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Assay of human plasma cortisone by liquid chromatography: normal plasma concentrations (between 8 and 10 a.m.) of cortisone and corticosterone

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ABSTRACT

A high-performance liquid chromatographic method using ultraviolet detection to quantitate human plasma concentrations of cortisone simultaneously with cortisol and corticosterone is described. The method is based on the use of an octadecyl silica column (100 mm × 2 mm I.D., 3 μm), an ultraviolet absorbance detector (242 nm) with a 10 mm path length flow-cell, and a mobile phase composed of water–tetrahydrofuran–acetonitrile (82:10:8, v/v) containing 5 ml/l triethylamine and citric acid to adjust the pH of the buffer to 6.5. Flumethasone is used as the internal standard. The detection limit of the method for the three steroids is 300 ng/l using a 1-ml sample. The average inter-assay coefficient of variation for cortisone is 3.3% and the average recovery is 100.8%. Possible interferences from common drugs and endogenous and exogenous steroids in the method have been studied. Plasma concentrations (drawn from 8 to 10 a.m.) of cortisone and corticosterone for 43 normal volunteers have been determined.

INTRODUCTION

Cortisone (E) is a physiologically inactive steroid formed by the oxidation of cortisol (F) by the enzyme 11β-hydroxysteroid-dehydrogenase (11β-OHSDH) present in various tissues. It is reduced back into active cortisol, and an equilibrium E$\leftrightarrow$F exists in many human tissues such as liver, kidney, adipose, brain, lung, testis, uterus, placenta, and parotid gland [1–3]. Human pharmacokinetics [4] and metabolism of F and E, their interconversions and equilibria in different pathologic conditions [5–7], and the dual nature (oxoreductase and dehydrogenase) of 11β-OHSDH [3,8] have all been studied quite extensively.

F and E are usually quantitated by independent methods only. In the early days E was analyzed

after initial extraction from plasma and purification by Sephadex LH-20 column chromatography, followed by competitive protein binding assay [9]. In 1973 Srivastava *et al.* [10] introduced a radioimmunoassay (RIA) for E. This method is the only available choice for human plasma and saliva and it requires initial extraction and purification by thin-layer or high-performance liquid chromatography (HPLC) [11]. Human urine contains fairly high concentrations of free E (54 $\pm$ 50 μg per 24 h) and F (22 $\pm$ 18 μg per 24 h) and both steroids have been simultaneously, accurately, and precisely measured by HPLC–UV [12]. Total human plasma and urine E concentrations can also be estimated by gas chromatography after enzymatic hydrolysis, extraction, purification, and derivatization [13]. Although HPLC is a readily available, powerful, and simple tool requiring no prior derivatization, very little is

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known about its use for determining the low levels (<30 μg/l) of free human plasma E concentrations. However, Wade and Haegele [14] published a HPLC–UV method for human saliva E and F assays. It has many weak features: the HPLC system is unnecessarily sophisticated, the analytical column is too long (250 mm $\times$ 2 mm I.D.), the nature of mobile phase mandates column thermostating at 50°C, and the extraction is cumbersome, involves deproteinization of saliva, and requires up to 6 ml of sample.

We recently developed a simple, sensitive (detection limit of 300 ng/l using 1 ml of plasma), and highly reproducible HPLC–UV method that distinctly separated E, F, 11-deoxycortisol (S), corticosterone (B), deoxycorticosterone (DOC), dexamethasone (D), 17-hydroxyprogesterone (17-OHP), and progesterone (P) [15]. In this paper we describe a sensitive, specific analysis of human plasma E by reversed-phase HPLC and provide comprehensive precision, accuracy, recovery, and linearity data for the method. We have also determined plasma concentrations (drawn from 8 to 10 a.m.) of B and E in 43 normal volunteers.

