[Chem. Pharm. Bull. 30(6)2228—2231(1982)]

Determination of Corticoids using Pyrrole. IV. Fluorometric Determination of Cortisol in Serum

Hiroshi Tokunaga,* Toshio Kimura, and Jiro Kawamura

National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo, 158, Japan

(Received November 11, 1981)

A fluorometric method for the determination of cortisol (CS) in serum was established. The keto-alcohol side-chain of CS was converted to keto-aldehyde with cupric acetate, and the product was reacted with pyrrole. The relationship between the fluorescence intensity and the concentration of CS was linear in the range of 0—50 ng. This method was applied to the determination of human serum CS and the analytical data were compared with those obtained by radioimmunoassay. The regression equation for the results of the two method was $y=1.29x-0.44$, and the coefficient of correlation for 14 samples with CS in the range of 6.78—14.56 μg/dl was 0.967. This method gave reliable results with high specificity and sensitivity.

Keywords——cortisol; serum cortisol; fluorometry; pyrrole; radioimmunoassay

Cortisol (CS) belongs to the group of corticoids, which are hormones secreted by the adrenal cortex. In various diseases such as Cushing's syndrome, adrenal insufficiency, Addison's disease, *etc.*, it is known that the concentration of CS in blood and urine varies markedly. For the determination of CS in blood and urine, various methods are available, for example, colorimetric assay,[1] fluorometric assay,[2] competitive protein binding assay,[3] radioimmunoassay (RIA),[4] high-performance liquid chromatography (HPLC),[5] and enzyme immunoassay.[6]

We have established a new fluorometric method using pyrrole for the determination of CS, prednisolone, cortisone, hydrocortisone acetate, and cortisone acetate in pharmaceutical preparations.[7] This fluorometric method has now been applied to the measurement of serum CS and has given reliable results with high specificity and sensitivity. Analytical values obtained by this method were compared with those obtained by RIA.

Experimental

Apparatus——Fluorescence excitation and emission were measured with a Hitachi 650-10S fluorescence spectrophotometer. Radioactivity was measured with an Aloka Auto Gammacounter.

Reagents and Solutions——CS, testosterone (TS), estradiol (ED), and cholesterol (CR) were purchased from Sigma Chemical Co. (USA). Progesterone (PR) and cholic acid (CA) were obtained from Wako Junyaku Co. (Tokyo). Deoxycorticosterone (DC) was supplied by Nakarai Chemical Co. (Kyoto). SPAK cortisol kit was obtained from Daiichi Radioisotope Laboratory (Tokyo). Bovine serum was from Chiba Serum Laboratory (Chiba). All other chemicals were of reagent grade.

Human Serum: Human serum was prepared from normal human blood.

Pyrrole Solution: Pyrrole (2.5 ml) was diluted to 100 ml with methanol.

Cupric Acetate Solution: Cupric acetate (25 mg) was dissolved in 100 ml of methanol.

Sodium Hydroxide Solution: Sodium hydroxide (0.8 g) was dissolved in 100 ml of water.

Standard Solution: Twenty milligrams of CS, accurately weighed, was dissolved with methanol to make exactly 100 ml. This solution was diluted with methanol so as to give a concentration ranging from 10 to 100 ng/ml.

Fluorometric Procedure——Into a glass-stoppered test tube, 0.2 ml of human or bovine serum was pipetted, and then 0.8 ml of water, 0.1 ml of sodium hydroxide solution, and 3.0 ml of dichloromethane were added. The mixture was vigorously stirred for one minute. After standing for a few min, 2.0 ml of the dichloromethane layer was transferred to another glass-stoppered test tube, and evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 ml of methanol, and 0.1 ml of cupric acetate solu-

tion was added to the solution. This solution was allowed to stand for 30 min, and 0.1 ml of methanol, 0.3 ml of hydrochloric acid and 0.05 ml of pyrrole solution were added. The mixture was left to stand for 20 min at 25°C, then 1 ml of water and 3.0 ml of benzene were added to the reaction mixture and mixed well. The benzene layer was transferred to a test tube, and dried over anhydrous sodium sulfate. The fluorescence intensity of the supernatant was measured at 615 nm with excitation at 592 nm.

