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# Liquid chromatography–electrospray ionization mass spectrometric analysis of corticosterone in rat plasma using selected ion monitoring

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**Abstract**

A simple and fast yet highly sensitive and specific method based on HPLC coupled to electrospray ionization mass spectrometry has been developed for the quantitation of corticosterone in rat plasma. After extraction of rat plasma (100 μl) with diethyl ether using 5-pregnen-3β-ol-20-one-16α-carbonitrile (Sigma) as internal standard, HPLC was performed on a short $C_8$ column (Zorbax-Eclipse, 50×4.6 mm I.D.) using a steep methanol–water gradient (methanol 54% to 90% in 6 min). Detection was performed on a single quadruple mass spectrometer in selected ion monitoring mode ($m/z$ 369 for corticosterone and 364 for the internal standard). The detection limit of the assay was 9 fmol (3 pg) of corticosterone on column. In vitro data were subjected to curve fitting (cubic, $r^2=0.9999$). Recovery of corticosterone after extraction ranged from 81 to 93%. The relative standard deviations for intra- and inter-assay precision ranged from 0.8 to 3.6% and 5.2 to 12.9%, respectively. Corticosterone did not undergo any appreciable degradation when stored in plasma at −20°C for 2 months. The assay is routinely used in our laboratory to examine corticosterone levels as a marker of stress in rats and may also be used for the determination of 18-hydroxy-11-deoxycorticosterone. 

*Keywords:* Corticosterone

## 1. Introduction

During our on going investigations in search of active metabolites of dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one) and 7-oxygenated-DHEA derivatives for the enhancement of the formation of liver mitochondrial sn-glycerol 3-phosphate dehydrogenase and cytosolic malic enzyme [1–3], we became interested in studying the amount of stress in experimental animals, particularly rats, leading to the development of a new highly sensitive and specific method for the estimation of corticosterone in rat plasma.

Corticosterone (I) is the principle glucocorticoid in the rat, which lacks adrenocortical 17α-hydroxylase activity, and therefore its plasma concentration is an indicator of stress [4]. Numerous chromatographic methods have been described for the determination of corticosterone, mainly in human and

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rat plasma, utilizing fluorimetry [5], high-performance liquid chromatography (HPLC) [6–11] and recently HPLC coupled with atmospheric pressure electrospray ionization mass spectrometry (MS-ESI) [12,13].

Although a few LC–MS methods were available in the literature for the estimation of corticosterone, none was found to be sensitive enough and rapid for the estimation of corticosterone in small amounts of plasma (100 μl or less). The method of Ghulam et al. [12], for the estimation of corticosterone by LC–ESI-MS using a microbore column (250×1.0 mm I.D.), requires an equilibration time of at least 5 h before a series of analyses could be performed, and the column had to be thoroughly rinsed for 2 h at the end of each series, making the method time consuming and hence it could not be used quickly for the estimation of corticosterone on demand. Moreover, the lower limit of detection defined as a signal-to-noise ratio of 3 could be reached only with 1550 pg in 25 μl (4.5 pmol) of corticosterone injected on column. Miksik et al. [13] have described an LC–ESI-MS method for the separation and identification of corticosterone metabolites produced by in vitro incubation with avian intestines. We did not find the method suitable because of the long (~1 h) run time.

We here describe a simple, rapid, yet highly sensitive and specific method based on HPLC coupled to atmospheric pressure ESI-MS for the quantitative determination of corticosterone in small volumes (≤100 μl) of rat plasma.

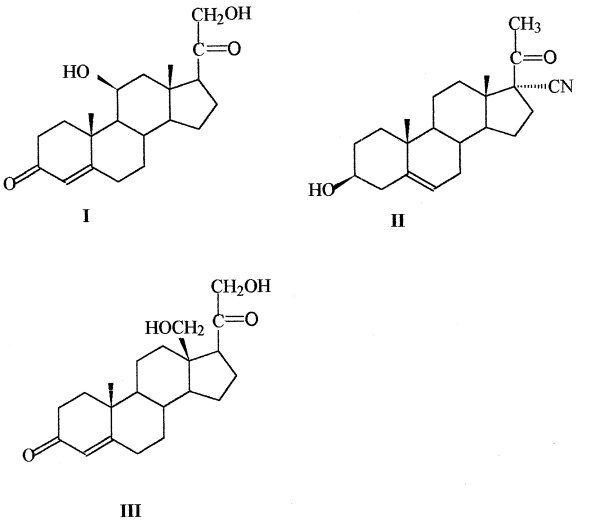


Fig. 1. Structures of corticosterone (I), 5-pregnen-3β-ol-20-one-16α-carbonitrile, (II, I.S.) and 18-hydroxydeoxycorticosterone (III).