EXPERIMENTAL

Reagents and materials

Cortisol (98.9%) was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA), and cortisone (98%) from Sigma (St. Louis, MO, USA). Flumethasone and corticosterone were from Steraloids (Wilton, NH, USA). HPLC-grade acetonitrile (ACN), methanol, and tetrahydrofuran (THF), were from Burdick & Jackson (Muskegon, MI, USA). Reagent-grade triethylamine and citric acid were from Fisher Scientific (Fair Lawn, NJ, USA). Steroid-stripped plasma was from Biocell (Carson, CA, USA). The all-PTFE C_{18} solid-phase extraction (SPE) columns (Tef Elutor), 2 ml capacity and containing 200 mg of material, were obtained from Versa Prep Lab Systems (Nanuet, NY, USA). SPE was carried out with Baker SPE systems (J. T. Baker, Phillipsburg, NJ, USA). The 10-ml all-PTFE tubes (Oak Ridge type from Nalgene) used throughout the extraction work

were from VWR Scientific (Chicago, IL, USA). (Note: PTFE tubes are not essential for the extraction work.)

Instrument and mobile phase

The HPLC unit consisted of a Model L-6000 pump (Hitachi, Tokyo, Japan), a Model 710B WISP autosampler (Waters, Milford, MA, USA), an octadecyl silica (ODS) 3-μm Shandon Hypersil column (100 mm $\times$ 2 mm I.D.) (Keystone Scientific, Bellefonte, PA, USA), a Model 480 ultraviolet detector (Waters) with a 10 mm path length flow-cell set at 242 nm, and a Model D-2000 integrator–plotter (Hitachi). The mobile phase was a mixture of water–THF–ACN (82:10:8, v/v), containing 5 ml/l triethylamine and citric acid to adjust the pH to 6.5. The flow-rate was 0.6 ml/min.

Standard solutions

A 250-ml aliquot of a primary stock solution was prepared by dissolving 25 mg of cortisone in methanol. Working standard solutions were prepared in either steroid-stripped serum or methanolic saline (20 ml of methanol and 8 g of sodium chloride per liter) and contained 1, 4, 10, 20, 40, 80, and 200 μg/l cortisone. The aqueous and serum-based standards were used in no set order. In all cases, serial standardizations were performed. The working internal standard solution contained 20 ng of flumethasone per 50 μl of solution.

Extraction

The extraction procedure for all the samples using Tef Elutor SPE C_{18} columns is described elsewhere [15]. The final nitrogen gas-evaporated residue was reconstituted in 50 μl of mobile phase, and 25 μl of the solution were injected into the HPLC system using an autosampler.

RESULTS AND DISCUSSION

Calibration

A series of standards covering the range 0–200 μg/l were used in daily work. The regression line between the ratio of peak heights (component

peak height/internal standard peak height) and the concentration of cortisol was calculated and used for determining the unknowns. A typical regression line was $y = 0.0009 + 0.2840x$ ($r^2 = 0.9997$).

Linearity

Steroid-stripped plasma samples were spiked with cortisone in the concentration range 0–200 $\mu g/l$ and analyzed several times ($n > 20$), and the absorbances of the standards were linear throughout the range.

Precision

Inter-assay and intra-assay coefficients of variation (C.V.) were determined using three plasma pools on the range 4.5–30 $\mu g/l$ (covering the normal human range). The data are presented in Table I. The average inter-assay C.V. for E was 3.3%.

Accuracy

E was added to steroid-stripped plasma in the concentration range (2–200 $\mu g/l$) we encountered in our work. We determined both inter-assay and intra-assay recoveries for these samples by the method (Table II). The average inter-assay recovery was 100.8%.

Limit of detection

The detection limit of the assay was 300 ng/l

TABLE I

PRECISION DATA FOR HUMAN PLASMA CORTISONE ASSAY ($n = 8$)

Concentration (mean ± S.D.) ($\mu g/ml$)	C.V. (%)
Inter-assay	
4.88 ± 0.19	3.9
15.99 ± 0.50	3.1
29.87 ± 0.87	2.9
Intra-assay	
4.76 ± 0.12	2.5
15.64 ± 0.45	2.9
29.15 ± 0.61	2.1

