro, J. Clin. Endocrinol Metab., 50 (1980) 355.
2 M.H.H. Al-Hakiem and S.J. Abbas, Clin. Chem., 33 (1987) 191.
3 J. Kolennowski, J. Steroid Biochem., 5 (1974) 55.
4 A.E. Kitabchi and L.C. Kitchell, Anal. Biochem., 34 (1970) 529.
5 K. Ichimura, H. Yamanaka, K. Chiba, T. Shinozuka, Y. Shiki, K. Saito, S. Kusano, S. Ameniya, K. Oyama, Y. Nozaki and K. Kato, J. Chromatogr., 374 (1986) 5.
6 F.J. Frey, B.M. Frey and L.Z. Benet, Clin. Chem., 25 (1979) 1944.
7 T. Matsuzawa, N. Sugimoto and I. Ishiguro, Anal. Biochem., 115 (1981) 250.
8 K. Shimizu, S. Amagaya and Y. Ogihara, J. Chromatogr., 272 (1983) 170.
9 C.J.H. Woodward and P.W. Emery, J. Chromatogr., 419 (1987) 280.
10 N.R. Scot, J. Chakraborty and V. Marks, Anal. Biochem., 108 (1980) 266.
11 V.K. Prasad, B. Ho and C. Haneke, J. Chromatogr., 378 (1986) 305.
12 T.J. Goehl, G.M. Sundaresan and V.K. Prasad, J. Pharm. Sci., 68 (1979) 1374.

13 E. Stoner, S. Loche, A. Mirth and M.I. New, J. Chromatogr., 374 (1986) 358.
14 S. Hara, M. Yamaguchi, Y. Takemori, T. Yoshitake and M. Nakamura, Anal. Chim. Acta, 215 (1988) 267.
15 M. Nakamura, S. Hara, M Yamaguchi, Y. Takemori and Y. Ohkura, Chem. Pharm. Bull., 35 (1987) 687.
16 S. Görög and G. Szepesi, Anal. Chem., 44 (1972) 1079.