## 2. Experimental

### 2.1. Materials and reagents

Corticosterone (I) and 5-pregnen-3β-ol-20-one-16α-carbonitrile (II, the internal standard, I.S.) were purchased from Sigma–Aldrich, and 18-hydroxy-11-deoxycorticosterone (III, Fig. 1) from Steraloids, USA. Stock solutions (1 mg/ml) were prepared in methanol and stored at −20°C. HPLC-grade methanol, acetonitrile and diethyl ether were purchased from Aldrich and used as such. Acetic acid, HPLC grade (Fisher, USA) was used to adjust the pH of the aqueous phase. Distilled water, deionized and purified by a Nanopure ultra pure water system from Barnstead (USA) was used (18.25±0.05 MΩ-cm). Glass tubes with PTFE lined screw caps (Pyrex brand) were silanized and used for extraction.

Male Sprague–Dawley rats (Charles River Labs., WI, USA) weighing 200–300 g were used in the present study. They were housed in pairs in clear Plexiglas cages with wood shavings in a temperature-controlled room (25°C) with a 12-h light cycle. Rats were acclimatized for 1 week prior to study. The rats were sacrificed by decapitation (between 10:00 and 12:00) and trunk blood was collected in heparinized beakers.

### 2.2. Instrumentation

The chromatographic system consisted of an Agilent 1100 series HPLC–MS system, comprised of a quaternary pump (G1311A) with an on-line degasser, a thermostated column compartment (G1316A), an autosampler (G1313A), a diode array UV detector (G1315A) and a mass detector (1946A). Data were acquired and processed using ChemStation version A.07.01 software from Agilent.

### 2.3. Chromatographic conditions

Chromatography was performed on a Zorbax-Eclipse $C_8$ (50×4.6 mm I.D.) analytical column (Agilent Technologies, USA) packed with 3.5 μm diameter particles. The analytical column was protected using a $C_8$ guard column (Zorbax-Eclipse $C_8$, 12.5×4.6 mm I.D. cartridge, 5 μm). The flow-rate was set at 0.8 ml/min. The column temperature was maintained at 40.0±0.5°C. The mobile phase consisted of methanol–water, each containing 0.002% (v/v) acetic acid. All solvents were filtered (0.45 μm) before use. A gradient elution was used. The mobile phase composition was methanol–water (54:46, v/v) at time $t=0$, (90:10) at $t=6$, and (54:46), i.e., the initial composition at $t=7$ min. A 5-min post run time was used to reequilibrate the column and for the baseline to return to the normal.

A single quadruple mass spectrometer was interfaced via an electrospray probe operated in the positive ion mode. The operating conditions were optimized by flow injection analysis (FIA) and were determined as follows: drying gas ($N_2$) flow 12 l/min; drying gas temperature 350°C; nebulizer pressure 40 p.s.i. (268 kPa); capillary voltage 3500 V; gain 5; and fragmentor 90 V. Chromatograms were monitored in the selected ion mode (SIM) at $m/z$ 369 [$M+Na^+$] for corticosterone from time $t=0$ to 3.6 min and then at $m/z$ 364 [$M+Na^+$] for the internal standard.

The dwell volume ($V_D$) of the system was determined graphically by replacing the column with a short piece (50 mm) of HPLC tubing and by running a gradient of water vs. 3% acetic acid (0–100% in 10 min) and recording the response at 254 nm.

The resolution factor, $R$, was determined using the standard equation: $R=2(t_2-t_1)/(W_2+W_1)$ in which $t_2$ and $t_1$ are the retention times of internal standard and corticosterone, respectively, and $W_2$ and $W_1$ are corresponding peak widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

### 2.4. Preparation of standards

Charcoal treated rat plasma was used to prepare standard samples so as to remove interference from endogenous corticosterone. Pooled plasma was stirred at room temperature with decolorizing charcoal (40 mg/ml) for 1 h, centrifuged at 5000 *g* for 30 min at 5°C and then filtered through a 0.5-μm filter protected with a 1.2-μm filter, to remove carbon particles. Methanolic solutions of corticosterone and the internal standard were added to 125×15 mm silanized glass centrifuge tubes and evaporated to dryness prior to addition of plasma. Blanks were prepared by spiking the tubes with solvent, evaporating it to dryness and then adding the plasma.