TABLE II

RECOVERY DATA FOR CORTISONE IN STEROID-STRIPPED PLASMA

Spiked ($\mu g/l$)	Measured (mean ± S.D.) ($\mu g/l$)	Recovery (%)
Inter-assay (n = 8)		
2.0	2.1 ± 0.1	105.0
8.0	8.1 ± 0.2	101.3
20.0	19.8 ± 1.2	99.0
40.0	40.2 ± 1.5	100.5
80.0	79.6 ± 2.8	99.5
200.0	198.7 ± 5.3	99.4
Intra-assay (n = 6)		
2.0	2.1 ± 0.1	105.0
20.0	20.1 ± 1.0	100.5
80.0	80.2 ± 2.2	100.3
200.0	199.2 ± 4.7	99.6

using 1 ml of sample, at a signal to noise ratio of 3:1. This detection limit is lower than the normal values observed in human populations.

Selectivity

The assay was reasonably more specific than the RIA method [10]. We determined the retention times of many endogenous and exogenous steroids and commonly used drugs [15].

Earlier HPLC methods for quantitating glucocorticoids in human plasma [16–22] had the following drawbacks. (1) Most of the analytical columns were long (≥ 250 cm) and contained 5- or 10-μm packing materials which were responsible for poor resolutions and increased assay times [16–22]. (2) Analytical columns were thermostated for improving the resolution and decreasing the assay time per sample [16,17,19,20]. (3) Earlier mobile phase compositions were not conducive for good resolution and short assay time [16–22]. (Note: progesterone is generally coextracted with the glucocorticoids and elutes very late in the reversed-phase method.) (4) It is not clear if the earlier workers [16–22] took advantage of the possible higher sensitivities in the assay by using a minibore column and longer path length

flow-cell as shown by Taylor *et al.* [23]. One of the most successful mobile phase compositions for the separation of glucocorticoids contains THF [20], a solvent of intermediate eluotropic behavior between methanol (low) and ACN (high). Greater flexibility of mobile phase optimization results when a binary mixture of THF and ACN is chosen. Dawson *et al.* [20] using a mobile phase of THF–ACN–water (12:10.5:77.5, v/v) and a C_8 column (5 μm, 250 mm × 4.5 mm I.D.) at 50°C resolved E, F, B, D, etc. very nicely. However, their detection limit was ≥ 15 μg/l. We modified the method of Dawson *et al.* [20] using a minibore ODS column (100 mm × 2 mm I.D., 3 μm, theoretical plate count of *ca.* 8000) and a detector with a 10-mm path length flow-cell. We also

