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DETERMINATION OF PLASMA AND URINARY CORTISOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DERIVATIZATION WITH DANSYL HYDRAZINE

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SUMMARY

A method is described for the determination of cortisol in human plasma and urine by high-performance liquid chromatography using fluorophotometric detection. After extraction with methylene chloride, cortisol is labelled with dansyl hydrazine, and then separated by high-performance chromatography. The eluate is monitored by a fluorophotometer at 350 nm (excitation) and 505 nm (emission). The optimum conditions for the determination, such as HCl and dansyl hydrazine concentrations, reaction time and reaction temperature, and for the eluent of high-performance liquid chromatography, are discussed. Linearity of the fluorescence intensity (peak height) with the amount of cortisol was obtained between 0.5 and 60 ng. The recoveries for 50 and 100 ng of added cortisol were 98.7 and 95.4% for plasma, and 96.4 and 90.6% for urine, respectively. Comparison with a radioimmunoassay gave a correlation coefficient of 0.978. The proposed method is suitable for the routine analysis of cortisol in plasma and urine.

INTRODUCTION

Cortisol is an important secretory product of the adrenal cortex, and represents a good parameter for adrenal activity. Many methods have been proposed for the determination of cortisol in biological samples, including thin-layer and gas—liquid chromatography [1], spectrophotometry [2], fluorimetry [3] and radioimmunoassay [4]. In certain instances, these methods may be considered lacking in either sensitivity, specificity, reproducibility or convenience of analysis. It has recently been demonstrated that high-performance liquid chromatography (HPLC) is suitable for the analysis of cortisol and corticosterone in biological materials [5—9]. On the other hand, fluorigenic labelling techniques have been used for several years in conjunction with thin-layer chromatography

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[10, 11], and are now being used also to good advantage in HPLC [12, 13]. In this study, dansyl hydrazine was used to derivatize the carbonyl group of cortisol. The fluorescent derivative was then separated and detected by HPLC equipped with a fluorimeter. This method has been applied to the quantitative determination of cortisol in human plasma and urine.

EXPERIMENTAL

Materials

All chemicals used were of reagent grade and obtained from commercial sources.

Apparatus

An Hitachi Model 634 high-speed liquid chromatograph and an Hitachi Model 204 fluorophotometer equipped with xenon lamp and flow cell were used.

Chromatographic conditions

A 250 mm × 4 mm I.D. stainless-steel column fitted with an on-column injection port was used. The column packing was Hitachi gel No. 3042 (silica gel, particle size 5 μm). The column was packed by a slurry method. The eluent system, dichloromethane—ethanol—water (948:35:17) was used according to the description of Hesse et al. [14, 15]. The eluent was prepared as follows: 17 ml of redistilled water and 35 ml of ethanol were made up to 1000 ml with dichloromethane. The mixture was shaken for 1 h at room temperature and then allowed to stand until the emulsion had cleared. After separation, the organic phase was ready for use. The column was equilibrated at 35° with the above eluent; the flow-rate was 1 ml/min, which corresponded to a pressure drop of 25 kg/cm^2 in the column used. The effluent was monitored with a fluorimeter at an excitation wavelength of 350 nm and emission wavelength of 505 nm.

Reagent solutions

Dansyl hydrazine solution: A 0.02% (w/v) solution of dansyl hydrazine was prepared by dissolving 2 mg of dansyl hydrazine (Sigma, St. Louis, Mo., U.S.A.) in 10 ml of ethanol, and stored at 4°.

Hydrochloric acid–ethanol solution: The solution was prepared by mixing 0.9 ml of concentrated HCl and 500 ml of ethanol.

Cortisol stock solution: One mg of cortisol (Merck, Darmstadt, G.F.R.) was dissolved in redistilled water and made up to 100 ml. The resultant stock solution was stored at 4°.

Cortisol working standard solution: One ml of the cortisol stock solution was transferred into a 50-ml or 100-ml volumetric flask and made up to volume with redistilled water before use.

Procedure

Extraction of cortisol from plasma and urine: Half a millilitre of well-mixed plasma or 1.0 ml of urine was transferred to a screw-cap culture tube. Half a

millilitre of water, 0.1 ml of 2 *N* NaOH solution, and 10 ml of methylene chloride were added to the tube, which was then capped tightly. The mixture was stirred vigorously for 1 min by a Vortex-type mixer, and centrifuged for 5 min at 10,000 *g*. The aqueous layer was removed with a Pasteur pipette, and the organic layer was washed with 1 ml of 0.1 *N* H_2SO_4 by stirring for 30 sec, after which the aqueous layer was removed again. After drying by the addition of 2 g of anhydrous Na_2SO_4, exactly 7.0 ml of the organic layer were evaporated to dryness at 40° under a stream of nitrogen gas.