### 2.5. Extraction procedure

The internal standard (2.0 ng in 20 μl methanol) was added to a silanized screw cap glass tube (15 ml) and evaporated to dryness under nitrogen. Rat plasma (0.1 ml) was added, diluted with water (0.1 ml) and extracted with diethyl ether (2.5 ml). The contents were vortex mixed for 2×15 s, centrifuged for 5 min at 1500 *g* and the ether layer was separated from aqueous layer by snap drying in an acetone–dry ice bath. The residue was dissolved in the mobile phase of initial concentration (100 μl), and a 10.0-μl aliquot was injected onto the chromatograph.

### 2.6. Effect of injection volume

Short columns can provide faster separations but they do not have the separating power (efficiency) of longer columns. The effect of injection volume is more pronounced on the early eluting peaks and may result in compromised column performance. Therefore, we considered it prudent to study the effect of injection volume on peak shape of corticosterone. Samples containing 1 ng of corticosterone in 2.0, 10.0, 20.0, 40.0 and 60.0 μl of mobile phase of initial concentration were injected onto the chromatograph and peak width and peak height were recorded. Each analysis was conducted in duplicate.

### 2.7. Standard curve

The standard stock solutions were prepared by dissolving separately 25 mg of corticosterone (I) and the internal standard (II) in 25 ml of methanol. From these stock solutions working solutions were prepared at final concentrations of 0.2, 1.0, 10.0, 20.0

and 40.0 ng of corticosterone (I) in 20 μl methanol. All solutions were stored at −20°C until use.

Calibration standards were prepared using 0.2, 1.0, 10.0, 20.0 and 40.0 ng concentrations of corticosterone (I) in charcoal treated rat plasma (100 μl). The standard samples were prepared by adding appropriate concentrations of corticosterone (I). These samples were processed as described earlier.

### 2.8. Stability study

In the present study, the stability of corticosterone (I) in plasma was assessed from spiked samples (0.2, 1.0 and 10.0 ng/100 μl), after bench top storage at room temperature for 4 h, at −20°C for 2 months, and after subjecting them to repeated (three times) freeze–thaw cycles. The samples were analyzed immediately after preparation and after storage as per the protocol. Prior to the analysis of the samples after cold storage, they were brought to room temperature (20°C) and vortex mixed. Each determination was performed in duplicate.

### 2.9. Calibration

The ratios of peak areas of the compound of interest to that of internal standard were correlated with the standard concentration over the range 0.2 to 40 ng/100 μl. After analysis, a standard curve for corticosterone was constructed by cubic regression analysis of peak area ratio ($y$-axis) and corticosterone concentration ($x$-axis) using SPSS 10.0.7 statistical software supplied by SPSS, USA.

### 2.10. Recovery

The recovery of corticosterone (I) was determined by comparing peak area ratios from plasma spiked with known amounts of corticosterone (I, 0.2, 1.0, 10.0, 20.0 and 40.0 ng/100 μl), processed as described earlier versus peak area ratios of the same concentrations obtained by spiking the extracted matrix with said concentrations. Each sample was analyzed five times.

### 2.11. Accuracy, precision and limit of quantitation

The intra-assay precision and accuracy of the method were evaluated by analyzing, during a single run, five replicates of spiked samples at five different concentrations (0.2, 1.0, 10.0, 20.0 and 40.0 ng/100 μl) against a separate calibration curve. The inter-assay precision and accuracy were assessed by analyzing spiked quality control samples at five different concentrations during different runs ($n=5$) against an independent calibration curve.

Accuracy was evaluated as percentage error (mean of measured−mean of added)/mean of added. The precision was given by inter- and intra-assay relative standard deviations (RSDs).

The limit of detection was defined as the sample concentration of corticosterone resulting in a peak height of three times $S/N$.

The limit of quantitation was defined as the sample concentration of corticosterone resulting in a peak height of 20 times $S/N$.

