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Analysis of corticosterone in rat plasma by high-performance liquid chromatography

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Abstract

A sensitive and specific high-performance liquid chromatographic assay for the determination of corticosterone in rat plasma using dexamethasone as the internal standard is reported. Rat plasma (0.5 ml) is extracted with methylene chloride, washed with 0.1 *M* sodium hydroxide and then with water. The extract is analyzed by HPLC on a C_{18} column with ultraviolet absorbance detection at 254 nm. Pooled rat plasma was treated with activated decolorizing carbon to remove endogenous corticosterone, and was then used to prepare standards for the assay. Using 0.5 ml plasma for extraction, the detection limit of the assay is 10 ng/ml. The standard curve is linear over the concentration range 10–500 ng/ml. The recovery of corticosterone after extraction was independent of concentration and ranged from 87 to 95%. The coefficient of variation for intra-day and inter-day precision ranged from 2.4 to 7.4% and 2.1 to 8.7%, respectively. In addition, for concentrations ranging from 10 to 500 ng/ml the accuracy is within 5% of the spiked standards. The assay was utilized to examine the circadian rhythm of plasma corticosterone, and to examine the effect of immobilization stress on corticosterone levels in rats.

1. Introduction

A major physiological response to stress in rats is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, leading to an increase in circulating levels of corticosterone (B). To assess the levels of B in rats after different stress paradigms, a reliable and efficient method of measuring plasma B is necessary. Most studies have employed radioimmunoassay (RIA) and fluorimetric methods to measure B. The commonly employed RIA techniques use antiserum which significantly crossreacts with precursors and metabolites of B, and with other endogenous steroids and their metabolites [1,2]. Consequently levels of B measured by RIA are probably an overestimate of the true levels. Chromatography of plasma samples prior to RIA analysis results in lowered estimates of B concentrations compared to direct RIA analysis of unchromatographed samples [2–4]. Furthermore, some RIA assays show poor precision with inter- and intra-assay coefficients of variation ranging from 10–25% [1,2].

Fluorimetric methods developed for the analysis of B require lengthy sample preparation, lack specificity, and have an inadequate detection limit of 50 ng/ml [5,6]. Fluorimetric assays also

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show a time-dependent increase in background fluorescence which complicates standardization of the assay and necessitates a correction for background fluorescence [5,7]. Chromatography or differential extraction (using organic solvents) of plasma samples, prior to fluorimetry, improves the selectivity of the assay [8,9].

Unlike RIA and fluorimetry, HPLC methods in combination with an autosampler easily lend themselves to automation and processing of a large number of samples. Also, while the initial capital costs of HPLC methods are high, the routine cost of processing samples is much lower compared to RIA assays. HPLC methods have been developed for the determination of plasma B levels in animals and in humans. However use of these methods is limited by the tedious extraction techniques with harmful solvents [10,11], lack of precision and accuracy data, or poor precision and accuracy, especially at concentrations of B between 10 and 100 ng/ml [10–14], and the use of the less cost efficient, and less stable normal-phase columns [13,15].

This report describes a specific, precise, and sensitive reversed-phase HPLC method for the determination of B levels in rat plasma. The assay has been used to examine circadian rhythms of B in rats, and to examine the effect of immobilization stress on plasma B levels in rats.

2. Experimental

2.1. Materials

Corticosterone and the internal standard dexamethasone were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and methylene chloride were obtained from Burdick and Jackson Labs (Muskegon, MI, USA). Decolorizing carbon (Norit) was purchased from Fisher (Fair Lawn, NJ, USA). All other reagents were of analytical reagent grade. The cone-shaped plastic bags used to immobilize rats were obtained from Braintree Scientific (Braintree, MA, USA).

2.2. Preparation of standards

Stock standard solutions of B and dexamethasone were prepared in methanol and stored at 4°C. Plasma used for the preparation of standards was first treated with decolorizing carbon in order to remove endogenous B. The decolorizing carbon was added to pooled rat heparinized plasma at a concentration of 0.04 g/ml of plasma. The suspension was stirred for 90 min at room temperature and then centrifuged at 10 000 *g* for 60 min at 4–6°C. The plasma layer was decanted and then filtered through a 0.80-μm Millipore filter to remove carbon particles. Decolorizing-carbon treated plasma was spiked with standard solutions of B to create plasma standards over the concentration range 10–500 ng/ml.

