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Short communication

Determination of corticosterone and 17-hydroxycorticosterone in plasma and urine samples by sweeping techniques using micellar electrokinetic chromatography

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Abstract

The analysis of corticosterone in mouse blood serum (metabolic-stress experiment) and 17-hydroxycorticosterone in human urine (exercise-stress experiment) samples by means of capillary electrophoresis/UV absorbance in conjunction with online sample concentration techniques is described. The use of normal MEKC had an analyte detection limit of 7 μg/ml (S/N = 3); whereas when online sample concentration methods, including sweeping-micellar electrokinetic chromatography (Sweeping-MEKC) and cation-selective exhaustive injection-sweep-micellar electrokinetic chromatography (CSEI-sweep-MEKC) were used, the detection limits could be improved to 3 and 5 ng/ml, respectively. In the analysis of actual samples from animal metabolic-stress experiments (39 mouse), chronically stressed animals showed a higher level (552 ± 152 ng/ml) and acute stressed animals showed an intermediate level (375 ± 105 ng/ml). In comparison, normal animals show a lower concentration level of corticosterone (153 ± 109 ng/ml). In addition, based on a human exercise-stress experiment (seven volunteers), the acute stressed humans (after exercise, 800 m of running) show a higher concentration of 17-hydroxycorticosterone (113 ± 55 ng/ml for males; 128 ± 25 for females) and the non-stressed humans (before exercise) show a lower concentration (63 ± 37 ng/ml for male; 60 ± 20 for female), respectively.

Keywords: Corticosterone; 17-Hydroxycorticosterone

1. Introduction

The most popular detection scheme used in capillary electrophoresis (CE) is UV absorption. Despite its popularity, the sensitivity of this is generally poor (10^{-5} to 10^{-6} M). Efforts have been made to increase the sensitivity of UV detection in CE including the use of a bubble cell, a Z-bend capillary or multiple reflection in a capillary. However, all of these techniques, when performing optimally, are capable of a achieving at most, a one order of magnitude improvement. Recently, a series of reports on online sample concentration techniques appeared by Terabe and co-workers [1–7], as well as other groups [8–14], concerning the so called "stacking" and "sweeping" technique. Using these techniques, an increase in sensitivity can be obtained. These techniques clearly have the capability of overcoming the drawbacks associated with UV absorption and to open new fields of investigation of molecules that exist in low levels, even in the parts per billion (ppb) range.

Typically, the concentration of steroids in blood or urine is very low and this continues to make their determination an analytical challenge. Hence, technology for quantifying the level of steroids in bio-fluids is an important issue. This is because in many physiological experiments, a change in hormone or steroid concentration can serve as an indicator of experimental treatment, such as the increased production of glucocorticoid or epinephrine in plasma that occurs in stressed animals. Corticosterone is a glucocorticoid that is produced by rodents; whereas 17-hydroxycorticosterone is produced by humans. Most previous studies have employed radioimmunoassay [15,16], fluorimetry [17,18], liquid chromatography-electrospray ionization mass spectrometry [19–21] and gas chromatography/negative ion chemical ionization mass spectrometry [22–25] for the measurement

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of corticosterone levels. Each of these methods has unique advantages and disadvantages with respect to sensitivity, precision and simplicity of use. Petersen et al. reported on the analysis of steroids by the MEKC mode in which the detection of limit of corticosterone was reported to be 500 ng/ml [26]. We previously demonstrated the application of sweeping-MEKC for the detection of corticosterone in mouse blood [27]. In this study, we report on an investigation of the UV absorption method in conjunction with online sample concentration techniques, including sweeping-MEKC (sweeping-micellar electrokinetic chromatography) and CSEI-sweep-MEKC (cation-selective exhaustive injection-sweep-micellar electrokinetic chromatography) for the detection of corticosterones in mouse blood and human urine during different stress-experiments. Several electrophoretic parameters, including buffer pH, SDS concentration, and the injection length required for the separation were optimized and these data are reported herein.