Labelling reaction: The residue in the test-tube was dissolved by adding 0.1 ml of HCl—ethanol and then mixed with 0.01 ml of dansyl hydrazine solution. After standing for 30 min, the solvent was evaporated to dryness under a stream of nitrogen gas. The labelled residue was dissolved in 50 μl of chloroform; 20 μl of this were injected into the chromatograph.

RESULTS

Fluorescence spectrum

Fig. 1 shows the excitation and emission spectra of the dansyl hydrazone of cortisol in chloroform which was prepared by preparative thin-layer chromatography after reaction. The excitation wavelength maximum was 350 nm and the emission maximum 505 nm. The fluorescence intensity of dansyl derivatives was affected by the polarity of the solvent. The solvent effects on the fluorescence were therefore examined before the analytical technique was

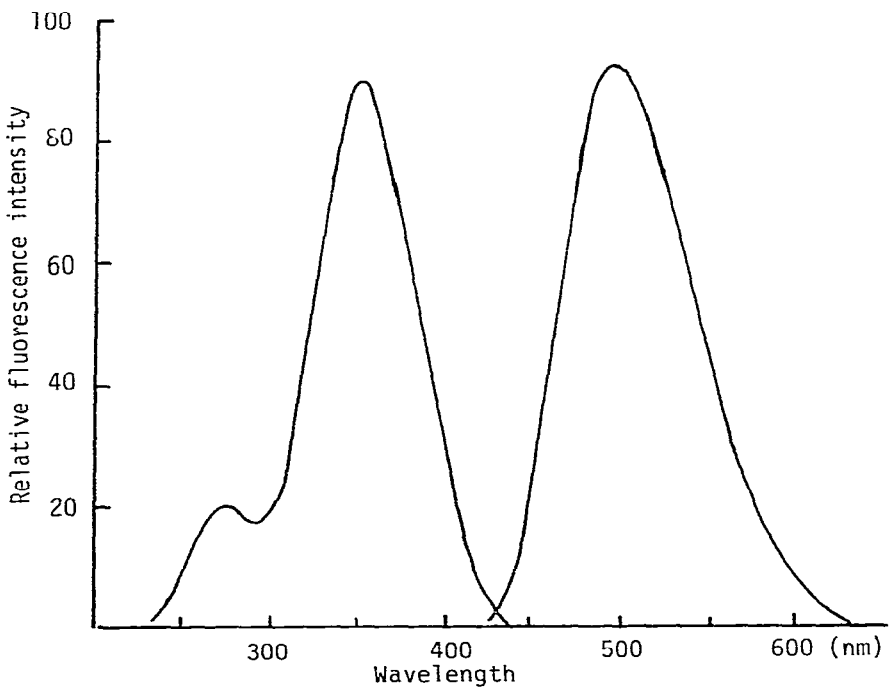

Fig. 1. Excitation and emission spectra of the fluorescent dansyl hydrazone of cortisol in chloroform. Excitation maximum, 350 nm; emission maximum, 505 nm.

developed. Among the solvent systems tested, methylene chloride gave the most intense fluorescence, and the polar solvents such as methanol, ethanol, etc., gave a lower fluorescence intensity. Although the eluate used in this paper contained a small amount of ethanol and water, the fluorescence intensity of the dansyl hydrazone of cortisol in it was almost the same as that in methylene chloride.

Concentration of HCl and dansyl hydrazine

Dansyl hydrazine reacted with cortisol to form hydrazone in acidic medium. As shown in Fig. 2, the reaction time to reach a maximum and constant peak