2.3. Extraction procedure

A 500-μl aliquot of plasma was added to a 20×125 mm screw-capped borosilicate glass tube containing 50 μl of dexamethasone solution (4 μg/ml). The mixture was extracted with 15 ml of methylene chloride on a horizontal reciprocating shaker for 15 min, and then centrifuged at 3000 *g* for 10 min. After removal of the plasma phase, the organic phase was washed with 2 ml of 0.1 *M* sodium hydroxide and then with 2 ml of water. After aspirating the aqueous phase, the organic layer was evaporated to dryness under nitrogen at room temperature. The residue was reconstituted in 250 μl of mobile phase and 100 μl was injected onto the HPLC system.

2.4. Chromatography

A reversed-phase column, LiChrospher 100 RP-18 (250×4.6 mm I.D., 5 μm particle size) was purchased from Merck (Darmstadt, Germany). The mobile phase was acetonitrile–water–glacial acetic acid (35:65:0.05, v/v) and was pumped through a Waters 501 pump (Waters, Milford, MA, USA) at a flow-rate of 1.0 ml/min. The eluent was monitored by a variable wavelength UV detector (Spectro Flow 783, Kratos Analytical Instruments, Ramsey, NJ,

USA) at 254 nm. Peak heights of B and the internal standard, dexamethasone, were measured using a SP4290 integrator (Spectra-Physics, Piscataway, NJ, USA). Plasma concentrations were calculated from a standard plot of peak-height ratio of B to dexamethasone versus B concentration.

2.5. Precision and accuracy

Spiked plasma standards were extracted as described above and used to determine the within-day ($n = 7$) and between-day ($n = 6$) coefficients of variation (C.V.) of the assay.

The percent accuracy of the assay was expressed as the difference between the spiked and analyzed concentration and was determined by the following equation:

Percent accuracy = [(observed conc. − spiked conc.)/spiked conc.] · 100

2.6. Extraction recovery

Plasma samples containing B at concentrations of 10, 20, 100 or 500 ng/ml were extracted as described above. Simultaneously, methanolic solutions of B at identical concentrations as in plasma were prepared and served as the unextracted standards. These solutions were evaporated to dryness under nitrogen, the residue reconstituted with 250 μl of the mobile phase and then injected onto the HPLC system. The extraction recovery for each concentration was calculated using the following equation:

Percent recovery = (peak height of extracted plasma/peak height of unextracted standard) · 100

2.7. Specificity

The specificity of the assay was verified by mass spectrometric (MS) analysis. A triple-quadruple mass spectrometer (Model API 111, Thornhill, Ont., Canada) interfaced with an ion-spray ionization source operated in the Q1 scanning mode was used. The MS–MS analysis was performed by setting the Q1 to admit only the protonated molecular ions at m/z 347. Q2 was used to induce fragmentation by collisions with argon gas at a gas thickness of 240×1012 atom/cm^2, and an ion energy of 24 eV. The product ions were analyzed by Q3. In all experiments, the orifice, ion-spray, and the electron-multiplier voltage were set at +50 V, +5500 V and −4000 V, respectively.

2.8. Animal studies

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA) weighing 250–300 g were used in the studies. They were housed in pairs in $24 \times 20 \times 40$ cm clear Plexiglas cages with wood shavings in a temperature controlled room (25°C) with a 12-h light cycle (lights on at 6:00 a.m.). Rats were acclimated for 9–10 days prior to the study. In the study designed to examine the circadian rhythm in plasma B levels, groups of eight rats were randomly assigned to either a day or a night group. Day group rats were decapitated between 10:00–12:00 a.m., and night group rats were decapitated between 10:00–12:00 p.m. Trunk blood was collected in a heparinized beaker for the determination of plasma B. To study the effect of immobilization stress on plasma B levels, a group of rats received a one-hour immobilization stress in the morning between 10:00–12:00 a.m. Physical immobilization stress was applied by enclosing the rats in cone-shaped plastic bags. Rats were decapitated immediately after the immobilization, and trunk blood was collected in a heparinized beaker for the determination of plasma B.