2. Experimental

2.1. Reagents

All chemicals used were of analytical grade. Corticosterone (4-Pregene-11β, 21-diol-3, 20-dione) was obtained from Fluka (Bauch, Switzerland). 17-Hydroxycorticosterone (11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione) and 2-deoxy-D-glucose (2-DG) were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate, methanol, ethyl acetate were purchased from Acros (Belgium). Phosphate acid and sodium hydroxide were purchased from J. T. Baker.

2.2. Apparatus

The CE set-up was fabricated in-house. Briefly, a high-voltage power supply (Model RR30-2R, 0–30 kV, 0–2 mA, reversible, Gamma, FL, USA) was used to drive the electrophoresis and a 50 μm i.d. fused silica capillary column (J&W Scientific, CA, USA) was used for the separation (total/effective length: 102/90 cm in the case of sweeping-MEKC and 80/70 cm in the case of CSEI-sweeep-MEKC, respectively). The sample was hydrodynamiclly injected by raising the reservoir 60 cm relative to the exit reservoir (at this height, the flow rate for the sample injection was 2.16 cm/min) to provide the injection length (depending on the specific situations). A UV detector (CE-971 UV, Jasco, Japan) was used for the determination of the analytes and the wavelength used for the detection was 247 nm. A laboratory-built operational amplifier (OPA) was used for the signal amplification, which contained a single integrated LM1458 circuit chip. The analog signal was converted to a digital signal by an A/D converter (ADAM-4012 module, Advantech Co. Ltd, Taiwan). Electropherograms were collected with a data acquisition system connected to a personal computer.

2.3. Blood and urine samples

2.3.1. Blood

The procedures used in the animal experiments have been described previously [27]. Briefly, stressed-mice received an injection of 2-DG (500 mg/kg body weight) which functions as a stressor because it stimulates central neuroglucopenia and peripheral hyperglycemia. Under ether anesthesia, whole blood (1 ml) was collected from the heart of each animal. The blood was transferred to 1.5 ml microcentrifuge tubes and plasma was collected after centrifugation at 12,000 rpm for 5 min. Plasma samples were stored at −18 °C until used for assay.

2.3.2. Urine

All urine samples were collected from seven adult volunteers. They were allowed to function in a normal manner before exercise, with free access to food and water for collection of the control-urine samples. They were then exercised, by running for 800 m in 5–10 min, after which, experimental urine samples were immediately collected. These samples represent the urine samples discussed below.

2.3.3. Extraction

A 25 μl of blood serum (or urine) was added 60 μl of ethyl acetate followed by shaking the mixture for 30 min. Following this, the mixture was centrifuged for 5 min at 5000 rpm. The upper layers (45 μl) were collected, transferred to clean tubes and then placed in a vacuum chamber to dry. The residues were acidified by the addition of 45 μl of 20 mM H_3PO_4 (pH: 2.1; conductivity: 3.5 mS/cm) for the subsequent CE separation.

3. Results and discussion

3.1. On-line sample concentration

Fig. 1A shows typical CE electropherograms of corticosterone and 17-hydroxycorticosterone, obtained by the normal MEKC method. Herein, in the case of blood serum samples, 17-hydroxycorticosterone was used as an internal standard; in the case of urine samples, corticosterone is treated as internal standard. A complete, optimal separation of the two analytes (both 300 μg/ml) was achieved by using phosphate buffer (30 mM) containing SDS (100 mM) in a methanol-water (1:3, v/v) solution ($pH^* = 2.4$; conductivity: 4.3 mS/cm); applied voltage, −28 kV (currents, −10 to −15 μA). Electropherogram a shows the result obtained using the Jasco-CE/UV detector, in which case, the signal intensity was ∼18 mV. Under exactly the same experimental conditions, a home-built OPA was used and the signal was significantly improved to 4.6 V, as shown in electropherogram b (limit of detection = 7 μg/ml). For a comparison of the efficiency of the laboratory-built OPA, a commercial voltage amplifier (Model: DLPVA-100-F-S, FEMTO