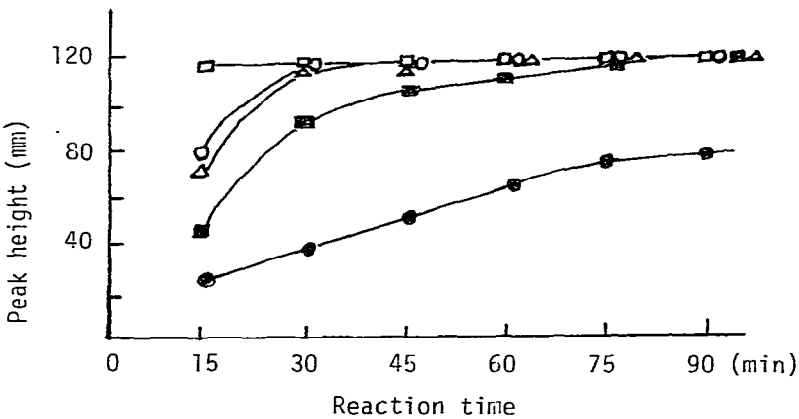

Fig. 2. Effect of HCl concentration and reaction time on peak height (fluroescence intensity). The amounts of concentrated HCl in 500 ml of ethanol were: 3.5 ml (□), 1.8 ml (○), 0.9 ml (△), 0.45 ml (■), and 0.225 ml (●). Injected volume: 5 μl of the final solution containing 35 ng of cortisol.

height decreased with increased HCl concentration in ethanol. Using 3.6 ml of concentrated HCl in 500 ml of ethanol, the peak height reached a constant value within 15 min, but unkown subpeaks appeared in the chromatogram. Therefore, the HCl—ethanol solution containing 0.9 ml of concentrated HCl in 500 ml of ethanol was used for the reaction to prevent the formation of by-products with the acid. The peak height reached a maximum at 30 min with this HCl—ethanol mixture so that the reaction time was held for 30 min. Fig. 3 shows the relationship between the fluorescence intensity (as peak height) and the ratio of dansyl hydrazine to cortisol. The peak height increased with increasing ratio, up to 3:1, and then became constant. In practice, the constant peak height of cortisol extracted from 0.5 ml of normal plasma was obtained by using more than 1 μg of dansyl hydrazine, whereas the peak of cortisol could not be separated from that of the excess dansyl hydrazine when 5 μg were used. Therefore, 0.01 ml of dansyl hydrazine solution containing 2 mg per 10 ml of ethanol was used for labelling.

Reaction temperature

The reaction temperature was set at room temperature, because the reaction rate was independent of temperature between 0° and 60°.

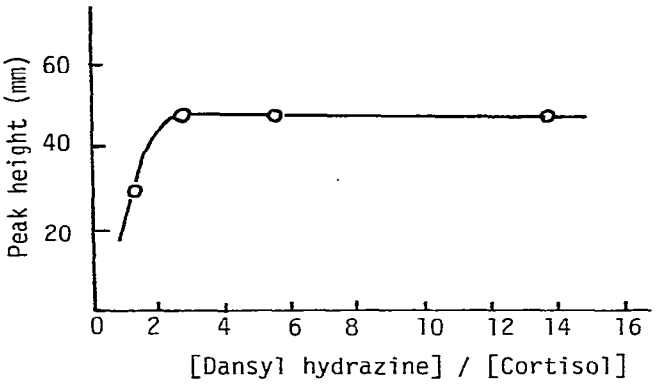

Fig. 3. Effect of dansyl hydrazine concentration on peak height (fluorescence intensity). Injected volume: 15 μl of the final solution containing 15 ng of cortisol.

Selection of eluent

Many solvent systems were examined in order to select the suitable eluent. An organic layer of methylene chloride—ethanol—water (948:35:17) was found to be suitable when used with an Hitachi gel No. 3042 column. The organic layer saturated completely with water by shaking at least for 1 h should be used as the eluent because the peak of cortisol splits into two peaks when unsaturated solvent is used. The chromatogram presented in Fig. 4 shows a good separation of cortisol and the other related steroids by this eluent.

Working curve and selectivity

Linearity of the fluorescence intensity (peak height) with the amount of cortisol injected was obtained between 0.5 and 60 ng. The detection limit of cortisol was about 0.2 ng from this working curve. Then, using 0.5 ml of plasma and 1.0 ml of urine as samples, the detection limits are 100 ng and 50 ng per 100 ml, respectively.