### 2.12. Specificity

To evaluate the specificity of the method, plasma samples were subjected to the assay procedure and the retention times of endogenous substances in plasma were compared with those of compounds of interest obtained at $m/z$ 369 for corticosterone and $m/z$ 364 for the internal standard. Interference from the internal standard on the retention time of corticosterone and vice versa was checked to rule out the presence of any interfering impurities.

Specificity of the method was further assessed by injecting different corticoids and related compounds and by evaluating the chromatogram obtained at $m/z$ 369 in the SIM analysis mode.

## 3. Results and discussion

### 3.1. LC–MS

A satisfactory resolution (resolution factor, $R>2$) of corticosterone (I) and the internal standard (II) was achieved by a gradient elution on a 5.0 cm reversed-phase column at $m/z$ of 369 and 364, respectively. The resolution factor, $R$, for the corticosterone and the internal standard was found to be 4.74 (RSD=3.7%, $n=10$). The overall run time, including the post run time for reequilibration of the column, for the chromatogram was 12 min.

The dwell volume of the system, i.e., the volume

of the mobile phase that passes through the head of the column before the actual gradient starts, attains significance for early peaks, which may elute in the dwell volume, thus essentially eluting isocratically. Dwell volume varies from instrument to instrument. It was measured using water vs. 3% acetic acid gradient and was found to be 1.18 ml. This high value of dwell volume was attributed to the use of the quaternary pump employing low-pressure mixing. This high value also prevented the use of a narrow diameter column, since corticosterone was found to elute in the dwell volume necessitating longer analysis time.

Chromatograms obtained from extraction of plasma treated with decolorizing-carbon, and charcoal treated plasma spiked with 10 ng/100 μl of corticosterone and 2.0 ng of the internal standard are shown in Fig. 2. Untreated plasma, which contained endogenous corticosterone showed a peak corresponding to retention time of corticosterone, having UV (diode array detection, DAD) and mass spectra similar to that of corticosterone and corresponding to the concentration, which varied depending upon the level of stress (Fig. 3). In charcoal treated plasma chromatograms, no interfering peaks were observed at the retention times of corticosterone and the internal standard. In stripped plasma spiked with corticosterone and the internal standard, these two peaks were well resolved from each other with retention times [min, mean±SD (RSD), $n=30$] of 3.16±0.12 (3.8%) and 4.05±0.13 (3.2%), respectively. However a positive shift of ~0.25 min was observed in the retention times when a diode array UV detector was used in line for the purpose of recording UV spectra.

The positive ion mass spectra of the analytes, obtained in scan mode, indicated the predominant presence of sodium ion adduct $[M+Na]^+$ for corticosterone ($m/z$ 369) as well as the internal standard ($m/z$ 364). Hence, the analysis was carried out by analyzing the samples in these selected ion modes (SIM). This adduct formation was very prominent, consistent and concentration dependent (within the assay limits) whenever methanol (HPLC grade, regardless of its source) was used in the mobile phase. Replacing methanol with acetonitrile invariably resulted in suppression of adduct formation.

A pregnene derivative containing odd number of nitrogen atoms (5-pregnen-3β-ol-20-one-16α-carbonitrile, II) was selected as internal standard since