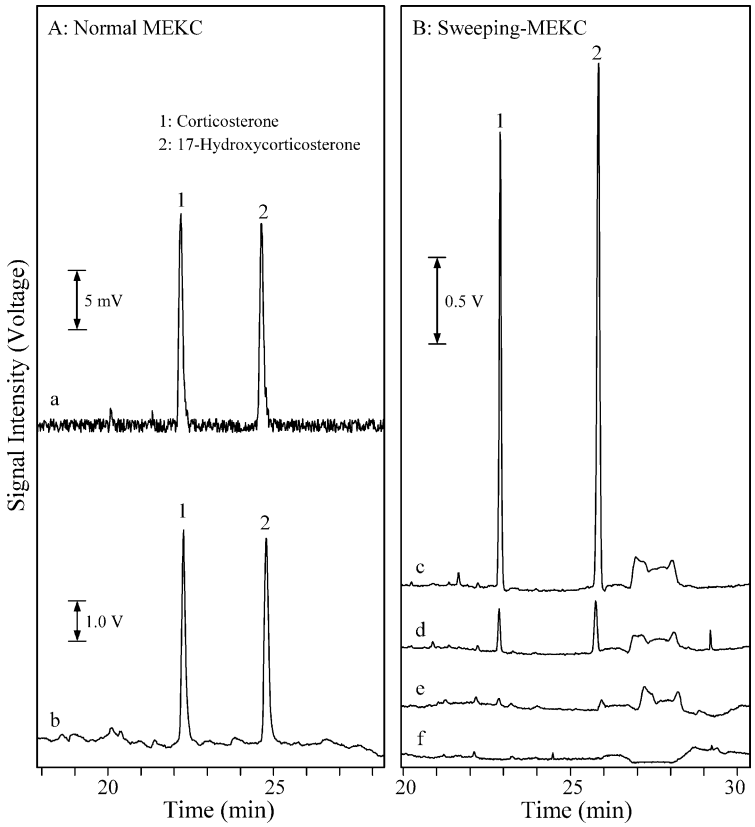


Fig. 1. (A) CE electropherogram of corticosterone and 17-hydroxycorticosterone (300 μg/ml each) obtained by the normal MEKC mode. Electropherograms a and b show the original data obtained from the UV detector and amplified by a OPA, respectively. (B) CE electropherogram of corticosterone and 17-hydroxycorticosterone (electropherograms c–f; 300, 30, 3 and 0 ng/ml) obtained by the sweeping-MEKC mode.

Messtechnik GmbH, Germany) was used and a similar result was obtained. Thus, in spit of the electronic technique used, it was still difficult to improve the limit of detection. However, after applying a sweeping-MEKC method, the detection limits of the two analytes were dramatically improved, as shown in Fig. 1B (electropherograms c–f: 300, 30, 3 and 0 ng/ml, respectively). Herein, the background solution consisted of 100 mM SDS and 30 mM H_3PO_4 in a mixed methanol–water solution (1:3, v/v). The pH of which was 2.4* (conductivity, 4.4 mS/cm). The two analytes were first dissolved in a phosphate buffer (pH = 1.8) resulting in a non-micelle buffer. Hydrodynamic injection was achieved by raising the reservoir 60 cm relative to the exit reservoir for 1440 s. Using this procedure, 52 cm column lengths of solution were injected into the capillary. When the injection was completed, −28 kV was applied to power the CE separation. This procedure permits the SDS-anionic surfactant micelles (in the inlet reservoir) to enter the sample zone. Thus, along the capillary axis, the samples were being swept and concentrated near the junction between the sample solution and the background solution. As in the following step, the samples were separated by the MEKC mode. As a result, a ∼600-fold improvement (electropherogram b, 300 μg/ml: 4.6 V; electropherogram c, 300 ng/ml: 2.8 V) was achieved. In order to investigate the effects of injection length and the corresponding signal intensity (voltage) when the sweeping-MEKC technique was used under exactly the same experimental conditions, various (10.8, 21.6, 32.4, 43.2, 54.0, 64.8 and 75.6 cm) column lengths of the sample solution were injected into the capillary and these results were plotted, as shown in Fig. 2. Basically the signal intensity increased with increasing injection length. The inset shows the relationship between the length of the sample injection and plate numbers; under either injection length, reasonable plate numbers ($(4–12) \times 10^5$) can be obtained. Furthermore, when the injection length was 75.6 cm (∼4/5 of the effective length), a ∼1500-fold improvement was obtained. However, when the injection length was longer, the separation zone of the capillary became shorter. This might pose a problem for the separation of actual samples because of the complicated matrix. For this reason, a 43.2 cm injection length was used in all subsequent experiments.