Recovery and reproducibility

The extraction procedure was carried out with methylene chloride as

TABLE I

RECOVERY OF CORTISOL ADDED TO PLASMA AND URINE PRIOR TO EXTRACTION

Sample	Added (ng)	Found (ng)	Recovery (%)	*n*	C.V. (%)
Plasma	0	1.8*	—	5	15.7
(0.5 ml)	50	51.1	98.7	8	3.3
	100	97.1	95.4	12	3.7
Urine	0	49.6*	—	10	4.9
(1.0 ml)	50	97.8	96.4	10	4.4
	100	140.2	90.6	10	6.2

*Value before spiking.

solvent. The cortisol-free plasma was presented by treatment of dexamethazone. In recovery tests the method was applied to samples to which had been added 50 ng and 100 ng of cortisol per 0.5 ml of cortisol-free plasma and 1.0 ml of normal urine. The reproducibility was determined by carrying out 8—12 analyses, with the results shown in Table I. Some typical chromatograms are shown in Fig. 5. The large peak appearing in front of cortisol is due to excess dansyl hydrazine and the fluorescent compounds in the sample, but no peaks interfering with cortisol were observed. The same chromatograms were also obtained with the urine samples.

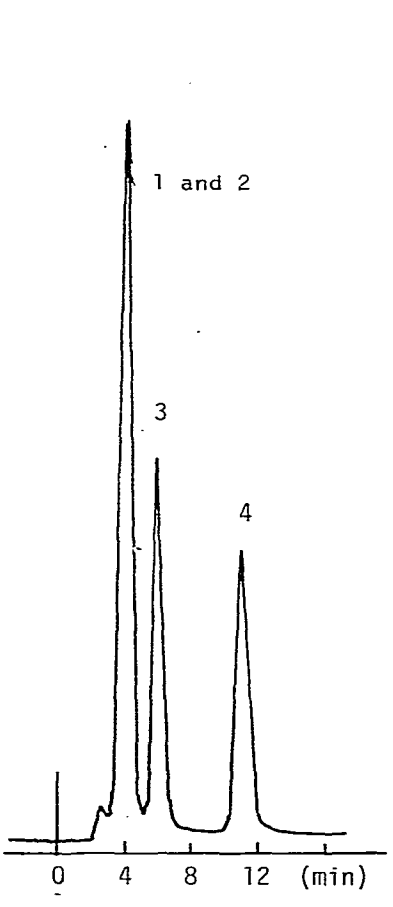

Fig. 4. Chromatogram of dansyl hydrazone derivatives of Δ^4-3-oxo-steroids. 1, Corticosterone; 2, 11-desoxy-17-hydroxycorticosterone; 3, cortisone; 4, cortisol. Column (250 mm × 4 mm I.D.) prepared with Hitachi gel No. 3042; flow-rate, 1 ml/min; solvent system, dichloromethane—ethanol—water (948:35:17); wavelengths, 350 nm (excitation) and 505 nm (emission).

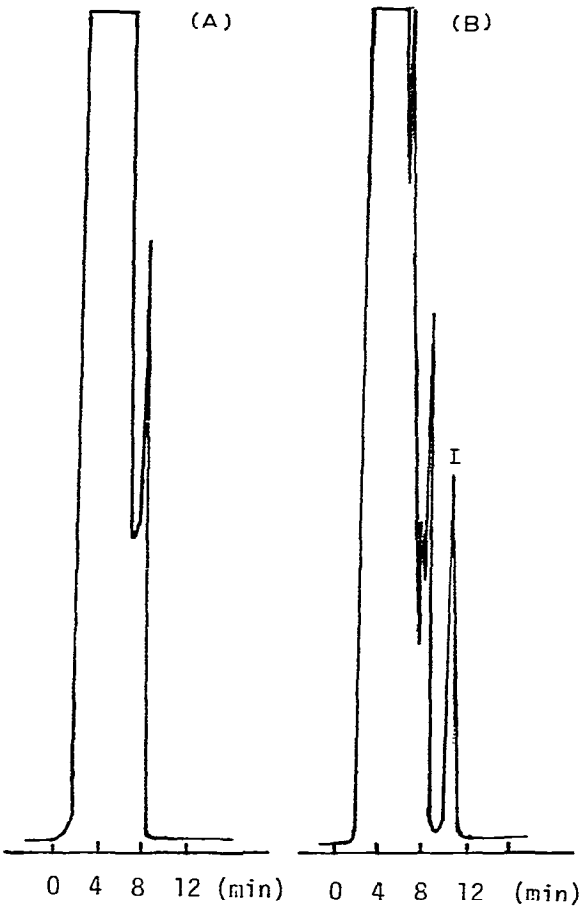

Fig. 5. Typical chromatograms of plasma samples. (A) Cortisol-free plasma presented by treatment of dexamethazone. (B) Normal human plasma. Peak I: cortisol. Assay procedure and chromatographic conditions are given in the text.