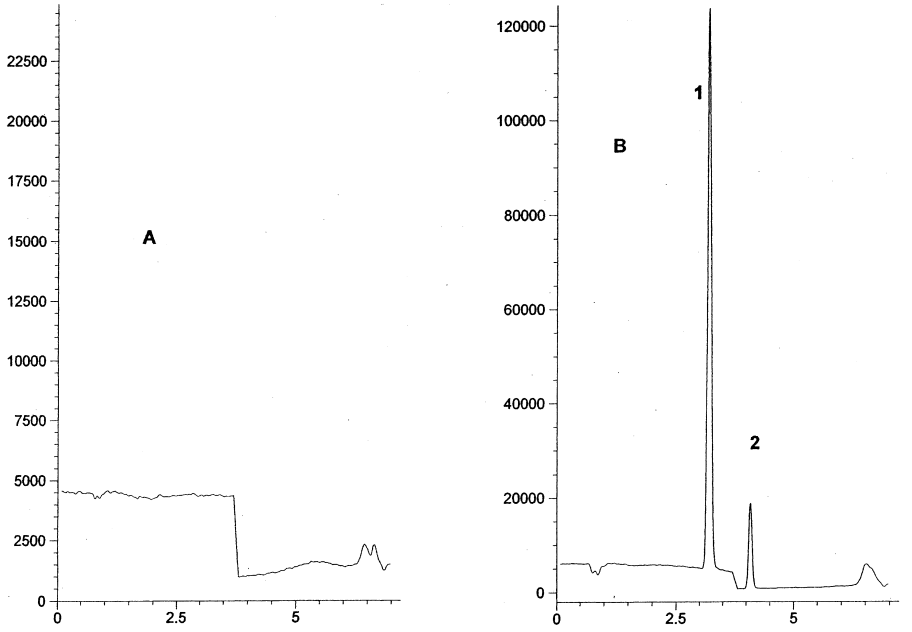


Fig. 2. Chromatograms of (A) charcoal treated rat plasma, (B) charcoal treated rat plasma spiked with 10.0 ng/100 μl of corticosterone, and 2.0 ng of I.S. Peaks: 1=corticosterone, 2=I.S. Chromatographic conditions as described in Section 2.3.

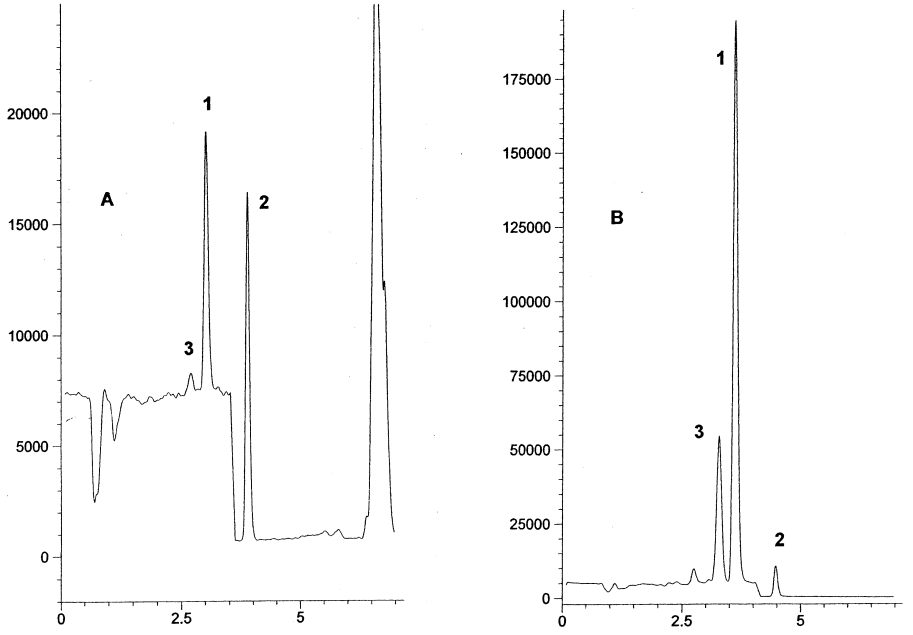


Fig. 3. Chromatograms of (A) untreated rat plasma (100 μl) with 2.0 ng/100 μl of I.S., concentration of corticosterone 0.99 ng/100 μl and (B) rat (stressed) plasma, concentration of corticosterone 26.6 ng/100 μl. Peaks: 1=corticosterone, 2=I.S., 3=18-hydroxydeoxycorticosterone. Chromatographic conditions as described in Section 2.3.

this yielded an even numbered $m/z$ ion, thus eliminating interference from normally found carbon-, hydrogen- and oxygen-containing steroid molecules in the SIM analysis mode, since the later yielded odd numbered $m/z$ ion peaks.

### *3.2. Effect of injection volume*

There were no significant differences (<5%) between the responses (peak width and height) when the same amount (1 ng) of corticosterone was injected in volumes ranging from 2.0 to 60 μl in spite of the fact that we used a short column (50 mm length) for the present analysis. The mean peak height of corticosterone for injection volumes of 2.0, 10.0, 20.0, 40.0 and 60.0 μl was 94.95±2.03 (mean±SD) with an RSD of 2.1%; the corresponding values for the peak width were 0.1253±0.0011 and 0.9%, respectively. The mean percentage difference between the peak height and peak width for 2.0 and 60.0 μl injection volumes was −4.3 and 0.9%, respectively. Therefore it was concluded that a 30-fold increase in the injection volume (from 2.0 to 60.0 μl) had no significant effect on the chromatographic performance. The use of a fast gradient (54 to 90% methanol in 6 min) was able to offset the effect of injection volume.

