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# Note

# Analysis of corticosterone in rat urine by high-performance liquid chromatography and fluorimetry using post-column reaction with sulphuric acid

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Stress is known to induce enhanced activity of the pituitary–adrenal axis, resulting in increased secretion of corticosteroids from the adrenal cortex. The plasma level and urinary excretion of corticosteroids have been measured for assessment of stress.

It has been demonstrated that corticosteroids in plasma are largely bound to proteins, mainly corticosteroid-binding globulin, and that these protein-bound corticosteroids are biologically inactive, whereas the non-bound fraction is active [1]. According to Beisel et al. [2], the level of urinary free corticosteroid reflects that of the non-bound fraction in plasma, because the free fraction in plasma is filtered out in the kidney whereas the protein-bound fraction is not. It is assumed, therefore, that the urinary free corticosteroid level is a useful index for evaluation of stress in various situations.

Our preliminary report [3] described a high-performance liquid chromatographic (HPLC) method for the determination of cortisol in human plasma or saliva. The aim of the present work was to apply this method for measurement of corticosterone in urine and plasma of rats under both basal states and stress situations. For this purpose, the conditions of HPLC and the post-column reaction were optimized and recovery throughout the procedure was corrected using an internal standard.

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## EXPERIMENTAL

### *Chemicals*

Corticosterone, cortisol, tetrahydrocorticosterone (5$\beta$-pregnane-3$\alpha$,11$\beta$,21-triol-20-one, THB) and other steroids used were obtained from Sigma (St. Louis, MO, U.S.A.). Standard solutions of steroids were made by weighing them and dissolving them in ethanol–water, followed by storage at 4 °C. Acetonitrile, dichloromethane, ethanol, sodium hydroxide and sulphuric acid were purchased from Wako (Osaka, Japan). Distilled and deionized water was used.

### *Chromatography and post-column reaction detection*

The mobile phase was delivered by a pump (L-6200, Hitachi) at a flow-rate of 0.6 ml/min. The mobile phase was acetonitrile–water (24.5:75.5, v/v). The volumes of the components were exactly measured and mixed to obtain complete separation of corticosterone from other fluorogenic substances. Samples were injected with an autosampler (KSST-120, Kyowa-Seimitsu, Tokyo, Japan) into an HPLC column (Capcell Pak CN, 250 mm × 4.6 mm I.D., Shiseido, Tokyo, Japan), which was maintained at 40 °C. Another type of HPLC column (Capcell Pak $C_8$, the same size) eluted with 28% acetonitrile is also useful for the separation of corticosterone in rat urine. Effluent from the column was continuously introduced into a post-column reactor to produce fluorescent derivatives, as shown in Fig. 1.

In the reactor, sulphuric acid was added and mixed step by step with the HPLC effluent, so that the stream of mixed reagents was not disturbed. Unsteady flow can cause fluctuation of the baseline in fluorimetric detection. The final concentration of sulphuric acid in the reagent mixture was 65%. The reaction mixture was allowed to pass through a 20-ft. glass coil at 65 °C for the fluorescence reaction, which took ca. 7.5 min. The fluorescence intensities of corticosteroids were dependent on the temperature of the reactor and on the

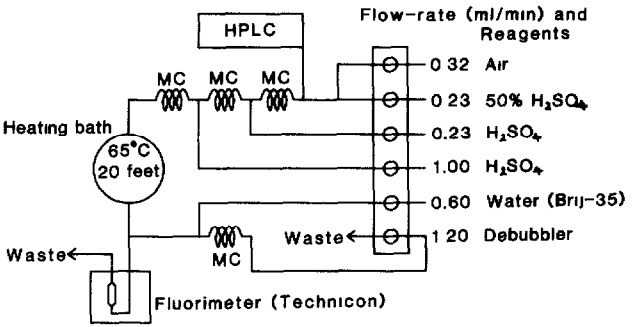


Fig. 1. Flow diagram of corticosteroid analysis by autoanalyser. MC = mixing coil; 50% $H_2SO_4$ = mixture of equal volumes of $H_2SO_4$ and water. Acidflex pump tubing was used for sulphuric acid. The fluorometer filters were No. 48 for excitation and No. 2A-12 for emission.