Another important online sample concentration technique, i.e. the CSEI-sweep-MEKC mode is also available. In this case, the background buffer consisted of only 30 mM H_3PO_4 in a mixed methanol-water solution (1:3, v/v); the sample was resolved in 20 mM of H_3PO_4 aqueous buffer. The capillary was initially filled with the background buffer, followed by a 10 cm length of capillary containing a 100 mM H_3PO_4 solution and, lastly, a plug

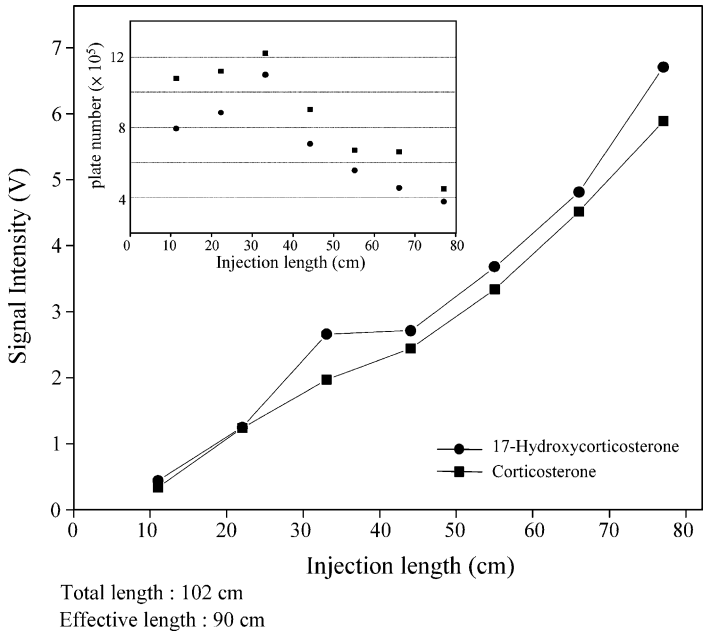


Fig. 2. Relationship between sample injection length and related signal intensity (mV), and plate numbers (inset) in sweeping-MEKC.

of water (∼1.6 mm) was injected. By providing +28 kV for a period of 25 min (electrokinetic injection for cation), the currents increased from ∼10 to ∼20 μA. Meanwhile, the samples are concentrated into the zone of the water. Following this, by quickly shifting the voltage to −28 kV (current, ∼−18 μA), the separation can be completed, as shown in Fig. 3. Herein, the sample concentrations of corticosterone and 17-hydroxycorticosterone were 300 ng/ml. As a result, a detection sensitivity of 5 ng/ml can be obtained. In order to examine the effects of electrokinetic injection, different injection times of 10, 15, 20 and 25 min were tested and the optimal injection time was found to be 25 min. The intensity of the signal increased in a non-linear manner, when the injection length was longer. This is because electrokinetic injection involves a mechanism that is different from hydrodynamic injection; the plate numbers for the CSEI-sweep-MEKC experiments were found to be (2–4) × 10^5. Although CSEI-sweep-MEKC provides better sensitivity in detection for cations, corticosterone and 17-hydroxycorticosterone only weakly positively charged and for this reason the LOD can not be further improved. For this reason, the sweeping-MEKC method was