A reagent blank, substituting water for serum, was processed in a similar manner.

A 0.5 ml standard solution containing 100 ng of CS per ml was pipetted into a glass-stoppered test tube, and evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 ml of water, and processed in the standard manner.

The content of CS was calculated according to the following equation, where F_s, F_t, and F_b, are the fluorescence intensity obtained from standard solution, serum and water, respectively.

$$\frac{F_t - F_b}{F_s - F_b} \times 25 = \mu g \text{ of CS per dl of serum}$$

RIA Method——Sera were assayed according to the manufacturer's instructions.

Results and Discussion

Effect of Various Steroids on the Fluorescence Intensity

Into each tube containing 500 ng of TS, ED, CR, PR, CA, or DC, 0.5 ml of standard solution of CS as a concentration of 100 ng per ml was pipetted, and fluorescence intensity was measured as described in "Experimental." The presence of TS, ED, CR, PR, or CA did not interfere with the measurement of CS, though DC affected it markedly. The presence of 500 ng of DC caused an increase in the fluorescence intensity of 50 ng of CS (about 1.5 fold). We have already reported that 17-deoxycorticoids react with pyrrole.[8] Therefore, this increase is presumably due to the reaction between DC and pyrrole.

Conditions of the Fluorometric Determination

Recovery——Into a glass-stoppered test tube, 0.5 ml of standard solution was pipetted. The solution was evaporated to dryness under reduced pressure, then 0.2 ml of bovine serum was added to each tube, and CS was determined by fluorometric determination. In the cases of the addition of 10 and 50 ng of CS, the recoveries were 99.3±4.5% (mean±S.D, n=6) and 100.9±2.6% (mean±S.D, n=6), respectively. It is suggested that CS was effectively extracted from serum. Rudd *et al.*[2a] and Trefy *et al.*[5a] reported that the mean recoveries of CS from serum were 79.6 and 78.0%, respectively. In regard to recovery, our procedure is thus superior to the known methods.

Working Curve——Into a glass-stoppered test tube, 0.5 ml of standard solution (10 to 100 ng per ml) was pipetted. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 ml of bovine serum, and subjected to the fluorometric procedure for serum.

Percentage fluorescence intensity was calculated according to the following equation, where F_{50}, F and F_0 are the fluorescence intensities due to 50 ng of CS, another concentration of CS, and bovine serum, respectively.

$$\frac{F - F_0}{F_{50} - F_0} \times 100 = \text{percentage of fluorescence intensity}$$

The working curve for CS was linear from 0 to 50 ng and passed through the zero point as shown in Fig. 1.

Identification of Fluorescence Products obtained from Human Serum CS and Bovine Serum CS

The fluorescence products obtained from CS were compared with those from human serum CS and bovine serum CS by the use of thin-layer chromatography (TLC). After the reaction of CS, human serum and bovine serum containing CS with pyrrole, each sample was developed on a silica gel TLC plate (Kieselgel G-60, Merck) with a mixture of dichloromethane

