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Determination of corticosterone in rat and mouse plasma by gas chromatography-selected ion monitoring mass spectrometry

Pin-Yen Shu[a], Shiu-Huey Chou[b], Cheng-Huang Lin[a,*]

[a]*Department of Chemistry, National Taiwan Normal University, 88 Sec. 4, Tingchow Road, Taipei, Taiwan*
[b]*Department of Life Science, Fu-Jen University, 510 Chung Cheng Rd., Hsinchuang, Taipei Hsien 24205, Taiwan*

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Abstract

A simple, highly sensitive and specific method based on gas-chromatography-selected ion monitoring (SIM) mass spectrometry has been developed for the quantitation of corticosterone in rat and mouse plasma. After extraction of the plasma with ethyl acetate, the residue was trimethy-silylated with pentafluorobenzyl hydroxylamine-trimethylsilyl (PFBO-TMS). Detection of the derivatives was accomplished by a quadruple mass spectrometer in the selected ion monitoring mode (m/z of 316, 648, 663 and 678). The detection limit of the assay was 0.1 pg on column. The results show that in the plasma of non-stressed animals, only minor amounts of corticosterone were found; whereas in the plasma of stressed animals, it was dramatically increased. The method developed here can be used to examine corticosterone levels as a marker of stress in rats and mice and may also be used for estimation of the effect of stress-release medications.

Keywords: Corticosterone

1. Introduction

In many physiological experiments, the change in hormone concentration can be regarded as an indicator of experimental treatment, for example, the production of glucocorticoid or epinephrine in plasma is increased in stressed animals [1,2]. Hence, the technology to quantify the level of hormones in samples during the experimental treatment became important. Glucocorticoids are adrenal steroid hormones which are made in the adrenal cortex. Stress will increase corticotropin-releasing factor (CRF) release and thus raises glucocorticoid level [3]. Corticosterone is a glucocorticoid that exists in rodents. Most previous studies have employed radioimmunoassay (RIA) to measure the concentration of corticosterone [4–7]. However, some RIA assays show poor precision. Furthermore, because of the radiation hazards associated with RIA, alternate methods have been developed for the determination of corticosterone, mainly in human and rat plasma. These methods include fluorimetry [8–11] and liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) [12–14]. Although these methods yield interesting results, the fluorimetric method requires a complex sample labeling procedure and only a few LC–ESI-MS methods are sufficiently sensitive and rapid for use in the de-

*Corresponding author. Tel.: +886-2-8931-6955; fax: +886-2-2932-4249.
E-mail address: chenglin@cc.ntnu.edu.tw (C.-H. Lin).

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termination of corticosterone in small amounts of plasma. Gas chromatography/negative ion chemical ionization mass spectrometry (GC-NIC-MS) has also been used to determine endogenous steroids in bovine samples [15–17]. This method can be used to detect endogenous steroids, but the detection limit for corticosterone is quite high (850 pg) [15]. The pentafluorobenzyl hydroxylamine (PFBH) reagent has been used previously as a derivatizing agent in the analysis of a number of ketosteroids by GC with electron-capture detection (GC/ECD), and has also found application in the analysis of low-molecular-mass ketones by GC/ECD. The use of pentafluorobenzyl hydroxylamine-trimethylsilyl (PFBO/TMS) derivatives in the analysis of corticosteroids by GC/NICI mass spectrometry was originally reported in 1989 [16]. The derivatives have been employed in the quantification of ~400 pg amounts of endogenous hydrocortisone present in samples of human aqueous humor.

In this study, we report a simple and highly sensitive method for the detection of corticosterone in rat and mouse plasma using GC-selected ion monitoring (SIM) mass spectrometry. The method requires only a simple derivatization procedure, using PFBO-TMS as the silylating agent, and was successful in detecting very small amounts of corticosterone in small volumes (less 100 μl) of rat and mouse plasma.

2. Experimental

2.1. Reagents

Corticosterone (95%) and anhydrous sodium sulfate (99.5%) were purchased from Sigma (St Louis, MO, USA). Pentafluorobenzylhydroxylamine hydrochloride (PFBO HCl) was obtained from Aldrich (Dorset, UK). Trimethylsilyl-imidazole (TMS IM, 98%) and ethyl acetate were purchased from Fluka (Derbyshire, UK) and Santoku (Japan), respectively.