3. Results

Chromatograms obtained from extraction of untreated plasma, decolorizing-carbon-stripped plasma, and decolorizing-carbon-stripped plasma containing 50 ng/ml of B are shown in Fig. 1. Untreated plasma which contained endogenous B showed a peak with a retention time of 10.8 min (Fig. 1A). Injection of a standard solution of

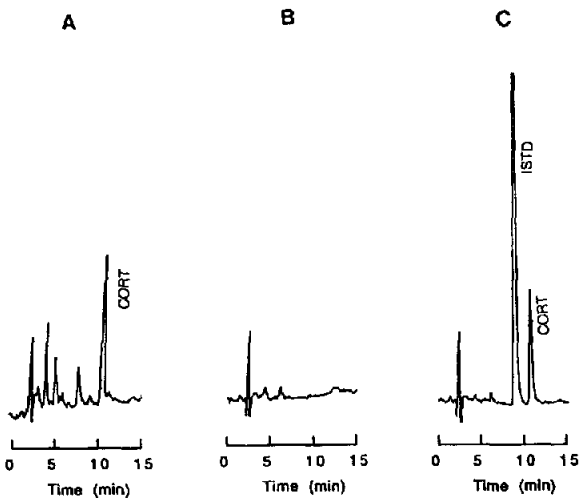


Fig. 1. Chromatograms of an extract of 0.5 ml untreated plasma (A), decolorizing-carbon-treated plasma (B), and decolorizing-carbon-treated plasma spiked with corticosterone at a concentration of 50 ng/ml (C). Dexamethasone is the internal standard (ISTD).

B indicated that the peak observed in untreated plasma had a retention time similar to B and corresponded to a concentration of 66 ng/ml. In the decolorizing-carbon-stripped plasma chromatogram, no interfering peaks were observed at the retention time of B or the internal standard (Fig. 1B). In stripped plasma spiked with B and the internal standard, the two peaks with retention times of 8.9 and 10.7 min, respectively were well separated (Fig. 1C).

In order to verify specificity of the assay, the eluent corresponding to the 10.7-min peak was collected from differentially processed samples, and subjected to MS analysis. Fig. 2A shows the mass spectrum of a pure standard of B in methanol. The m/z 347 was represented by the $(M+H)^+$. Fig. 2B is the spectrum of an extract of decolorizing-carbon-stripped plasma containing 100 ng/ml of B. The fragmentation pattern of this sample was identical to that of the standard solution of B. Figs. 2C and 2D show mass spectra of plasma extracts of naive rats and rats immediately after a one-hour physical immobilization stress, respectively. The fragmentation pattern of these two samples are similar to that of the standard solution of B.

In our assay, two standard curves were constructed on each day. The low-concentration standard curve included concentrations ranging from 10 to 100 ng/ml, while the high-concentration standard curve included concentrations ranging from 100 to 500 ng/ml. The correlation coefficients for both standard curves were usually 0.990 or better. As shown in Tables 1 and 2, the within-day ($n = 7$) and between-day ($n = 6$) C.V. of the assay ranged from 2.4 to 7.4% and 2.1 to 8.7%, respectively. The accuracy of the assay ranged from within −1.3 to 4.5% of the spiked standard over the entire validated concentration range (Table 2). As indicated in Table 3, the mean extraction recoveries of B ranged from 87 to 95%. Based on a 0.5-ml plasma sample and a signal-to-noise ratio of 7 to 1, the detection limit of the assay was 10 ng/ml.

Fig. 3 shows the circadian rhythm of plasma B levels in rats. Night-time levels of 81 ± 11 ng/ml (mean ± S.E.M.) were significantly higher than day-time levels of 36 ± 8 ng/ml. Fig. 4 shows chromatograms of extracts of plasma of naive rats and of rats immediately after a one-hour immobilization stress. The mean plasma B concentration immediately after the stress of 336 ± 10 ng/ml (mean ± S.E.M.) was found to be significantly higher [$p < 0.001$] than the mean plasma B concentration in naive unstressed animals of 36 ± 8 ng/ml (Fig. 3).

4. Discussion

A reversed-phase HPLC assay utilizing liquid extraction was developed for the quantitation of B in samples of rat plasma. The specificity of the assay was evaluated using MS analysis. The similarity of the fragmentation patterns of plasma samples of naive rats and of rats subjected to a one-hour immobilization stress to that of a methanolic solution of pure B confirmed the specificity of the assay.