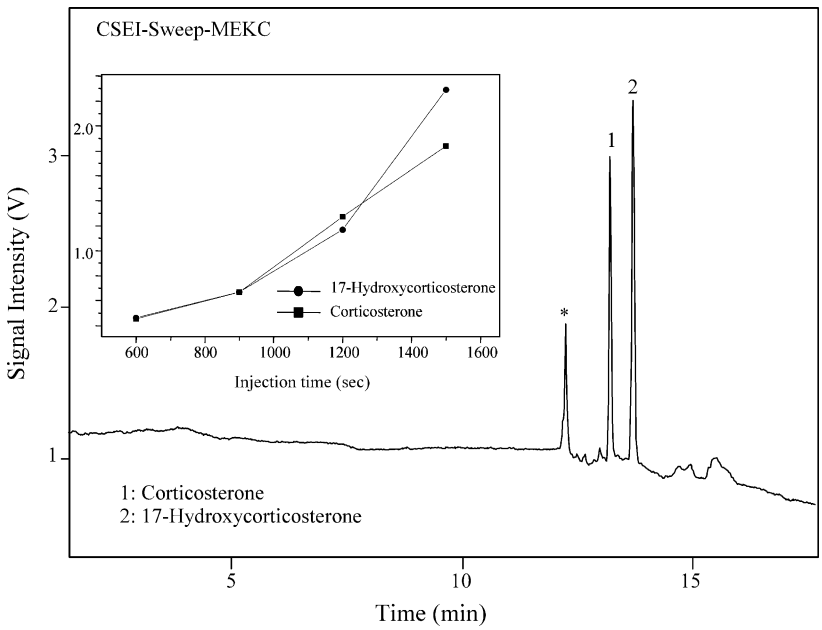


Fig. 3. CE electropherogram of corticosterone and 17-hydroxycorticosterone (300 ng/ml each) by the CSEI-sweep-MEKC mode. The inset shows the relationship between electrokinetic injection times and related signal intensity (mV).

Table 1
Limit of detection (LOD) values and plate numbers for corticosterone and 17-hydrocorticosterone using the sweeping-MEKC/CSEI-sweep-MEKC

	Corticosterone	17-Hydrocorticosterone
A. Normal MEKC		
LOD (S/N = 3)	6 μg/ml (1.7×10^{-5} M)	7 μg/ml (0.9×10^{-5} M)
Plate number	1.8×10^5	1.9×10^5
B. Sweeping-MEKC		
Equation of the line	$y = -0.0024 + 0.009x$	$y = 0.0012 + 0.01x$
Coefficient of variation	0.99995	0.9963
LOD (S/N = 3)	3 ng/ml (8.6×10^{-8} M)	3 ng/ml (8.3×10^{-8} M)
Plate number	6.5×10^5	4.9×10^5
C. CSEI-Sweep-MEKC		
LOD (S/N = 3)	5 ng/ml (1.4×10^{-7} M)	4 ng/ml (1.1×10^{-7} M)
Plate number	3.9×10^5	2.2×10^5

UV absorption at 247 nm.

used exclusively in all subsequent experiments. Using the conditions described in Figs. 1 and 3, the equation for the calibration curves, limit of detection values for signal intensity and plate numbers are summarized in Table 1.

3.2. Analysis of corticosterone in mouse blood

Fig. 4 shows a typical CE electropherogram of an extract of a blood serum sample from non-stressed, acute stressed and chronic stressed animals (electropherograms a–c), respectively, by applying the sweeping-MEKC technique. These extracts were spiked with 300 ng/ml of 17-hydroxycorticosterone as an internal standard prior to extraction. Corticosterones (arrows) appear to the left of the internal standard. We assigned these peaks (indicated by arrows in electropherograms a–c) to corticosterone and their concentrations were determined to be 14, 219 and 386 ng/ml for the non-stressed, acute stressed and chronic stressed mouse, respectively. Using the same procedure, 39 animals were examined and the results are shown in Fig. 5.