2.2. Apparatus

A gas chromatograph (GC 5890 Hewlett-Packard, Avondale, PA, USA) equipped with a mass spectrometer (Hewlett-Packard 5973 mass selective detector) was used. A capillary column (30 m×0.25 μm I.D.) with an HP-5MS (cross-linked 5% PH ME siloxane) bonded stationary phase film 0.25 μm thickness (Agilent Technologies, USA) was used. The inlet temperature was maintained at 200 °C. The column oven was held at 190 °C for 1 min, then programmed from 190 to 300 °C at 10 °C/min and, finally, held for 20 min. Helium at a constant flow-rate of 1 ml/min was used as the carrier gas. Data were collected using the Hewlett-Packard Chem-Station software. The mass spectrometry conditions were as follows: SIM mode; ionization energy, 12 eV; ion source temperature, 230 °C. The mass selective detector was operated in the SIM mode at a scan rate of 1.25 scans per s.

2.3. Mice and rats

Inbred male mice of the BALB/c strain (10–12 weeks) or rats of the Lewis strain (220–250 g) were purchased from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Upon arrival, the mice were caged in a colony room where a 12-h light–dark cycle was maintained through artificial illumination. The rats were maintained in a 12-h reversed light–dark cycle. They received free access to both food and water throughout the experiment except as noted and a 2-week acclimation period prior to experimental manipulation. Additionally, animals were physically handled on a bidaily basis to prevent the hyperactivity that occurs when they are left untouched for a prolonged period. All animal manipulation was followed according to the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996).

2.4. 2-Deoxy-D-glucose (2-DG)-induced stress experiment

In current stress experiments, 2-deoxy-D-glucose (2-DG) was used as stressor. 2-DG is a non-metabolizable structural analog of glucose [18]. The difference between 2-DG and glucose is a substitution of hydrogen (–H) on the second carbon rather than hydroxyion (–OH). 2-DG stimulates as central neuroglucopenia and a peripheral hyperglycemia. Sole and Crane (1954) demonstrated that the glucose configuration at carbons 3–5 was essential for the

inhibitory properties of 2-DG [19]. Hence, 2-DG produces intracellular hypoglycemia by: (1) acting as a competitive inhibitor of the hexokinase and phosphor hexoisomerase enzymes in the cell membrane, which is essential for the transport of glucose into the cell, and (2) inhibiting glycolysis. When 2-DG enters the cell, it is converted to the phosphate ester.

Mice were assigned to one control (non-stress) and one experimental group (stress). Injections were carried out 2 h after the lights went on. For each injection, a total inoculum of 0.28 ml/mouse was administered intraperitoneally. Control rats received an injection of sterile phosphate buffered saline solution (PBS). The experimental groups of animals received an injection composed of 2-DG (500 mg/kg body wt).

Rats were assigned to one control (non-stress) or one of two experimental groups (stress). Each control or experimental animal was injected a total of five times, and the subjects were manipulated so that groups of rats differed only in the content of the inoculum. Injections were performed at 48-h intervals, 2 h after the lights went off (reverse light cycle). For each injection, a total inoculum of 0.28 ml/rat was administered subcutaneously. Control rats received a total of five injections of sterile phosphate buffered saline solution (PBS). The experimental groups of animals received a total of five injections composed of either four PBS followed by one 2-DG (acute stress) or five (chronic stress) exposures to 2-DG (500 mg/kg body wt.). Thus, two groups of rats received 2-DG on the final test day.

For 1 h following each injection of 2-DG or PBS, the animals were deprived of food and water. One hour following the last injection, each subject was rapidly sacrificed by cervical dislocation.

2.5. Blood plasma

2.5.1. Mice

Under ether anesthesia, the animal was placed on its back. Whole blood (1 ml) was collected from each animal's heart by means of a heart puncture through a 22-gauge needle into a syringe that contained 100 U/ml heparin. Blood was transferred into 1.5-ml microcentrifuge tubes and plasma was collected after centrifugation at 12 000 rpm for 5 min. Plasma samples were stored at −70 °C until used for assay.