improved upon earlier SPE methods by ensuring > 90% extraction efficiencies [15], and optimized the composition of the mobile phase of THF ACN–citrate buffer (10:8:82, v/v) for a good isocratic resolution at ambient temperature of many endogenous and exogenous steroids. The cumulative effects of our modifications of the method resulted in a detection limit of 300 ng/l for the glucocorticoids E, F, and B [15]. Slight modifications of the mobile phase would enable the separation of any combination of synthetic and endogenous steroids. We investigated the possibility of using still shorter columns (75 or 50 mm × 2mm I.D.) in the hope of reducing the analysis time per sample, but found that the optimal mobile phase composition that would
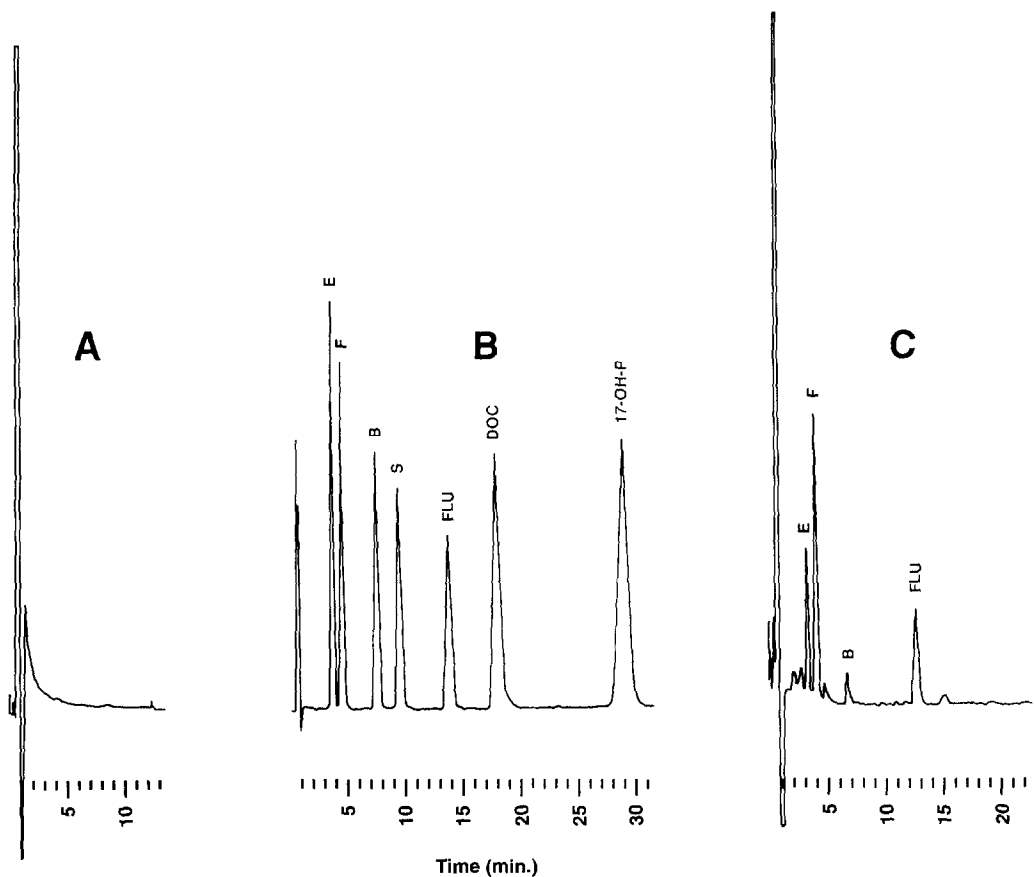

Fig. 1. Representative liquid chromatograms of (A) reagent water blank, (B) 25 ng each of E, F, B, S, flumethasone (FLU) and 50 ng each of DOC and 17-OHP and (C) 1-ml plasma extract containing 12.2 μg/l E, 23.76 μg/l F, 3.1 μg/l B, and 20 μg/l FLU (internal standard). Refer text for other abbreviations. Integrator attenuation is 2^3.

resolve the different glucocorticoids will preclude it. However, the assay time per sample can be reduced to less than 20 min if progesterone (especially in female patient samples) is removed by including a wash step of methylene chloride and hexane (5:95, v/v) either at the beginning of the extraction or just prior to elution at the end with methanol in the SPE [15].

Fig. 1 shows representative chromatograms obtained in this work. The peaks are sharp and baseline-resolved. The assay time per sample is *ca.* 20 min. The present work, in conjunction with our earlier paper [15] for the simultaneous analysis of F and B, would form a good and comprehensive HPLC–UV method for the three most relatively abundant glucocorticoids (E, F, and B) in human plasma.

