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Note

# Analysis of corticosterone in the serum of mice and rats using high-performance liquid chromatography

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Corticosterone is an endogenous glucocorticoid which is secreted mainly in mice and rats. While a number of workers have published assays for endogenous glucocorticoids in rats and humans by means of radioimmunoassay [1, 2], quantitative analysis by chromatographic methods is very rare [3, 4]. Also, the determination of corticosterone in the serum of mice has not been reported previously. The purpose of the present work is the application of high-performance liquid chromatography (HPLC) in the quantitative analysis of serum corticosterone in a reproducible, accurate and rapid manner and comparison of corticosterone levels between mice and rats to develop an assay for corticosterone in mouse serum as a preliminary experiment in investigating the biochemical response to the administration of drugs. This paper describes the identification and the determination of minimum amounts of corticosterone in the serum of mice and rats by HPLC and gas chromatography combined with mass spectrometry (GC—MS).

## EXPERIMENTAL

### *Standards and reagents*

Corticosterone and dexamethasone, the internal standard, were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, methylene chloride, sodium hydroxide, sulfuric acid, pyridine and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Nakarai Chemical Co. (Kyoto, Japan).

### *Apparatus*

A gas chromatograph combined with a mass spectrometer (GCMS9020DF,

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SCAP1123; Shimadzu) was used. The glass column (1 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (80—100 mesh) was used. The column temperature was 290°C, the injection port and detector temperature 330°C. The carrier gas was helium (30 ml/min). The conditions of the mass spectrometer were: electron energy 30 eV; ion source electron impact; temperature of ion source 250°C; current 60 μA; acceleration voltage 3.5 kV; gain 50; scan speed 8. Samples were derivatized to give the 21-mono-trimethylsilyl (TMS) derivative by BSTFA—pyridine (1:1) for 30 min at room temperature; 1 μl was injected.

For HPLC a Shimadzu Model 4A chromatograph with a Shimadzu Model SPD-2A UV detector was employed. A stainless-steel column (25 cm × 4 mm I.D.), packed with reversed-phase Fine SIL $C_{18}$-5 (5 μm; JASCO, Tokyo, Japan), was used. The mobile phase was acetonitrile—0.03% sulfuric acid solution (36:64). The column temperature was 45°C, the flow-rate was 1.2 ml/min, detection wavelength was 240 nm and the sensitivity was 0.005 a.u.f.s. Peak area was measured using a Shimadzu C-R1A computing integrator.

*Animals*

Male Wistar rats weighing 180—200 g and male ddY mice weighing 20—25 g were used. They were kept in an air-conditioned room (24°C) under a light—dark cycle (light phase, 06:00—18:00 hours) for seven days. They were given commercial diet (CLEA, Tokyo, Japan) and water ad libitum, unless otherwise specified. To avoid stress-induced release of corticosterone, they were calmed by daily handling once a day in the morning for seven days. Rats were decapitated every 2 h between 10:00 and 08:00 hours to investigate the rhythm of corticosterone secretion over 24 h.

*Extraction*

Blood samples were collected and allowed to stand for 1 h at room temperature. After centrifugation at 7400 *g* for 10 min, 200 μl of serum were transferred to a 10-ml separating funnel and internal standard solution of dexamethasone (5 μl = 50 ng) was added. Then 0.05 ml of 0.25 *M* sodium hydroxide and 4 ml of methylene chloride were added. The mixture was shaken by hand for a minute. The organic layer was washed with water, transferred to a 5-ml flask and evaporated in vacuo at 30°C. The residue was dissolved in 100 μl of methanol and 60 μl were injected into the HPLC column.

*Calibration graph*

Corticosterone at concentrations varying from 5 to 30 μg per 100 ml and internal standard at a fixed concentration of 10 μg per 100 ml were dissolved in 3% albumin solution and a calibration graph was obtained using the procedure described above.

## RESULTS AND DISCUSSION

Typical chromatograms obtained from mouse and rat serum spiked with dexamethasone (internal standard, 10 μg per 100 ml) are shown in Fig. 1. The retention times of corticosterone and dexamethasone are 18 min and 9