2.5.2. Rats

After cervical dislocation, the animal was placed on its back and a midabdominal incision was made to expose the abdominal aorta. Blood (5 ml) was collected from all animals into heparinized syringes through 21-gauge needles. A 1-ml aliquot was then placed in a microcentrifuge tube to separate plasma for use in the assay. The plasma preparation was the same as used for the mouse plasma preparation.

2.6. PFBO-TMS derivatives of standard

The procedure for the derivatization of corticosterone is shown in Fig. 1. Corticosterone (0.1 ml) was treated with PFBO–HCl (100 mg/ml; in dry pyridine; 60 μl) at 60 °C for 15 min. TMS (80 μl) was then added and heated at 60 °C for 6 h. The mixture was then kept in the dark at room temperature for 48 h to complete the reaction. The reaction mixture was then diluted with ethyl acetate (100 μl) and anhydrous sodium sulfate (10 mg) to remove moisture, and was then centrifuged (5000 rpm for 2 min) to remove the precipitate. The supernatant was passed through a filter (0.45 μm) and 1 μl was then injected into the GC–MS.

Plasma samples (500 μl) were centrifuged (18 000 rpm for 20 min at 4 °C) to remove the proteins. The upper layer (100 μl) was removed and ethyl acetate (100 μl) was added, followed by shaking for 5 min (1200 times/min). After shaking, the samples were frozen at −20 °C for ~30 min. When the aqueous layer was frozen, the organic layer (100 μl), con-

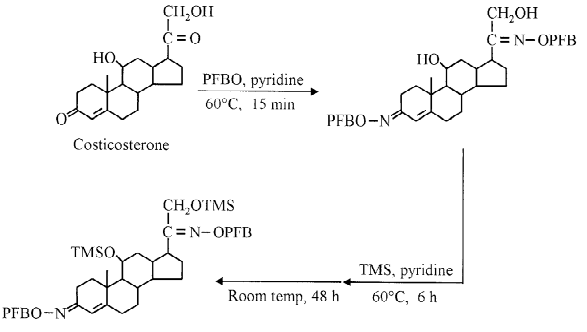

Fig. 1. Derivatization of the corticosterone standard.

taining corticosterone, was removed for derivatization using the same procedure as described above.

3. Results and discussion

3.1. GC-SIM analysis for corticosterone

The GC-SIM chromatogram of the derivatized

Table 1
Recovery and precision for corticosterone (ng/ml) spiked to the plasma of a control mouse (non-stressed)

Spiked	Recovery (mean±SD)	Precision (RSD, %)
10	24.1±1.89	7.8
100	36.8±4.71	12.8
1000	36.4±4.83	13.2

$n=3$.