Cortisone is a pharmacologically inactive steroid (the 11-OH group is essential for activity) but the equilibrium E↔F lies predominantly towards the right (reduction) in the liver. Liver is the largest site for the reduction of cortisone, and the magnitude of this reduction is greater than the magnitude of oxidation in kidney or testis [3]. In cirrhosis the equilibrium in the liver is maintained but the half-life of F is increased from *ca.* 1.0 h to 5 h [24]. The kidney, parotid gland, placenta, and testis are major sites where F is oxidized predominantly to E [1,25]. Among the oxidative tissues kidney ranks highest and testis second [25]. It has been shown that the renal 11β-OHSDH protects the kidney from the mineral corticoid actions of F [25,26]. In kidney failure, the plasma level of E is much lower than in normal controls [27]. Hyperthyroid individuals convert F more readily to E [6] and the opposite is true in hypothyroidism and inflammatory diseases [5,6]. In pregnancy and during oral contraceptive use the total concentrations of F and E are higher than normal but the concentrations of the free compounds change little. Thus, the disposition of E↔F equilibrium is different in different tissues and it can vary depending upon its pathological status [5,6,24,27] and the drugs the person is using [28]. According
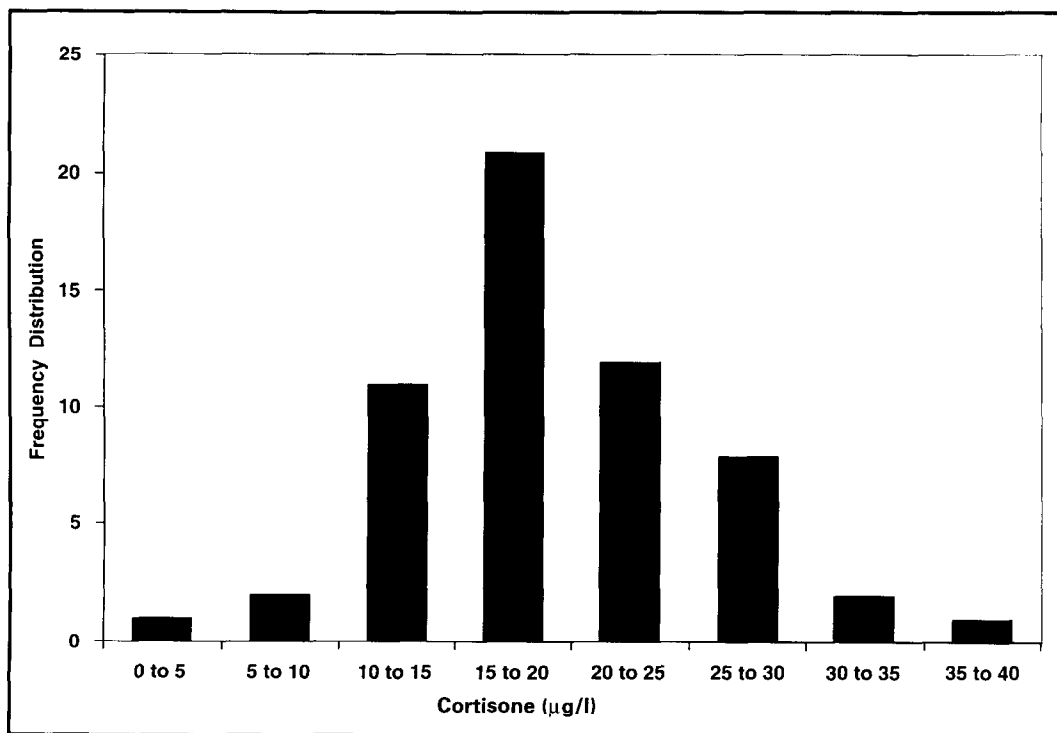

Fig. 2. Histogram of human plasma cortisone of 43 normal volunteers (drawn between 8 and 10 a.m.).

to Monder [25] the selectivity, specificity, and extent of glucocorticoid responses in tissues is modulated by the reversible function of the enzyme 11β-OHSDH and the time variable needs of the tissue.

In the light of all the facts on the E↔F equilibrium and the enzyme 11β-OHSDH, it seems that a better way to monitor the gluco-corticoid activity would be to determine simul-taneously, by this HPLC–UV method, the con-centrations of the three relatively abundant com-pounds E, F, and B rather than the most abun-dant F alone by RIA. From our limited study we find that the plasma concentration of E is about 25% of the concentration of F while that of B is about 5–10%. Thus a possible additional 30–35% glucocorticoid activity would be of much signif-icance. Lewbart and Elverson [12] suggested that the urinary free E concentration cannot be ignor-ed because of its variable interconversion with cortisol *in vivo*.

Normal values

Cortisone. The values for 43 normal volunteers (aged 20–70 years; drawn between 8 and 10 a.m.; non-fasting) are plotted as a histogram (Fig. 2). The histogram shows a Gaussian distribution. The concentration (mean ± S.D.) of the sample is 19.36 ± 6.6 μg/l (drawn between 8 and 10 a.m.). This value compares quite favorably with those reported by Sippell *et al.* [29] and determined by RIA following Sephadex column chromatogra-phy (17.3 ± 3.1 μg/l for 12 males; 13.5 ± 2.9 for 12 females; samples drawn between 9 and 10 a.m.).