A 10.0-μl injection volume was found to be sufficient for the analysis of corticosterone in the present study. The lowest concentration of corticosterone, encountered by us in the plasma of unstressed rats was ~4 ng/ml, corresponding to ~40 pg/ml of corticosterone on-column for a 10.0 μl injection (based on 100-μl matrix volume), which was well within the range of assay. However injection volume can be increased several fold without sacrificing precision and accuracy if warranted by lower corticosterone concentrations (~1–2 ng/ml or less) and/or availability of lower matrix volumes (<100 μl).

### *3.3. Curve fitting*

ESI-MS has a relatively narrow linear dynamic range [14]. It has been our experience as well as

reported in the literature that ESI response levels out even at sub-ng concentrations leading to calibration plots (straight lines to markedly curved plots) which vary from compound to compound [14] and even for the same compound depending upon LC–MS conditions. In the present study we observed a marked deviation in the straight line plot at ~200 pg on-column injections. Therefore, a range of non-linear curve fitting routines was investigated to ensure that response curves were accurately defined. A cubic curve model ($x=ay^3+by^2+cy+d$) was found to best fit the data of the present study in the concentration range of 0.2 to 40.0 ng/100 μl. The representative cubic equation was $x=0.000775y^3-0.014996y^2+1.26917y-0.035296$. The value of $r^2$ (adjusted) was always found to be ≥0.9999. It may be emphasized that regression models in general and cubic regression models in particular must not be used to predict $x$ values associated with $y$ values outside the range of $y$ values used to fit the model since the model may not apply outside the studied range and the presence of cubic and quadratic terms may result in highly erroneous results. It may not be out of place to quote what some statisticians have said, ''all models are wrong, some models are useful'' [15]. Models are ''wrong'' because they do not apply to all situations and to every detail. Those that are useful yield insight in relevant situations. Models are not responsible for errors that result from applying them to situations in which they are not tested [15]. That is, in our opinion, the responsibility of the chromatographer.

### 3.4. Limit of quantitation and limit of detection

The limit of quantitation was 20 pg of corticosterone on-column regardless of the volume of injection (2.0 to 60.0 μl) as discussed in Section 3.2; the corresponding RSD ($n=5$) was 3.6%. At this level, the mean percentage error was found to be 9.9%.

The limit of detection was 3 pg of corticosterone on-column. A representative chromatogram is shown in Fig. 4.

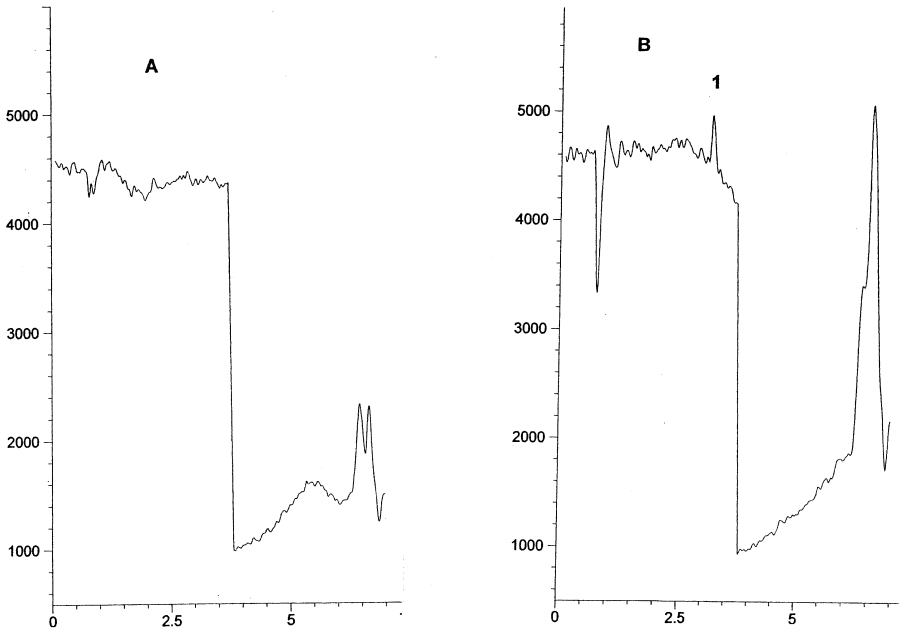


Fig. 4. Limit of detection. Chromatograms of (A) blank charcoal stripped rat plasma, (B) blank charcoal stripped rat plasma spiked with 30 pg/100 μl of corticosterone (3 pg corticosterone on column). Peak: 1=corticosterone. Chromatographic conditions as described in Section 2.3.