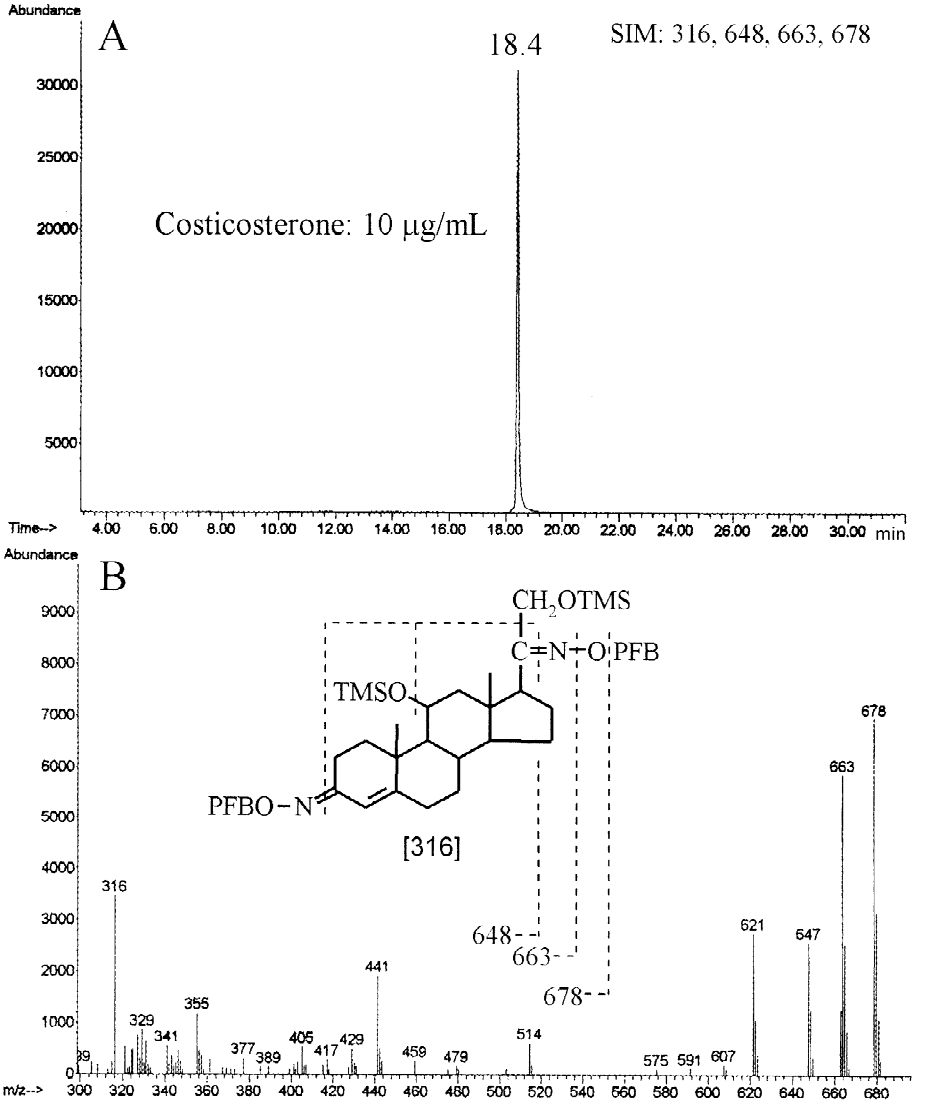

Fig. 2. (A) SIM (m/z of 316, 648, 663 and 678) chromatogram of the corticosterone derivative. (B) The mass fragmentation spectrum of the peak with a retention time of 18.4 min. The concentration of corticosterone standard was 10 μg/ml.