sulphuric acid concentration in the reaction mixture, as described by Zenker and Bernstein [4]. If the concentration of sulphuric acid and the temperature of the heating bath had been higher, a higher sensitivity for corticoid analysis would have been obtained with this detection system. In the present study, however, the conditions described above were chosen for technical and safety reasons. The fluorescence intensity of the reaction mixture was monitored using a fluorimeter (Technicon) with a No. 48 excitation filter (peak at 460 nm) and a No. 2A-12 emission filter (sharp cut-off below 510 nm). The chromatogram was recorded on a two-pen recorder. The concentration of corticosterone was calculated from the peak heights of both the steroid and the internal standard. Samples were injected into the HPLC system every 30 min. Forty-eight measurements could be performed in a day when the autosampler was used.

*Sample preparation*

Each rat was housed in a cage for urine collection and kept under a 12-h light–dark regime. Urine samples were collected separately in glass tubes containing 0.5 *M* sulphuric acid. Under sodium pentobarbital anaesthesia, blood was drawn from the abdominal aorta and heparinized. Plasma was separated after centrifugation. Urine and plasma were stored at −20°C until analysis was performed.

For analysis, an aliquot of the urine (usually 2 ml) or plasma (0.5 ml) was transferred to a 50-ml stoppered glass tube and THB was added as an internal standard. The mixture was extracted with 15 ml of dichloromethane according to the procedure described by Ratliff and Hall [5]. The dichloromethane extract was washed with 2 ml of 0.1 *M* sodium hydroxide and then with 2.5 ml of water. A 10-ml portion of the washed extract was evaporated to dryness at 45°C. The residue was dissolved in 30% acetonitrile and analyzed by the method described above.

## RESULTS AND DISCUSSION

The chromatographic separation of authentic steroids is shown in Fig. 2A. The peak heights of the steroids were proportional to the steroid concentrations [3]. Table I shows the relative values of retention times and fluorescence intensities of the steroids. The method was highly sensitive for cortisol and corticosterone, and 0.5 pmol of corticosterone was detectable at a signal-to-noise ratio of 5. Some corticosteroid metabolites were positive by this method, but their fluorescence intensities were considerably lower than those of cortisol and corticosterone. Synthetic corticoids, such as prednisolone, 6$\alpha$-methylprednisolone, dexamethasone and betamethasone, were also detectable by this method, although with less sensitivity.

Fig. 2B shows a typical chromatogram of an extract of rat urine. There is no

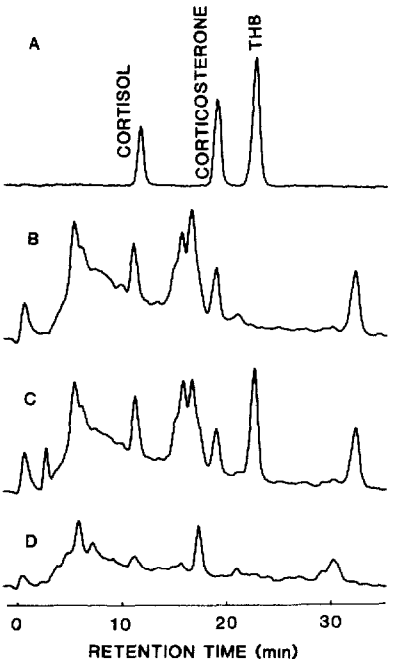


Fig. 2. Chromatograms of authentic standards and rat urine extracts. (A) authentic standards (cortisol 8 pmol, corticosterone 8 pmol, THB 0.4 nmol); (B) rat urine extract; (C) rat urine extract plus THB; (D) extract of urine of an adrenalectomized rat. Column: Capcell Pak CN.

apparent peak at the retention time of THB in the chromatogram. No THB was recognizable in the chromatogram of rat plasma extract either. The mean ($\pm$S.D., $n=8$) recovery of THB (4 nmol) added to rat urine was $100.0 \pm 8.1\%$, taking into account the volume of the extract. It is, therefore, considered that THB can be used as an internal standard for correction of recovery throughout the procedure. In the present method, 4–5 nmol of THB are added to the examined samples for use as an internal standard. The recovery of corticosterone (0.1 nmol) added to rat urine was 100.4% with a coefficient of variation of 3.2% ($n=8$).