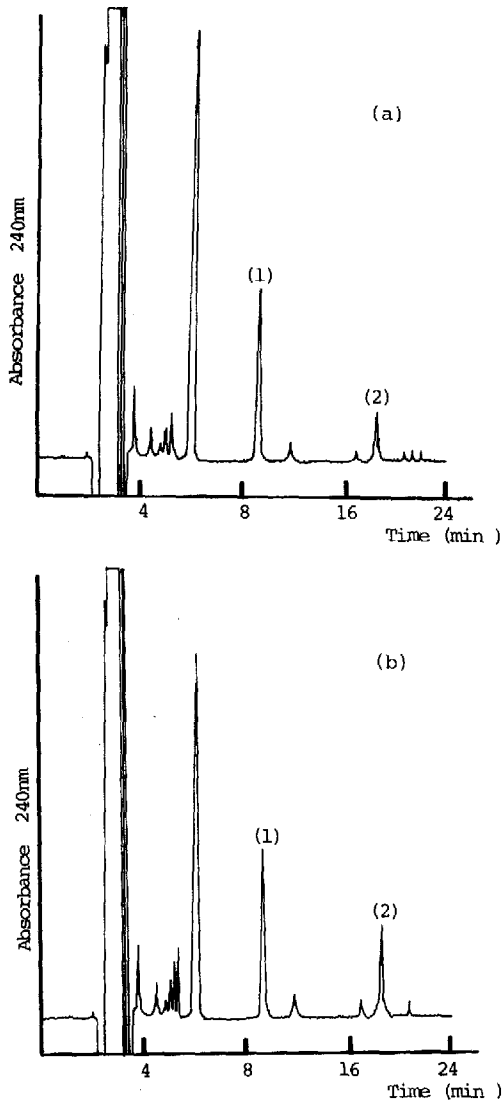


**Fig. 1. High-performance liquid chromatograms of (a) mouse serum and (b) rat serum spiked with 10 μg per 100 ml internal standard. Peaks: 1 = dexamethasone (internal standard); 2 = corticosterone.**

min, respectively. The patterns of the chromatograms are very similar. The identity of the separated mouse corticosterone was confirmed by GC—MS. Mild trimethylsilylation using BSTFA—pyridine (1:1) for 30 min at room temperature gave the 21-mono-TMS derivative selectively. To obtain the di-TMS derivative, it was necessary to stand for 1 h at 60°C. The GC peak was analyzed by mass spectrometry and selected ion monitoring of parent ions at *m/e* 418, 403, 315, 269, 251 and 227 for 21-mono-TMS corticosterone. A mass spectrum and chromatograms are shown in Fig. 2. This chromatogram corresponds well with that of corticosterone standard. From these results, the peak separated by HPLC was recognized as pure corticosterone.

A calibration graph for serum corticosterone obtained by plotting the ratio

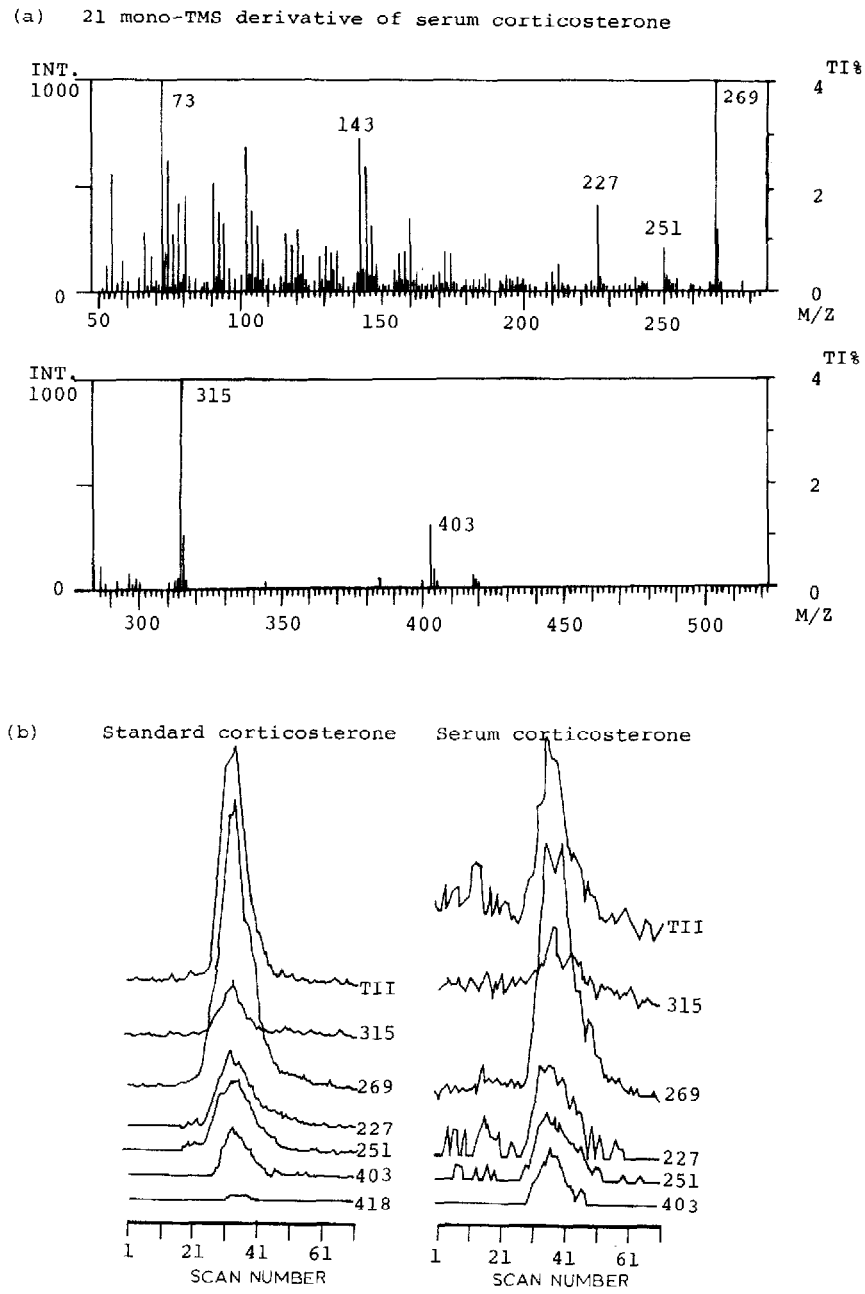


**Fig. 2. Mass spectrum (a) and mass chromatogram (b) of 21-mono-TMS derivative of mouse corticosterone separated by HPLC.**

of the peak area of corticosterone to that of internal standard against the concentration of corticosterone in mouse and rat serum showed linearity in the range 1—60 ng (8.3—500 ng/ml serum). The minimum measurable level was 1 ng (8.3 ng/ml serum).