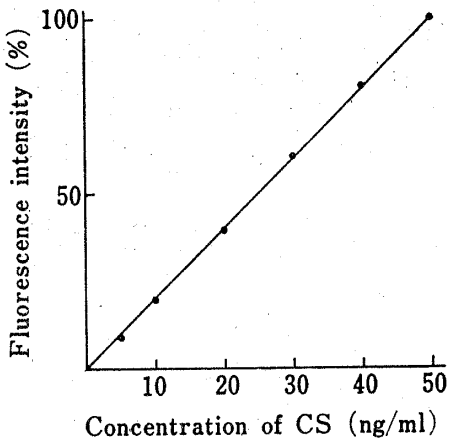

Fig. 1. Working Curve for CS obtained by the Fluorometric Method

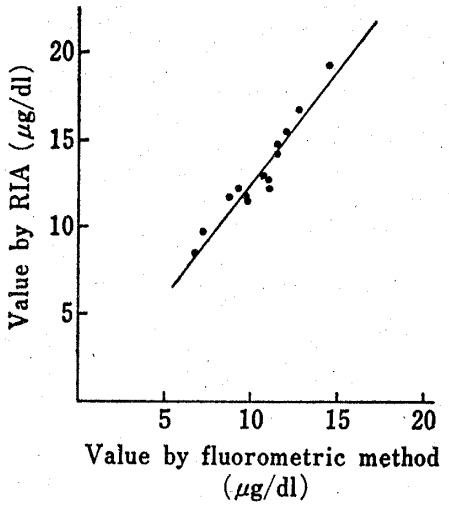

Fig. 2. Correlation of Values of CS in Human Serum determined by the Fluorometric Method and by RIA

and methanol (9: 1) as the solvent. After development of the plate, the fluorescence was detected under a long-wavelength mercury lamp. CS gave a single spot with reddish fluorescence on the TLC plate, and human serum and bovine serum containing CS each two spots, one with reddish and one with blue fluorescence. The *Rf* value of the reddish fluorescent spot from CS, human serum and bovine serum was 0.57, and that of the blue fluorescent spot from human serum and bovine serum was 0.89. In order to examine the excitation and emission spectra of the blue and reddish fluorescent products obtained from human serum and bovine serum, the silica gel with each spot was collected, and the blue and reddish fluorescent products were extracted with a mixture of dichloromethane and methanol (1: 1). After the filtration of extract, the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in benzene containing a little hydrochloric acid, and the excitation and emission spectra of blue and reddish fluorescent products were measured. The maximum excitation and emission wavelengths of the blue fluorescent product were 290 and 335 nm, respectively. Those of the reddish product were 592 and 615 nm, respectively, corresponding to those of the reddish product obtained from CS. Therefore, we conclude that the concentration of CS in human serum and bovine serum could be determined without interference from the former product.

Application to the Determination of CS in Human Serum

CS in human serum was determined by the method described in this paper. The analytical values for 14 samples were in the range of 6.78—14.56 μg/dl. These values are consistent with those hitherto reported for normal human blood (12—18 μg/dl).

The analytical values were compared with those obtained by RIA (Fig. 2). The regression equation for the results of these two method was $y=1.29x-0.44$, and the coefficient of correlation was 0.967. The values from this method were slightly lower than those by RIA.

References

1) C.C. Porter and R.H. Silber, *J. Biol. Chem.*, **185**, 201 (1950); R.E. Peterson, A. Karrer, and S.L. Guerra, *Anal. Chem.*, **29**, 144 (1957).
2) *a*) B.T. Rudd, P. Sampson, and B.N. Brooke, *J. Endocrinol.*, **27**, 317 (1963); *b*) B.R. Clark and R.T. Rubin, *Anal. Biochem.*, **29**, 31 (1969); *c*) R. Horikawa, T. Tanimura, and Z. Tamura, *Anal. Biochem.*, **85**, 105 (1978).
3) J. Kolanowski, *J. Steroid Biochem.*, **5**, 55 (1974); G.P. Klein, M. de Levie, and C.J.P. Girod, *Steroids*,

19, 275 (1972).
4) D.M. Laurence and D.D. Dennis, *Clin. Biochem.*, **10**, 104 (1977).
5) *a*) F.K. Trefy, D.J. Byrd, and W. Kochen, *J. Chromatogr.*, **107**, 181 (1975); *b*) T. Kawasaki, M. Maeda, and A. Tsuji, *J. Chromatogr.*, **163**, 143 (1979).
6) L.J. Winfrey, L. Johons-Stephens, and H.M. Greenwood, *Clin. Chem.*, **25**, 1151 (1979); H. Arakawa, M. Maeda, and S. Tsuji, *Bunseki Kagaku*, **26**, 322 (1977).
7) H. Tokunaga, T. Kimura, and J. Kawamura, *Yakugaku Zasshi*, **100**, 200 (1980).
8) H. Tokunaga, T. Kimura, and J. Kawamura, *Bunseki Kagaku*, **6**, 392 (1976).