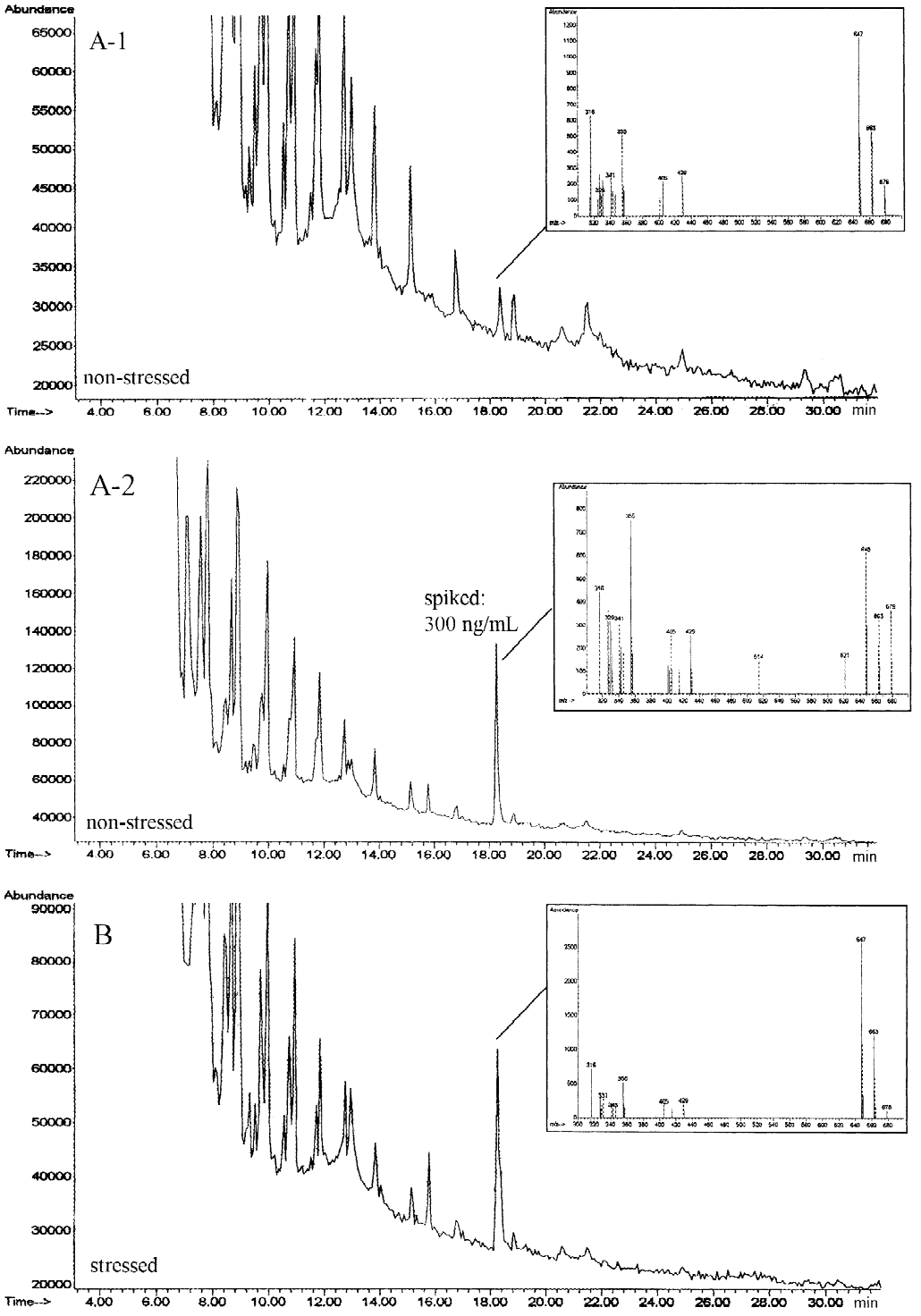

Fig. 3. Total ion chromatograms of mouse plasma extracts. (A-1) Non-stressed mouse; (A-2) non-stressed mouse spiked with 300 ng/ml of corticosterone standard; (B) stressed mouse. The insets correspond to the mass fragmentation spectrum of the peak at 18.4 min.

corticosterone standard is shown in Fig. 2A as analyzed by GC–MS in the electron impact (EI) mode. The detected peak, with a retention time of 18.4 min permitted the specific characterization of corticosterone which represents a potentially useful procedure for the direct identification of corticosterone. The presence of specific fragmentations, such as m/z of 316, 648, 663 and 678, etc., as shown in Fig. 2(B) permitted this characterization. The inset shows the possible fragmentation of the derivation of corticosterone. It is obvious that the GC-SIM method simplifies the chromatogram very efficiently and provides a single peak for identification. Herein, the concentration of corticosterone was 10 μg/ml. The selected ion current profile for the corticosterone derivative has a linear range over concentration ranges 10 μg/ml–10 ng/ml ($y=0.05024x+5.52253$; $r^2=0.9988$). A linear relationship between peak area and concentration exists and is very useful for the quantitation of corticosterone in the plasma samples. The detection limit was ~0.1 ng/ml (~0.1 pg/1 μl-injection). In general, plasma levels (basal

