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Note

Determination of plasma corticosterone using high-performance liquid chromatography

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Several methods have been reported for determining cortisol in human plasma by high-performance liquid chromatography (HPLC) [1–4]. In contrast with humans, the major glucocorticoid in laboratory rodents is corticosterone. To our knowledge, only two previous reports have described corticosterone analysis by HPLC. Yamada and Aizawa [5] used normal-phase chromatography and obtained a separation of limited resolution in about 5 min. With reversed-phase HPLC, Shimizu et al. [6] obtained higher resolution, but with an analysis time of about 20 min. The present report describes a rapid reversed-phase assay suitable for analyses of plasma samples.

EXPERIMENTAL

Reagents

HPLC-grade methanol, water and dichloromethane and chromatography-grade diethyl ether were obtained from BDH (Poole, U.K.). Corticosterone and 19-nortestosterone were supplied by Sigma (Poole, U.K.). Reactivials (3 ml capacity) were purchased from Pierce (Cambridge, U.K.).

Equipment

Separations were made using a Beckman 344 gradient liquid chromatograph (Beckman-RIIC, High Wycombe, U.K.) fitted with a Beckman 165 variable-wavelength detector. A Waters Nova-Pak C_{18} reversed-phase column (particle size 4 μm; column size 150 $\times$ 3.9 mm; Millipore, Harrow, U.K.) was used together with a guard column filled with pellicular C_{18} sorbent (Whatman Labsales, Maid-

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stone, U.K.). Results were analysed using a Shimadzu Chromatopak C-R1B data processor obtained from Beckman-RIIC.

Plasma extraction

Plasma (1 ml) was pipetted into screw-capped culture tubes together with 100 μl 19-nortestosterone solution (5.0 μg/ml in methanol) as internal standard. A 150-μl volume of sodium hydroxide solution (0.3 *M*) was added to prevent organic extraction of phenolic contaminants. Corticosteroids were extracted into 5 ml diethyl ether–dichloromethane (60:40, v/v) by vortex mixing for 30 s. The tubes were immediately centrifuged at 600 *g* for 5 min. Supernatant (4 ml) was transferred to a second culture tube and vortex-mixed with 1 ml HPLC-grade water. After recentrifugation, supernatant (3 ml) was transferred to a Reactivial and evaporated at room temperature under nitrogen. The residue was redissolved in 100 μl of methanol–water (55:45, v/v).

Chromatography

HPLC-grade methanol and water were degassed and filtered before use and delivered separately using a dual-pump system. The column was equilibrated using methanol–water (55:45, v/v) at a flow-rate of 1 ml/min. Separations were made at ambient temperature and the eluate was monitored by UV detection at 250 nm. The controller was programmed to incorporate a 1-min methanol wash after each separation. Standards containing corticosterone (5.00, 2.50, 1.25 and 0.50 μg/ml) together with 19-nortestosterone (2.3 μg/ml) were made up in methanol–water (55:45, v/v). The standard solutions were stable for at least one month when kept in the deep freeze. They were injected directly, without extraction. Samples and standards were injected manually using a 20-μl loop.

RESULTS

Chromatography

Typical chromatograms for standard steroids and rat plasma are shown in Fig. 1. Corticosterone and 19-nortestosterone had retention times of 4.7 and 7.4 min respectively. After 9 min, the absorbance increased due to the methanol wash; however, by 12 min the trace returned to baseline. Plasma samples showed a minor peak at about 0.5 min in front of corticosterone. Although it was not always completely resolved from corticosterone, the integrator processed the two peaks separately.

The standard curve obtained with corticosterone solutions containing 0.5–5.0 μg/ml (equivalent to plasma hormone concentrations of 80–800 ng/ml) was linear. The regression of peak-area ratio (corticosterone/19-nortestosterone) on corticosterone concentration was $y=0.356x+0.010$ ($r=0.9997$). Corticosterone was detectable in concentrations as low as 0.1 μg/ml, equivalent to a plasma concentration of about 20 ng/ml, although at this level precision was reduced (coefficient of variation of 11.3%).