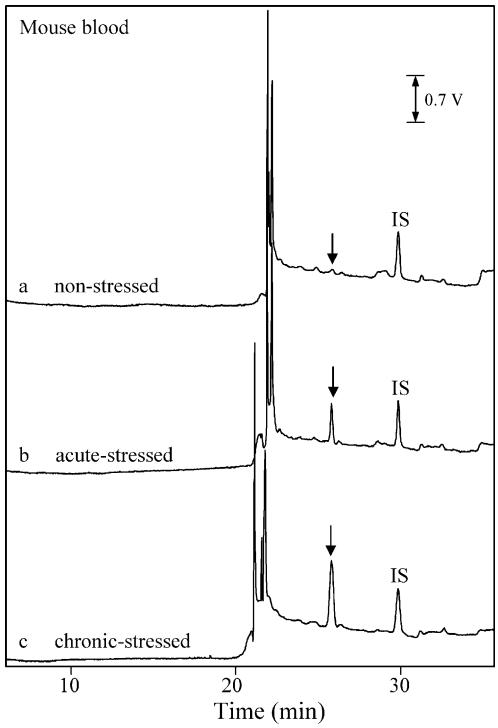
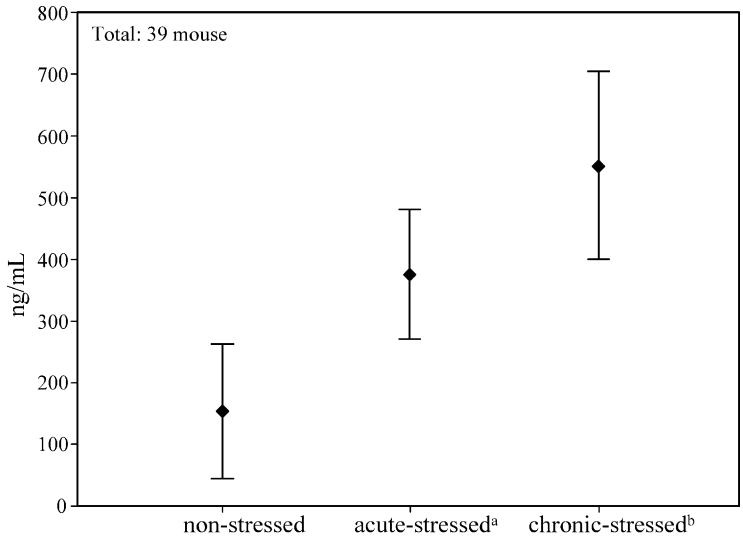


Fig. 4. CE electropherogram of blood serum extracts (electropherograms a–c, non-stressed, acute stressed and chronically stressed mouse), spiked with 300 ng/ml of 17-hydroxycorticosterone as an internal standard.



[a]: 2-DG exposure: one injection (500 mg/kg body wt)
[b]: 2-DG exposure: five injections, 2 days intervals (500 mg/kg body wt)