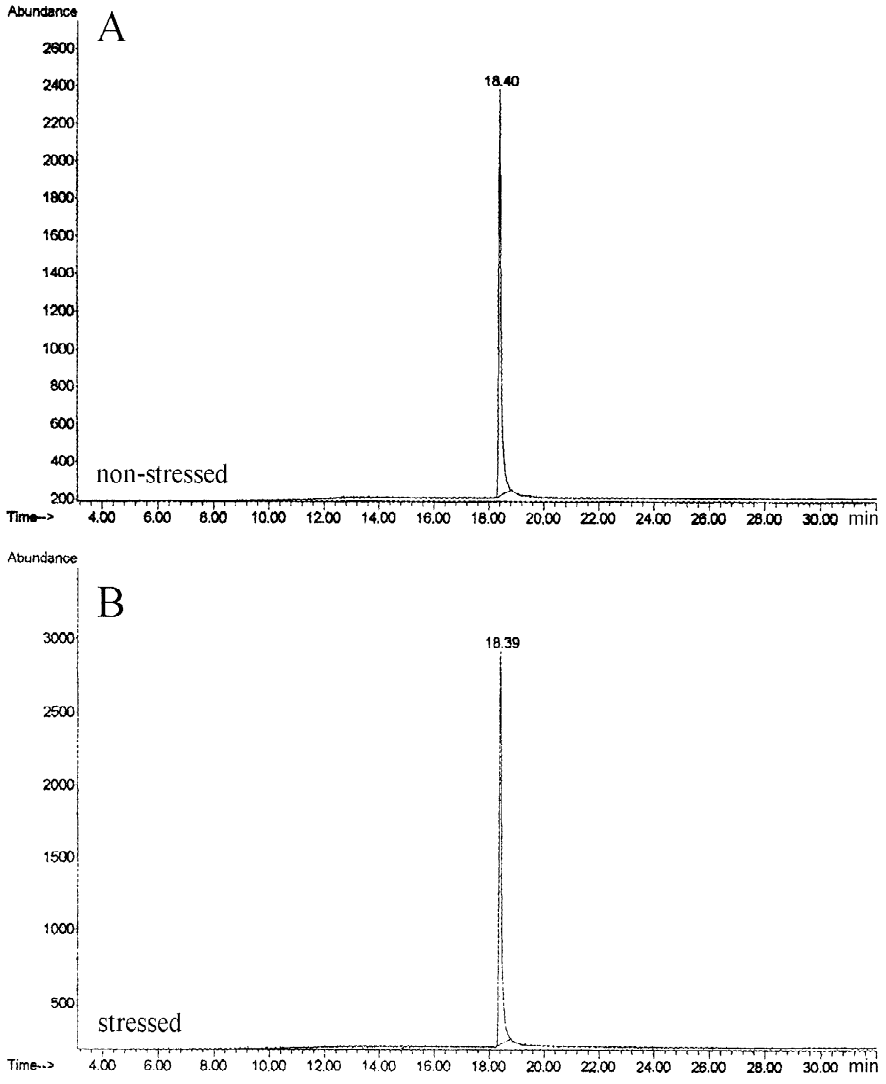

Fig. 4. SIM (m/z of 316, 648, 663 and 678) chromatogram of mouse plasma extracts). (A) Non-stressed mouse; (B) stressed mouse.

morning) of corticosterone in unstressed rats ranged from 4 to 12 ng/ml [12], and were much lower than values found in previous reports [20,21]. Obviously, our method provides sufficient accuracy and sensitivity for the detection of corticosterone. However, for an accuracy quantitation, it is necessary to estimate the recovery under different situations. Table 1 shows a summary of the recoveries for samples spiked with different concentrations of corticosterone (10, 100 and 1000 ng/ml, $n=3$) from a normal mouse (blank experiment). The recoveries were in the range of 24 –36% for various concentrations of corticosterones. For a higher spiked concentration, the recoveries were ~36%; whereas with lower spiked concentration, the recovery was poor. Corticosterone did not undergo degradation when stored in plasma at −20 °C for periods of up to 2 months. However, it degraded very rapidly at room temperature (100 ng/ml, 87% difference in 4 h) [16]. Because of the poor stability of corticosterones at room temperature, the liquid–liquid extraction process was kept at room temperature only for 5 min during the ethyl acetate extraction (shaking for 5 min; 1200 times/min). This is the reason for the low recovery.

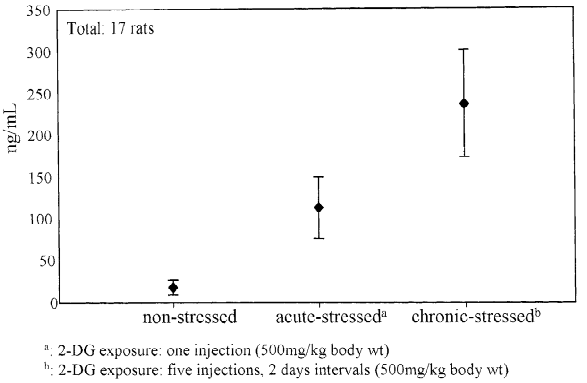

Fig. 5. Different concentration ranges of corticosterone from non-, chronic- and acute stressed rats.