Decolorizing carbon was used to strip the endogenous B in plasma used for the construction of standard curves. This improved the accuracy and precision of the assay, especially at the low end of the standard curve. Our attempts

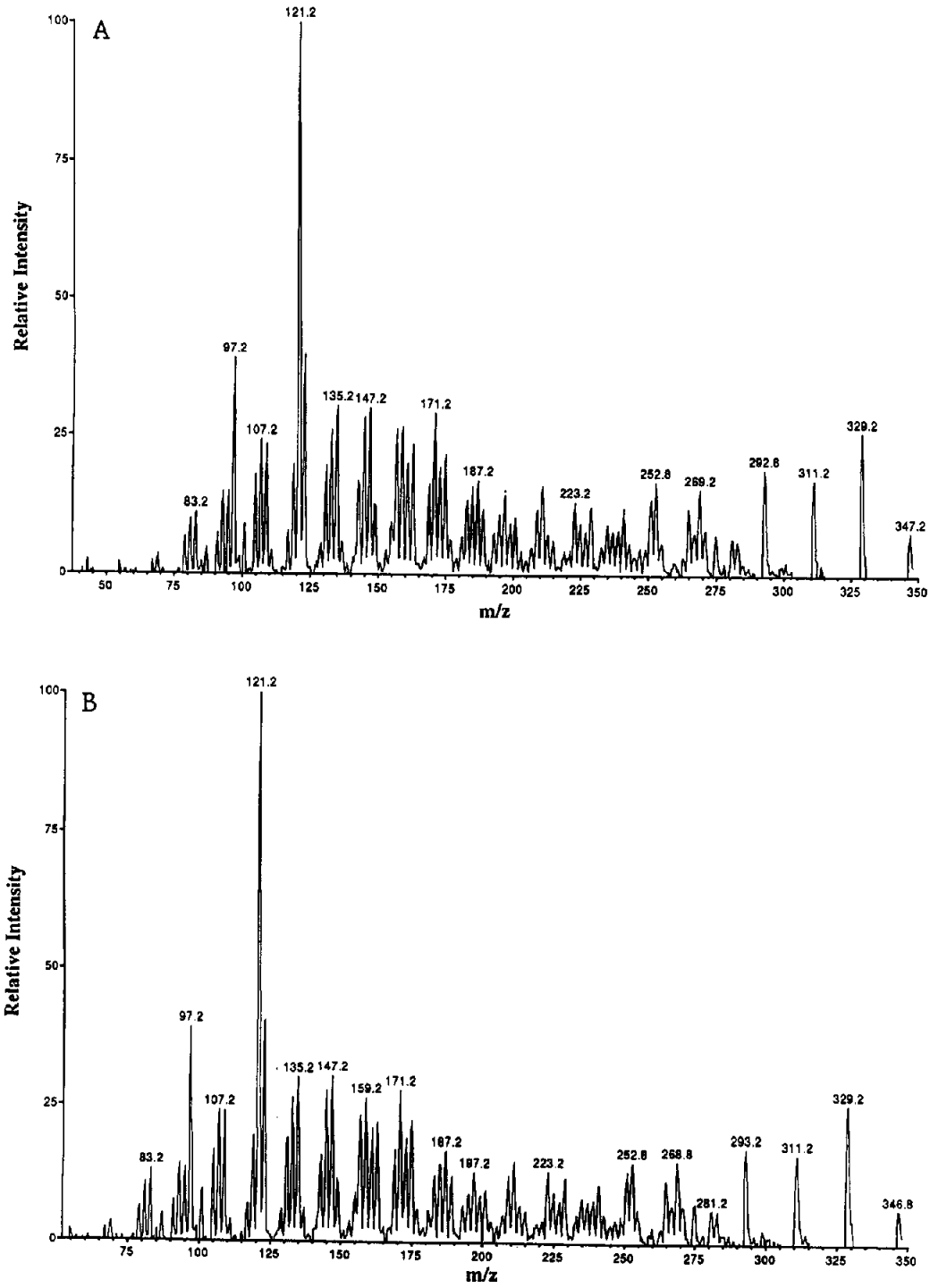
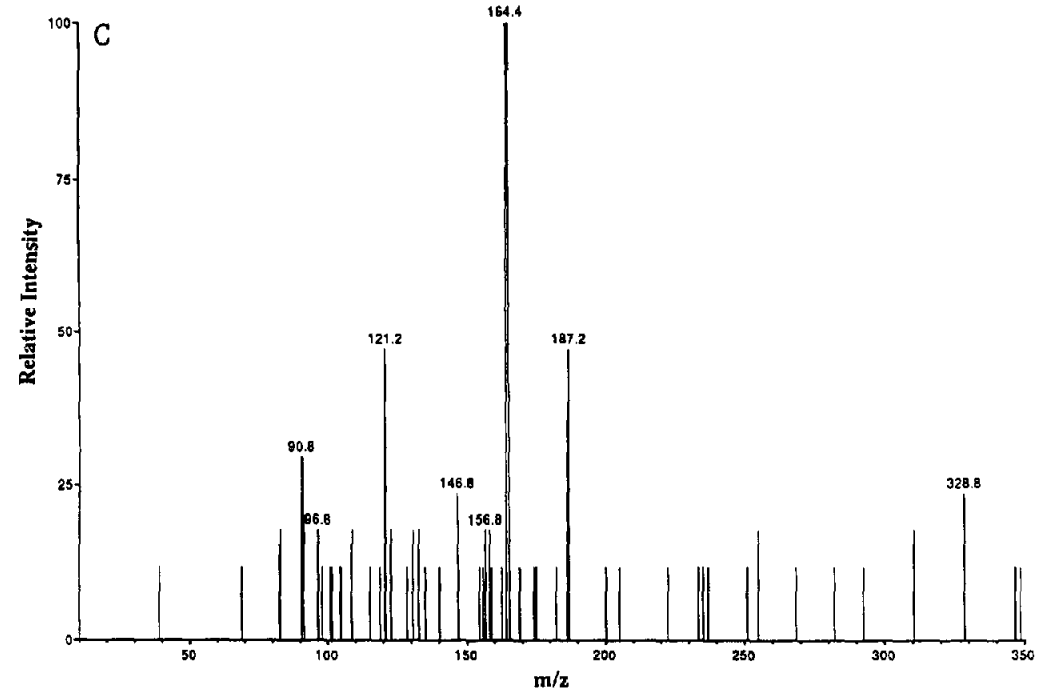