### 3.5. Matrix volume

The present assay was developed using 100 μl of the rat plasma. However, high sensitivity of the method coupled with the fact that several-fold increases in injection volume has little effect on chromatographic performance, permits the use of lower volumes of matrix. In the case of stressed rats, which have higher plasma corticosterone (I) levels (Section 3.10) 10 μl of plasma was found to be sufficient for the assay.

### 3.6. Extraction recovery

The mean extraction recovery of corticosterone was satisfactory, mean±SD=87.1±4.1 ($n$=5, RSD=4.7%), range 81–93%, and was consistent in the range of assay.

### 3.7. Accuracy and precision

The intra-assay precision for corticosterone was evaluated by analysis of plasma samples ($n$=5) along with an independent standard curve for quantitation. The RSD for within-run precision, was 3.6% for 0.2 ng/100 μl, 0.9% for 1.0 ng/100 μl, 0.8% for 10.0 ng/100 μl, 2.8% for 20.0 ng/100 μl and 1.2% for 40.0 ng/100 μl. The corresponding values for between-run precision were 12.9, 8.2, 7.9, 5.8, and 5.2%, respectively. The mean recovery for corticosterone observed during a within-run experiment was 109.4% for 0.2 ng/100 μl, 90.5% for 1.0 ng/100 μl, 101.4% for 10.0 ng/100 μl, 96.6% for 20.0 ng/100 μl and 89.8% for 40.0 ng/100 μl concentration. The percentage error ranged from 1.4 to 10.2%. The percentage recovery for between-run analyses ranged from 92.1 to 104.3% with a percentage error of 0.01 to 4.3%. Assay reproducibility was observed (RSD values <15%) over the range of 0.2 to 40 ng/100 μl. The results of accuracy and precision [within-run precision (repeatability) and between-run] for the analysis of corticosterone are given in Table 1.

### 3.8. Specificity

No endogenous substance interfered at the retention time of corticosterone (I). There was no interference from the corticosterone on the retention time of internal standard and vice versa. The following compounds were found not to interfere with the analysis of corticosterone in the present study: cortisone, hydrocortisone, cortexolone, deoxycorticosterone, 11-dehydrocorticosterone, progestrone, 20α-hydroxyprogestrone, 20β-hydroxyprogestrone, 17α-hydroxyprogestrone, pregnenolone, 17α-hydroxypregnenolone, estrone, 17β-estradiol and estriol.

### 3.9. Stability

Corticosterone (I) was stable in plasma for 4 h at room temperature (recoveries 88–100%) and for 2 months at −20°C (recoveries 93–108%). No signifi-

Table 1
Within- and between-run accuracy and precision in the measurement of corticosterone in rat plasma ($n$=5)

| Spiked concentration (ng/100 μl) | Measured concentration (ng/100 μl) | Precision (RSD, %) | Accuracy (%) |
|---|---|---|---|
| *Within-run* | | | |
| 0.2 | 0.22 | 3.6 | 9.9 |
| 1.0 | 0.91 | 0.9 | −9.5 |
| 10.0 | 10.14 | 0.8 | 1.4 |
| 20.0 | 19.31 | 2.8 | −3.5 |
| 40.0 | 35.90 | 1.2 | −10.2 |
| *Between-run* | | | |
| 0.2 | 0.18 | 12.9 | −7.9 |
| 1.0 | 0.97 | 8.2 | −3.4 |
| 10.0 | 9.63 | 7.9 | −3.7 |
| 20.0 | 20.00 | 5.8 | 0.0 |
| 40.0 | 41.72 | 5.2 | 4.3 |

cant deviation was found from the nominal values. At least three freeze–thaw cycles can be tolerated without losses higher than 7%. The values are given in Tables 2 and 3.