3.2. Analysis of actual samples

Fig. 3A-1 shows the total ion current (TIC) spectrum for a plasma sample from a non-stressed mouse, after the extraction and derivatization described above. A minor peak was detected at the same retention time (18.4 min) as the standard. The inset shows the mass spectrum of this peak. Corticosterone could be characterized on the basis of

Table 2
Amounts (ng/ml) of corticosterone detected in 17 different plasma samples

Rats	Non-stressed	Acute stressed	Chronic stressed
1	3.4±0.5		
2	16.1±2.5		
3	16.7±3.1		
4	9.1±1.3		
5	21.7±5.4		
6	38.9±6.7		
7		68.2±3.4	
8		110.0±16.8	
9		108.9±14.4	
10		70.7±11.5	
11		202.1±26.9	
12			117.6±6.4
13			258.5±28.5
14			229.4±22.2
15			297.2±20.4
16			288.3±37.1
17			228.6±29.1
Average	17.6±9.56	111.9±36.8	236.6±63.1

Table 3
Analysis of corticosterone in different biosamples and detection methods used

Specimen	Corticosterone		Method	LOD (ng/ml)	Ref.
	Non-stressed	Stressed			
Mouse blood	~20	112–237	GC–MS	0.1	In this study
Mouse blood	52	214	CE/UV-sweeping-MEKC	5	Unpublished data
Mouse blood	8	176	LC–ESI-MS	0.3	[12]
Human urine	–	–	HPLC/ESI-MS	178	[13]
Human blood	–	–	GC/NICI/MS	425	[15]
Rat plasma	–	–	HPLC–UV	96	[22]
Rat plasma	56	100	RIA	2	[23]
Human urine	–	–	HPLC/UV	37	[24]
Rat urine	3–18	–	HPLC/SPE/UV	3.8	[25]
Mouse blood	35	261	HPLC–UV	–	[26]

fragment peaks at m/z 316, 648, 663 and 678. As further proof of this peak, the same sample of mouse plasma was spiked with 300 ng/ml of corticosterone standard, and the results show that the intensity of this peak was increased, as shown in Fig. 3A-2. Due to the same retention times and these specific characterizations, this peak is assigned to corticosterone. Fig. 3B shows the TIC spectrum of plasma from a stressed mouse. A major peak was detected at 18.4 min and the inset shows the mass spectrum of this peak. The chromatographic separation of corticosterone in the mouse plasma extracts was achieved as a single peak when the SIM profile was selected, as shown in Fig. 4 (A, non-stressed mouse; B, stressed mouse). Based on the calibration curve and recoveries, the concentrations of corticosterone in the non-stressed and stressed mouse were 6.5 and 205 ng/ml, respectively. Using the same procedure, we examined 17 animals, both stressed and non-stressed (mice and rats). The results are shown in Table 2. Fig. 5 shows the different concentration ranges of corticosterone from non-, chronic- and acute stressed animals in this study. The chronic stressed animals show a higher concentration (236.6 ± 63.1 ng/ml) and acute stressed animals show intermediate concentration (111.9 ± 36.8 ng/ml). The normal animals show a very low concentration of corticosterone (17.6 ± 5.9 ng/ml). Thus, we conclude that the GC–MS-SIM method was successful in detecting very small amounts of corticosterone in the plasma samples from stressed and non-stressed animals (rats and mice), after a simple derivatization procedure using PFBO-TMS as the silylating agent. Table 3 summarizes some analytical data relative to corticosterone in different biosamples and their detection methods. This represents the successful detection of corticosterone in blood by GC-SIM-MS. This is a very useful and important finding and should be of interest to investigators who are interested in studying the amount of stress in experimental animals as this relates to an adequate medicine for releasing the stress.

4. Conclusions

A simple, highly sensitive and specific method for the qualitative and quantitative analysis of corticosterone in rat and mouse plasma by gas chromatography-selected ion monitoring (SIM) mass spectrometry is described. Corticosterone in the rat and mouse plasma extracts appeared as a single peak by chromatographic separation when four prominent fragmentation peaks (m/z of 316, 648, 663, 678) were selected as markers. The detection limit of corticosterone was ~0.1 ng/ml. Furthermore, this method requires only small amounts of plasma (100 μl) and can be used as a routine tool for pharmacological study based on different types of drugs, whether derived from natural herbs or by synthesis.

Acknowledgements

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