Fig. 2. Mass spectra of corticosterone samples. (A) 100 ng/ml Methanolic solution of corticosterone. (B) Extract of decolorizing-carbon-stripped plasma containing 100 ng/ml of corticosterone. (C) Plasma extract from a naive rat. (D) Plasma extract from a rat subjected to a one-hour physical immobilization stressor.



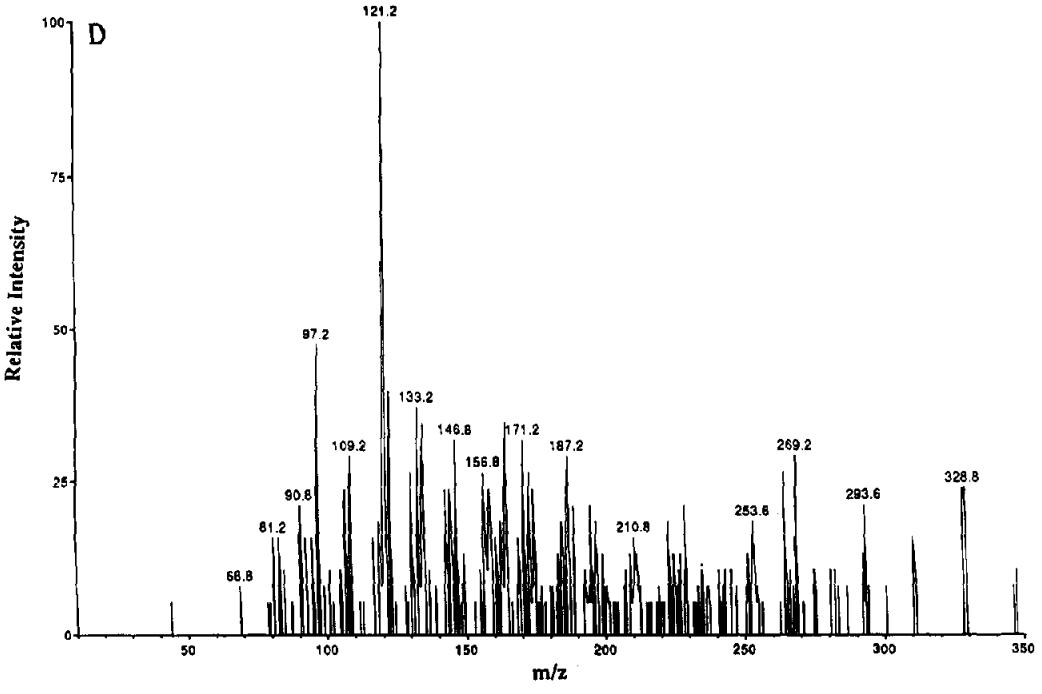


Fig. 2. (*Continued*).

Table 1
Intra-day variability for corticosterone in rat plasma

Spiked concentration (ng/ml)	Observed concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (% difference)
10	9.9 ± 0.7	7.4	−0.7
20	20.0 ± 0.8	4.1	0.0
50	51.6 ± 2.2	4.2	3.2
100	100.1 ± 2.5	2.4	0.0
200	197.4 ± 5.7	2.9	−1.3
500	500.9 ± 14.6	2.9	0.2

Number of replicates $n = 7$.

Table 2
Inter-day variability for corticosterone in rat plasma

Spiked concentration (ng/ml)	Observed concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy (% difference)
10	9.9 ± 0.9	8.7	−1.3
20	19.9 ± 1.7	8.4	−0.7
50	49.9 ± 3.1	6.2	−0.2
100	104.5 ± 3.4	3.3	4.5
200	199.1 ± 4.2	2.1	−0.4
500	493.9 ± 12.3	2.5	−1.2

Number of replicates $n = 6$.

to use unstripped plasma as a standard to measure low basal levels of B resulted in markedly inaccurate estimates. Earlier HPLC assays have used unstripped plasma to construct standard curves. The total concentration of B in the standards was determined and the endogenous level of B in the unstripped plasma was subtracted in order to estimate the actual concentration of the standards. The actual concentrations of standards were then used to compute accuracy and precision data of these assays [10–14]. This procedure might have contributed to the less than optimum precision and accuracy observed in the earlier HPLC assays. The inter- and intra-day coefficients of variation of our assay were lower than those reported for the RIA assays.

Another feature of our assay which improved the accuracy in the low-concentration range was the construction of a separate low-concentration standard curve. In our assay, the low basal levels of B in the morning (less than 100 ng/ml) can be measured within a 9% C.V. and with an accuracy of within 4.0% of the spiked standard. Use of stripped plasma also increased the sensitivity of the assay. The detection limit of our assay was 10 ng/ml, and was better than that reported for other HPLC assays or using fluorimetry.

Table 3
Extraction recovery of corticosterone in rat plasma

Concentration (ng/ml)	Recovery (mean ± S.D.) (%)
20	87 ± 1
50	95 ± 9
100	88 ± 3
500	93 ± 5

Number of replicates $n = 8$.

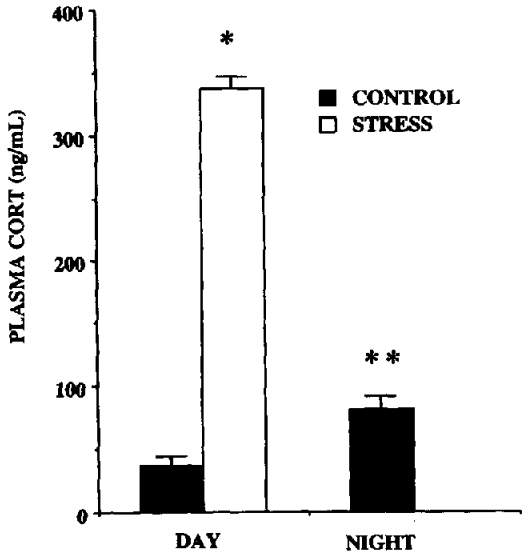


Fig. 3. Circadian rhythm of plasma corticosterone and effect of immobilization stress on plasma corticosterone in rats. Data in each group represents the mean ± S.E.M. of 8 rats. (*) Significantly different from respective non-stress control group ($p < 0.001$, student unpaired t-test); (**) significantly different from day non-stress control group ($p < 0.02$, student unpaired t-test).