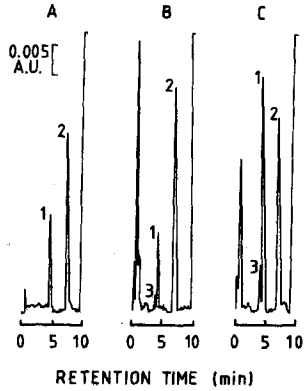

Fig. 1. Chromatograms obtained from (A) a standard solution containing 1.25 μg/ml corticosterone, (B) a plasma extract from fed rat containing 0.84 μg/ml corticosterone, equivalent to 165 ng/ml plasma before extraction, and (C) a plasma extract from fasted rat containing 2.80 μg/ml corticosterone, equivalent to 616 ng/ml plasma before extraction. Peaks: 1 = corticosterone; 2 = 19-nortestosterone; 3 = minor peak eluting ahead of corticosterone.

Precision

The precision of the determination was measured from the diference in corticosterone concentration between duplicate injections. Using eighteen plasma extracts, the coefficient of variation was 4.0%. Precision of the extraction procedure was measured from the difference between duplicate plasma extracts. For five paired samples, the coefficient of variation was 1.0%. These results indicate that the analytical error arose more from chromatography than from extraction of the samples.

Recovery

Recovery of the internal standard after plasma extraction was found to be $94.6 \pm 5.8\%$ (mean $\pm$ S.D.; $n = 8$). The recovery of corticosterone was also determined by adding a known amount of this steroid to one of two duplicate plasma aliquots, and measuring the increase in peak-area ratio (corticosterone/internal standard). With additions in the range 108–540 ng, the recovery measured was $96.7 \pm 7.7\%$ ($n = 8$ pairs of plasma samples).

Plasma corticosterone levels in fasted rats

The procedure was tested in a physiological application by measuring plasma corticosterone in fasted rats. Twelve female Sprague–Dawley rats were caged and accustomed to handling. Six rats were then fasted for two days, after which all the animals were killed by decapitation at 10.00–11.00 a.m. Trunk blood was collected in heparinised tubes and plasma was separated by centrifugation. Corticosterone was determined as described above. The results (Table I) showed that fasting caused a highly significant 5.5-fold increase in plasma corticosterone concentration.

TABLE I

PLASMA CORTICOSTERONE CONCENTRATIONS IN FED AND FASTED RATS

Plasma was collected after two days fasting and corticosterone was determined as described in the text. Corticosterone concentrations in the two groups differed significantly ($p < 0.01$ by Student's t-test).

Group	n	Concentration (mean ± S.D.) (ng/ml)
Fasted	6	761 ± 348
Fed	6	139 ± 140

DISCUSSION

While the present procedure is generally similar to those used for cortisol determination [1–4], several changes were required for accurate measurement of corticosterone. For example a higher proportion of methanol was needed in the mobile phase, because corticosterone is less polar than cortisol. Since basal corticosterone levels in rats are often very low, a relatively large sample size was used (equivalent to 0.1–0.2 ml extracted plasma). The electronic digital filter on the UV detector was also essential to reduce background noise to an acceptable level.

Repeated sample injections led to a rapid deterioration of column performance. This could be prevented by incorporating a 1-min methanol wash at the end of each cycle. In addition, the column was flushed with 50 ml methanol after each day. Use of a guard column provided further protection against build-up of contaminants. Sep-Pak cartridges have been recommended for preliminary purification of samples before HPLC [7,8]. However, this approach may also reduce assay sensitivity [9].

In plasma samples a minor peak eluted slightly ahead of corticosterone and was incompletely separated from it. Because the average area of the minor peak was only 10% that of corticosterone, its presence does not result in any substantial inaccuracy. The area of the minor peak was increased by fasting, suggesting that it is a corticosteroid metabolite.

Measurements were made on fasted rats to test whether the method could detect physiological changes. The corticosterone level of fed rats in mid-morning was similar to that reported by others using HPLC [5,6]. A two-day fast led to a highly significant 5.5-fold increase in plasma corticosterone level.

A plasma volume of 1 ml has been used routinely, since this provides ample extracted material. However, by increasing the volumes of supernatant carried over during extraction as well as reducing the volume of final suspension, it is feasible to start with only 100–200 μl plasma. Similarly, the lower detection limit can be extended by starting with 1 ml plasma and adjusting the extraction conditions.

In conclusion, the method provides a rapid and accurate means of determining plasma corticosterone for laboratories equipped with an HPLC system.

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