To test the specificity of the present method, blood and urine of adrenalectomized rats were analyzed. No peak was found at the retention time of corticosterone in the chromatograms of the extracts (Fig. 2D). This result indicates that the peak considered to be corticosterone in the present analytical method is derived from the adrenal gland.

Urinary excretion of free corticosterone in the rat under a 12-h light–dark cycle is illustrated in Fig. 3. The figure clearly shows a circadian rhythm of urinary free corticosterone in the rat, which is characterized by a steep rise before the beginning of the dark phase and a fall before the light phase. The

TABLE I

RETENTION TIMES AND PEAK HEIGHTS OF CORTICOSTEROIDS BY HPLC-FLUORIMETRY

The values of retention time and peak height (cm/pmol) represent values divided by those of cortisol. Column: Capcell Pak CN.

| Steroid | Retention time relative to cortisol | Peak height relative to cortisol |
|---|---|---|
| Aldosterone | 0.78 | 0.001 |
| Prednisolone | 0.98 | 0.075 |
| Cortisol | 1 | 1 |
| Cortisone | 1.09 | 0.001 |
| 6$\alpha$-Methylprednisolone | 1.41 | 0.063 |
| Betamethasone | 1.55 | 0.027 |
| Corticosterone | 1.60 | 1.65 |
| Dexamethasone | 1.61 | 0.005 |
| 21-Deoxycortisol | 1.64 | 0.49 |
| 11-Deoxycortisol | 1.85 | 0.063 |
| Tetrahydrocorticosterone | 1.91 | 0.036 |
| 11$\beta$-Hydroxyprogesterone | 3.15 | 0.002 |
| 11-Deoxycorticosterone | 3.29 | 0.43 |

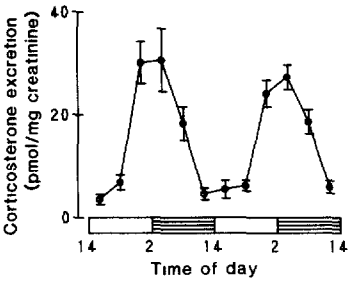


Fig. 3. Circadian rhythm of urinary free corticosterone in rats in a basal state. Urine samples (4 h) from eight male Wistar rats, weighing 130–170 g, were collected over two consecutive days and analysed using the present method. Each point represents the arithmetic mean value, and vertical bars indicate the standard errors of the mean. The shaded area indicates the dark phase of the light–dark cycle.

pattern of the rhythm is in good accordance with that of the rhythm of corticosterone excretion reported by Hausler et al. [6], who used radioimmunoassay for steroid analysis. The values of urinary excretion of free corticosterone in the rat are also similar to the results of Kley et al. [7].

The urinary level of free corticosteroid appears to be extremely low in comparison with the plasma level of total corticosteroid [1]. Furthermore, urine in general contains a wide variety of contaminating substances. Methods have been developed by several works [8] for measurement of unconjugated cortisol

in human urine. For corticosterone in rat urine, however, no simple practical method seems to be currently available. Thus, many of the endocrinological studies performed on the effect of stress in rodents have dealt with measurement of plasma corticosterone. In order to evaluate stress, however, urine analysis rather than blood testing is preferable, because blood sampling may cause psychological and physiological stress reactions. Such stress would be serious in the case of repeated blood sampling required for intra-individual experiments. These difficulties could be eliminated by measurement of free urinary corticosteroid. The present HPLC method would thus be useful for corticoid analysis in stress research and in studies of circadian rhythm.

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