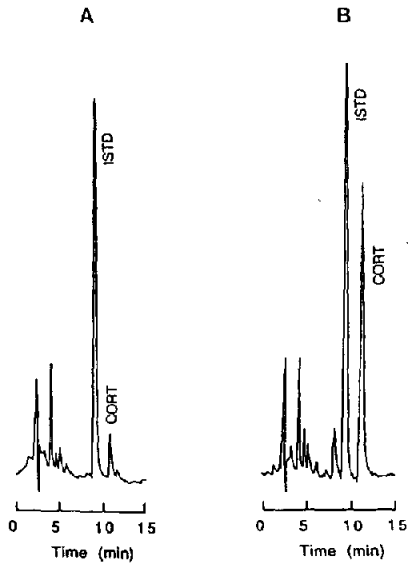


Fig. 4. Chromatograms of an extract of 0.5 ml plasma collected from control unstressed rat (A), or from a rat immediately after a one-hour immobilization stressor (B). Corticosterone peaks correspond to a concentration of 35 ng/ml in the naive animal and a concentration of 261 ng/ml in the stressed animal.

Similar to reports in the literature [16], we observed a circadian rhythm in basal plasma B levels in rats. In our study, the basal B levels in the morning ranged from 10 to 60 ng/ml and were at the low end of the range reported in the literature [17,18]. This probably reflects the increased specificity of the assay, and the relatively quiet and stress-free environment of the vivarium. A variety of stressors are known to increase B levels. As reported in the literature [18], a one-hour immobilization stressor produced significant increases in the levels of B.

In summary, the proposed HPLC method is specific, sensitive, and has good reproducibility. It has been used to examine the circadian rhythm of plasma B levels in rats, and to examine the effect of immobilization stress on plasma B levels.

References

[1] C.D. Lodson, S.F. Akana, C. Meyers, M.F. Dallman and J.A. Williams, *Endocrinology*, 121 (1987) 1242.
[2] H.J. Ruder, R.L. Guy and M.B. Lipsett, *J. Clin. Endocrinol. Metab.*, 35 (1972) 219.
[3] E.H. Mougey, *Anal. Biochem.*, 91 (1978) 566.
[4] L.C. Krey, K.H. Lu, W.R. Butler, J. Hotchkiss, F. Piva and E. Knobil, *Endocrinology*, 96 (1975) 1088.
[5] R.H. Silber, D. Busch and R. Oslapas, *Clin. Chem.*, 4 (1958) 278.
[6] D. Glick, D.V. Redlich and S. Levine, *Endocrinology*, 74 (1964) 653.
[7] D.A. Mattingly, *J. Clin. Pathol.*, 15 (1962) 374.
[8] M.L. Sweat, *Anal. Chem.*, 26 (1954) 1964.
[9] J. Van der Vies, *Acta Endocrinol.*, 38 (1961) 399.
[10] R.N. Sargent, *J. Anal. Toxicol.*, 9 (1985) 20.
[11] J. Mathew, V.L. Sallee, J. Curtis and J. Mrotek, *Steroids*, 46 (1985) 697.
[12] B.T. Hofreiter, J.P. Allen, A.C. Mizera, C.D. Powers and A.M. Masi, *Steroids*, 39 (1982) 547.
[13] M. Alvinerie and P.L. Toutain, *J. Pharm. Sci.*, 71 (1982) 816.
[14] N. Imaizumi, I. Yamamoto, M. Kamei, I. Yoshida, E. Miyauchi, T. Kigoshi, H. Hosojima, K. Uchida and S. Morimoto, *Horm. Res.*, 27 (1987) 53.
[15] D.B. Haughey and W.J. Jusko, *J. Chromatogr.*, 430 (1988) 241.
[16] D. Guillemin, W.E. Dean and R.A. Liebelt, *Proc. Soc. Exp. Biol. Med.*, 101 (1959) 394.
[17] W. Engeland, J. Shinsako, C.M. Winget, J. Vernikos-Danellis and M.F. Dallman, *Endocrinology*, 100 (1977) 138.
[18] M.F. Dallman and M.T. Jones, *Endocrinology*, 92 (1973) 1367.