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CLINICAL APPLICATIONS OF THE RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SERUM CORTISOL

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SUMMARY

We report a rapid and specific analytical method for the measurement of serum cortisol by reversed-phase high-performance liquid chromatography (HPLC). The method allows the diagnostic measurement of cortisol in emergency clinical circumstances of glucocorticoid deficiency or excess. Results obtained by HPLC are comparable to those by radioimmunoassay. The lower limit of sensitivity is 6 ng cortisol per ml of serum.

INTRODUCTION

We have utilized a high-performance liquid chromatography (HPLC) method for the measurement of serum cortisol which is readily applicable for the rapid clinical assessment of adrenal glucocorticoid function. Many analytical methods are currently available for the determination of serum cortisol, including photometry, fluorimetry, thin-layer chromatography, competitive protein binding assay, and radioimmunoassay (RIA). However, all of these methods have revealed some lack of either sensitivity, specificity, reproducibility, or convenience of analysis. In particular, interference with other steroids is a problem with all these methods. Fluorimetric analysis of cortisol, although commonly employed for routine analysis, may yield falsely elevated values due to the interference of corticosterone and certain fluorogenic drugs, such as spironolactone[1]. RIA is also not entirely specific, because anti-cortisol antibodies may cross-react to varying degrees with other steroids, such as cortisone, 11-deoxycortisol, 17-hydroxyprogesterone, corticosterone, prednisone and deoxycorticosterone[2]. Similar cross-reactivity is also observed with the competitive protein

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binding assay, as steroids other than cortisol possess affinity for corticosteroid binding globulin[3]. This lack of specificity may yield falsely elevated values, particularly in the evaluation of newborns, pregnant women, or patients with endocrine disorders such as adrenal hyperplasia. It is under these circumstances that a more specific assay for cortisol is necessary. In addition, certain emergency clinical circumstances of glucocorticoid deficiency or excess may require rapid diagnostic determination of cortisol, of which the above methods are not capable.

A number of methods have been reported for the quantification of serum cortisol by HPLC, demonstrating its suitability for this analysis[4–12]. We now present a report which demonstrates the value of HPLC analysis of serum cortisol in pediatric patients. The method described is rapid, sensitive, and specific. Requiring less than 1 ml of serum, it is ideal for use in the pediatric population.

EXPERIMENTAL

Equipment

A Waters Assoc. HPLC system was used, consisting of a data module Model 730, a system controller Model 720, a WISP automatic injector Model 710 B, two pumps, Model M6000A, and a UV detector Model 441, fitted with a mercury lamp at a fixed wavelength of 254 nm. A Waters reversed-phase radial compression C_{18} column (10 cm × 5 mm I.D. long, 10 μm particles) fitted with a Waters Bondapak C_{18} corasil pre-column, was employed for all assays.

Reagents

Methanol (HPLC grade) was purchased from Fisher Scientific, Fairlawn, NJ, U.S.A.; Mallinckrodt diethyl ether anhydrous was purchased from American Scientific Products, Edison, NJ, U.S.A. A continental water purification system was used to obtain distilled and deionized water. All steroids were purchased from Steraloids, Wilton, NH, U.S.A.

Blood samples

Blood was obtained after informed consent from patients who were undergoing laboratory and clinical evaluation for various endocrine diseases. Blood was drawn by venipuncture and centrifuged. Serum was stored at −20°C until assayed.

Serum sample extraction

A volume of 0.5 ml serum was extracted with eight volumes of diethyl ether in conical tubes. The aqueous phase was frozen with acetone–dry ice and discarded. The supernatant was evaporated under air at 37°C. The residue was reconstituted with 300 μl of water–methanol (45:55, v/v), and 100-μl aliquots were injected in duplicate into the HPLC column.

Method

Chromatographic separation was performed with water–methanol (45:55) as the mobile phase at a flow-rate of 1 ml/min for 10 min. An external standard was used for sample quantitation. Integration was performed by the Waters data module.

Radioimmunoassay

Serum cortisol was also determined by RIA following Celite column chromatography with ethylene glycol as the stationary phase and isooctane–ethyl acetate (60:40, v/v), as the mobile phase, according to methods previously described[13].

Statistical analysis

Specificity was evaluated by comparing the retention time of the serum samples to standard purified cortisol. In addition, purified cortisol was added to serum samples from various subjects in order to verify the retention time of cortisol in serum. Precision was evaluated by duplicate HPLC determinations. Accuracy was evaluated by comparing the HPLC assay with the RIA of the same specimen. The differences between the two techniques were analyzed statistically by the paired "*t*" test and the one-way analysis of variance (ANOVA). Sensitivity was measured by assessing the lower limits of detection of pure cortisol and of a standard addition of cortisol to serum.

RESULTS

Applicability of the HPLC method for the determination of cortisol was utilized in the clinical evaluation of several states of altered glucocorticoid funct ion. Specifically, serum cortisol was measured in two patients with Cushing's disease secondary to pituitary adenoma prior to and following treatment, in three patients with suspected adrenal insufficiency, in two patients with 21-hydroxylase deficiency congenital adrenal hyperplasia, and in two pregnant women. The HPLC values were compared with the RIA determination. The HPLC method yielded comparable results within 2 h after venipuncture.