Comparison with radioimmunoassay

The reliability of this method for the determination of cortisol in plasma or urine was assessed by comparing the results with those obtained by radioimmunoassay. As illustrated in Fig. 6, the values obtained by each method were in excellent agreement, the coefficient of correlation for cortisol samples in the range 5—250 ng/ml of plasma being 0.978. The values obtained by this method were slightly higher than those obtained by radioimmunoassay.

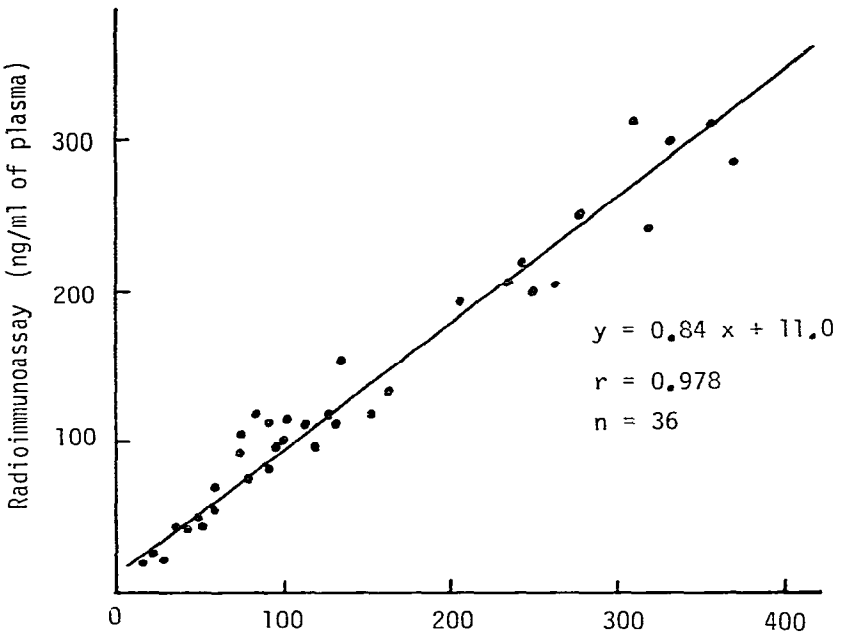

Fig. 6. Comparison of results obtained with fluorescence HPLC and radioimmunoassay.

DISCUSSION

Several reports on the use of HPLC to assay cortisol in biological fluids [5—9] have been published, but the sensitivities of most of them were too low due to the use of an UV detector. Fluorophotometric detection is more sensitive than the UV detection method. Fluorophotometry of cortisol could not be used in conjunction with HPLC because concentrated acids were used as reaction media. Recently, a fluorophotometric determination of Δ^4-3-oxo-steroids using aluminium salt and isonicotinylhydrazine has been developed by Horikawa et al. [16]. They stated that the reaction would be utilized in the detection of Δ^4-3-oxo-steroids in the eluate after separation by HPLC.

The advantages of fluorescence derivatization in liquid chromatography of trace amounts of substances in biological fluids are evident. Frei and Lawrence [17] used dansyl hydrazine as a fluorescent labelling reagent for carbonyl compounds. Chayen et al. [18] developed a thin-layer chromatographic determination of oxo-steroids, such as testosterone and cortisol, by using dansyl hydrazine. However, both methods have not been applied to biological samples.

In this paper, we have developed a clinically useful method for the determination of plasma or urine cortisol by HPLC with a pre-labelling technique using dansyl hydrazine. The optimum conversion to the fluorescent dansyl hydrazone was obtained by carrying out the reaction with 0.02% dansyl hydrazine—ethanol solution and HCl—ethanol (containing 0.9 ml of concentrated HCl per 500 ml of ethanol). The chromatographic conditions were chosen to give acceptable resolution between dansyl hydrazone of cortisol and the fluorescent co-extractives from plasma or urine samples in the shortest possible analysis time.

The detection limit of this method is 0.2 ng or better, depending on the efficiency of the fluorescence detector and the final injection volume. The sensitivity of the method is superior to those of other HPLC methods using an UV detector. Trefz et al. [6] reported that the cortisol values in plasma obtained by HPLC were slightly higher than those obtained from radioimmunoassay. Although the slightly higher values than those of radioimmunoassay were also obtained using our method, there was no interference in the clinical application being continued in our laboratory. The results will be reported in the near future.

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