Corticosterone. The values for the 43 samples determined by this HPLC method do not fall into a Gaussian distribution pattern (Fig. 3). We cal-culated the concentrations (mean ± S.D.) using statistical methods to be 1.78 ± 0.60 μg/l. The values are lower than those in earlier reports [29] perhaps due to the higher proportion of males (*ca.* 85%), the wider range of the age of our volun-teers, the slightly different blood drawing times
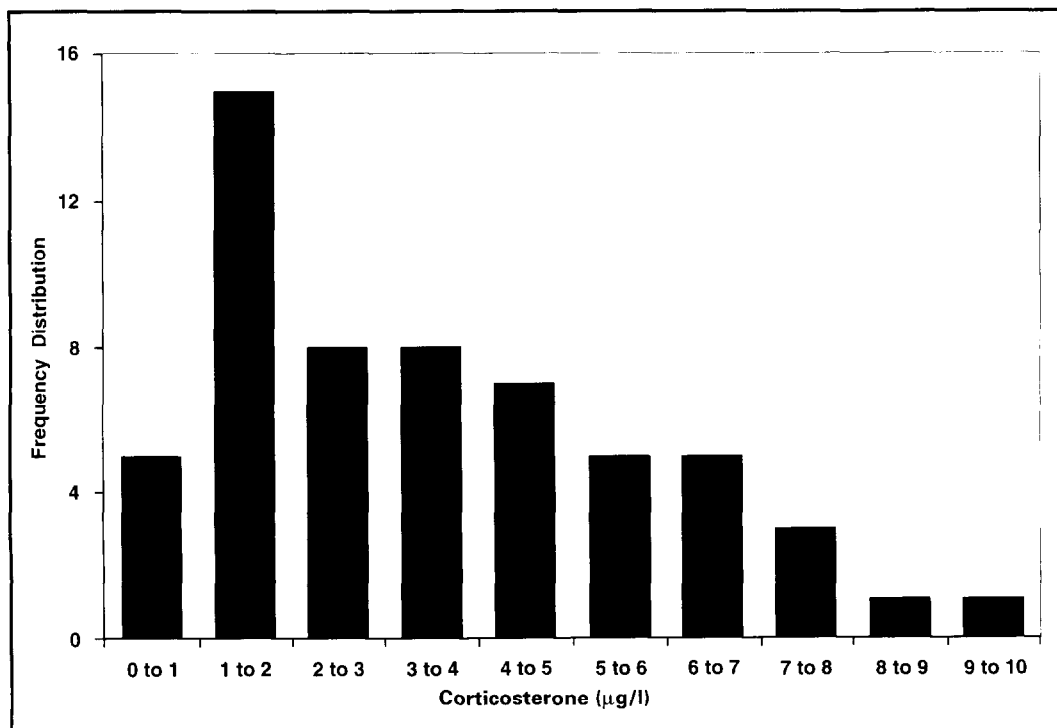

Fig. 3. Histogram of human plasma corticosterone of 43 normal volunteers (drawn between 8 and 10 a.m.).

(8–10 a.m.) and the larger population size of our samples compared to earlier methods.

CONCLUSION

The outlined reversed-phase ambient-temperature HPLC method is simple, sensitive, specific, accurate, and precise and a viable alternative to the present and only RIA method for human plasma E measurement. The method is economical since it cuts down the amount of the mobile phase used (flow-rate of 0.6 ml/min) and the assay time per sample (compared to earlier methods [16–22]). The big advantage of this HPLC method is that one can simultaneously measure many endogenous and exogenous steroids. An endogenous steroid profile consisting of E, F, B, S, DOC, 17-OHP, and P is quite useful in a detailed study of different types of adrenal hyperplasia as shown by Ji-qing *et al.* [30]. Also a knowledge of the plasma concentrations of the glucocorticoids (E, F, and B) instead of just the F level could be more informative in investigating the pathological conditions of different organs (liver, kidney, adrenal, and thyroid gland), inflammatory diseases and hypothalmic–pituitary–adrenal axis dysfunctions.

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