Cortisol is well separated from other steroids. Table I indicates the retention times of various steroid standards, demonstrating the separation of cortisol from other steroids. The mean retention time of cortisol for the determination of 50 samples was found to be 5.74 ± 0.19 min. Purified cortisol was added to serum samples and injected in eight different and increasing concentrations (1–60 ng) and assayed

TABLE I

RETENTION TIMES OF VARIOUS STEROID STANDARDS

Steroid	*Retention time (min)*
6β-Hydroxycortisol	< 3.00
Aldosterone	3.90
Cortisol	5.72
21-Desoxycortisol	8.45
Dexamethasone	9.66
Cortisone	11.29
Corticosterone	11.58
11-Deoxycortisol	12.50
17-Hydroxyprogesterone	29.01
Deoxycorticosterone	31.87
4-Androstenedione	37.57
Progesterone	40.00

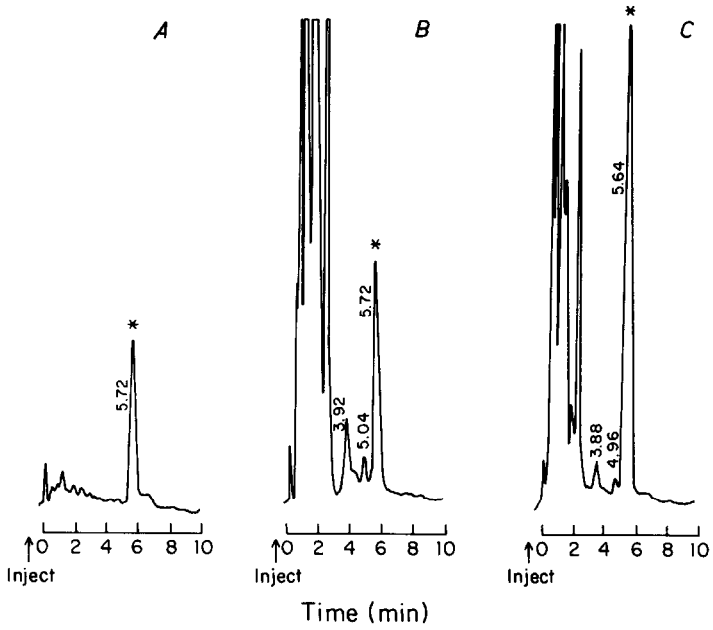

Fig. 1. Chromatograms of (A) pure cortisol standard; (B) normal serum sample; (C) same sample plus 40 ng of pure cortisol. * = Cortisol.

in quintuplicate. The equation of the regression line of ng measured versus ng injected was calculated to be $F(x) = 0.9986x - 0.1105$, $r = 0.9997$, $p > 0.001$. The lowest detectable amount was found to be 1 ng. The recovery for the complete assay (extraction and HPLC) was calculated to be 91.9%. All data reported in this paper are corrected by this factor.

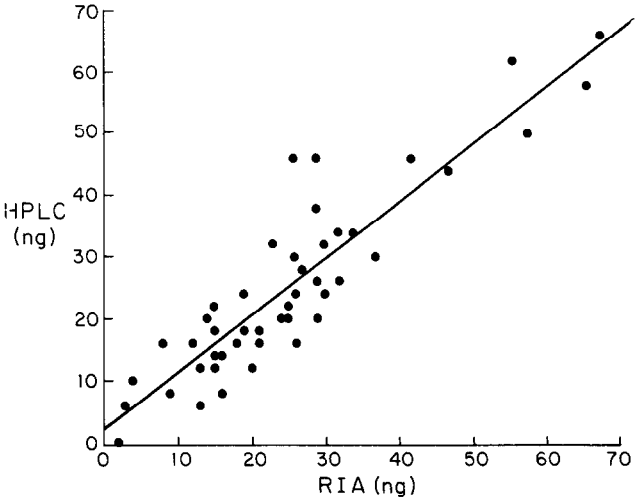

Fig. 2. Comparison of results obtained by RIA and HPLC.

Fig. 1a shows the chromatogram of pure cortisol; Fig. 1b is an example of a typical serum sample, Fig. 1c is a chromatogram of the sample shown in Fig. 1b but with the addition of 40 ng of pure cortisol. In all three panels, the retention time of cortisol was virtually identical.

Fig. 2 demonstrates the correlation between the mean HPLC values of each duplicate analysis and the corresponding RIA values. In more than 50 samples in which cortisol was measured by both RIA and HPLC (cortisol concentration was 1.2–46 μg/dl), no statistically significant difference was found between the two methods, as calculated by the paired "t" test ($p = 0.88666$) or the one-way ANOVA ($p > 0.25$). The intra- and interassay coefficients of variation were 7.87% and 8.78%, respectively.

DISCUSSION

Our study describes a precise and specific method in which reversed-phase HPLC is used for the simultaneous separation and quantitation of serum cortisol. The method permits reliable critical diagnostic decisions within 2 h. The use of the reversed-phase column is highly effective in separating cortisol from other steroids; this provides further support for its suitability in the separation of steroids from complex mixtures, as already reported[14–17]. The resolving capacity of the column was not reduced after the chromatography of as many as 48 samples, as demonstrated by the reproducibility of the retention times (5.74 ± 0.19 min). The method reported here proved to be more sensitive than others previously reported[4,5,10–12] as demonstrated by our ability to detect consistently as little as 6 ng of endogenous cortisol per injection. No significant differences were found between HPLC and RIA values. This may be due to the fact that time-consuming chromatographic purification is carried out in our laboratory before the radioimmunoassay to eliminate possible overestimation by RIA due to cross-reacting compounds[8].

The rapidity with which samples can be analyzed (each chromatographic analysis requires only 10 min), and the small amount of blood required make the method described suitable for a large number of samples in either mass screening or routine analysis. HPLC analysis offers the advantage of suitability for the determination of small samples or large batches of sample without excessive standardization procedures. It is also cost-effective, the cost being less than one-third that of RIA.

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