Fig. 3 shows the circadian rhythm of serum corticosterone in rats. The concentration of serum corticosterone between 02:00 and 14:00 hours exhibits a

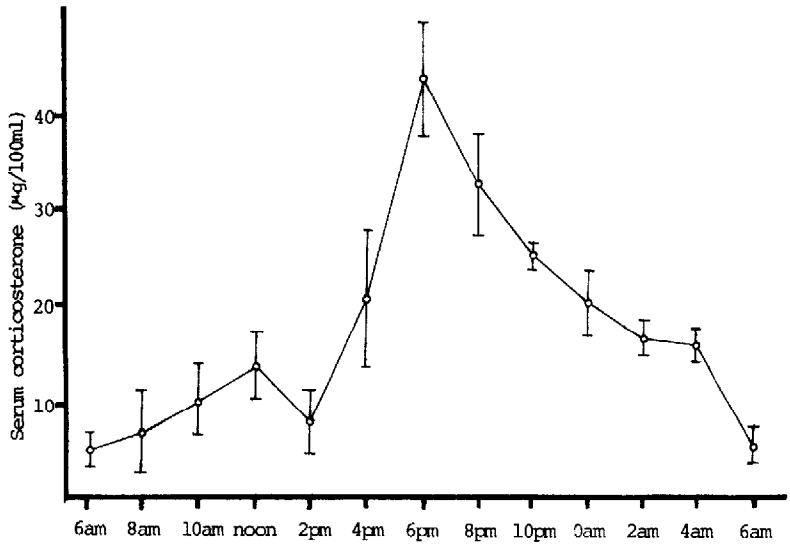


**Fig. 3. Circadian rhythm of serum corticosterone in rats. Each point represents the mean of six rats and vertical bars indicate the S.E.**

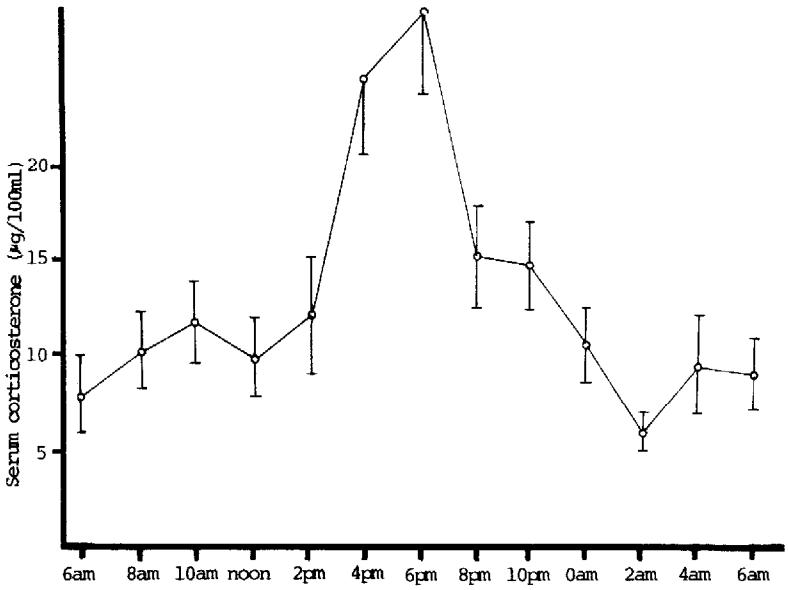


**Fig. 4. Circadian rhythm of serum corticosterone in mice. Each point represents the mean of six mice and vertical bars indicate the S.E.**

low level of around 5 μg per 100 ml; the level then increased from 15:00 hours and reached a maximum, 44 μg per 100 ml, at 18:00 hours. After that, the level decreased gradually until morning.

Fig. 4 shows the circadian rhythm of serum corticosterone in mice. The pattern of the rhythm is very similar to that of rats. The minimum level is 5 μg per 100 ml, which is equal to that of rats, and the maximum level is 15 μg per 100 ml which is lower than that of rats.

From these results, it is seen that the serum corticosterone levels and the circadian rhythm of the two animals are very similar, and mice can therefore be used for the assay of serum corticosterone instead of rats. The analysis of serum corticosterone by HPLC was reproducible, the most outstanding point in the method being the rapidity and accuracy in analysis of serum corticosterone above 1 ng (8.3 ng/ml serum) in comparison with radioimmunoassay.

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