### 3.10. Animal studies

Plasma corticosterone levels in rats can be provoked by a number of stressors such as immobilization, electrical shock, etc. [6,16,17]. We observed a 22-fold increase (range 10 to 35) in the plasma corticosterone levels (within 15 min) of rats (Fig. 5) that were witness to the sacrifice of the earlier rats. The increase was greater in those killed late in a series. This can be attributed to the fear of death. In our study, basal morning (10:00–12:00) plasma levels of corticosterone in unstressed rats ranged from 4 to 12 ng/ml, and were much lower than the ranges reported in the literature [6,16,17]. This is probably because of increased specificity of the assay due to selectivity provided by the use of the mass detector in the SIM mode as well as good laboratory practice.

In plasma samples of unstressed rats (Fig. 3), a minor peak eluted ahead of corticosterone, and had an average area of 5–10% that of corticosterone. Further studies indicated that it had the same molecular mass (346) and similar UV (DAD) as that of corticosterone. However in the case of stressed rats its area ratio to that of corticosterone was much higher (even up to ~40%). It was subsequently identified as 18-hydroxy-11-deoxycorticosterone (18-hydroxy-DOC, III) by comparison of its retention time (using a slower gradient system of 2% methanol/min), UV (DAD) and mass spectra obtained

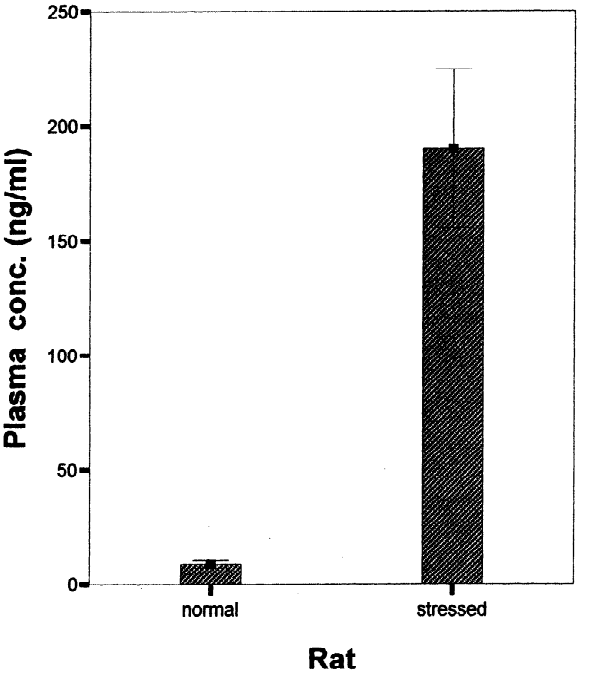


Fig. 5. Plasma corticosterone (ng/ml) in normal ($n$=6) and stressed ($n$=9) rats. Data in each group represents the mean±S.E.

Table 2
Stability studies on corticosterone in rat plasma ($n$=2)

| Concentration (ng/100 μl) | Room temperature 4 h (% difference) | −20°C for 2 months (% difference) |
|---|---|---|
| 0.2 | 0.18 (−7.7) | 0.19 (−3.5) |
| 1.0 | 0.99 (0.5) | 0.93 (−7.2) |
| 10.0 | 87.53 (12.5) | 10.76 (7.6) |

Table 3
Freeze–thaw stability for corticosterone in rat plasma ($n$=2)

| Concentration (ng/100 μl) | Freeze–thaw | | |
|---|---|---|---|
| | Cycle 0 | Cycle 3 | % Difference (3 and 0 cycle) |
| 0.2 | 0.1735 | 0.1753 | 1.0 |
| 1.0 | 1.0729 | 1.0020 | −6.6 |
| 10.0 | 8.8187 | 8.9580 | 1.6 |

under scan mode with an authentic sample purchased from Steraloids. 18-Hydroxy-DOC (III) is a major component of rat adrenocortical secretion, and significant differences in the 18-hydroxy-DOC:corticosterone ratios between normotensive and hypertensive strains of rats have been reported [4].

## 4. Conclusions

The described LC–MS method is very sensitive, highly specific, reproducible, accurate, and requires only small amounts of matrix (100 μl), which can be further reduced in the case of stressed rats or by increasing injection volume. The short run time coupled with quick equilibration of column permitted us to analyze rat plasma for corticosterone on demand. Moreover, the method can be used to estimate 18-hydroxydeoxycorticosterone in rat plasma as well. This method combines the universality of HPLC separation with the sensitivity, specificity and selectivity of mass spectrometry detection in the SIM mode.

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