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

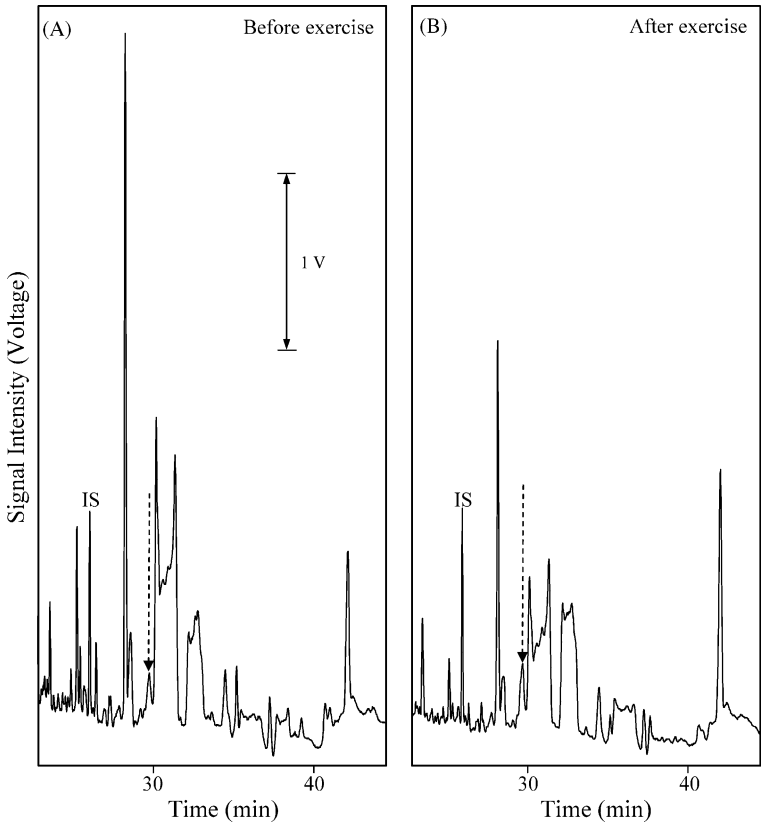


Fig. 6. CE electropherograms of urine sample extracts (frame A, before exercise; frame B, after exercise), spiked with 300 ng/ml of 17-hydroxycorticosterone as an internal standard.

The chronically stressed animals show a higher concentration (552 ± 152 ng/ml) and acute stressed animals an intermediate concentration (375 ± 105 ng/ml). The control (normal) animals show a very low level (153 ± 109 ng/ml).

3.3. Analysis of 17-hydroxycorticosterone in human urine

Fig. 6 shows a typical CE electropherogram of urine sample extracts from non-stressed (before exercise) and acute stressed (after exercise for 800 m running) adult male humans (electropherograms A and B), respectively, by applying the sweeping-MEKC technique. These extracts were spiked with 300 ng/ml corticosterone as an internal standard prior to extraction. 17-Hydroxyorticosterone (arrows) appears to the right of the internal standard. We assigned these peaks (indicated by arrows in electropherograms A and B) to 17-hydroxyorticosterone and their concentrations were determined to be 80 and 104 ng/ml for the non-stressed and

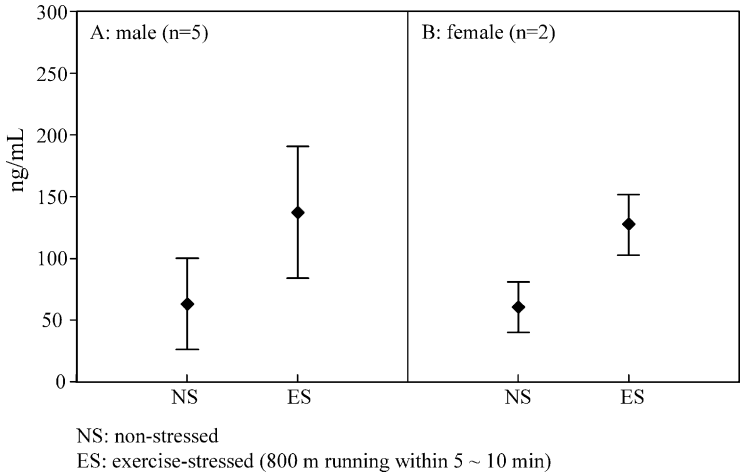


Fig. 7. Different concentration ranges of 17-hydroxycorticosterone from human urine samples.

acutely stressed male human, respectively. Using the same procedures, five adult male and two female volunteers were tested and the results are shown in Fig. 7. The results for the acutely stressed human (after exercise, 800 m running) show a higher concentration of 17-hydroxyorticosterone (137 ± 55 ng/ml for male; 128 ± 25 for female), whereas urine samples form non-stressed volunteers (before exercise) show lower concentrations (63 ± 37 ng/ml for male; 60 ± 20 for female), respectively. Thus, we conclude that the sweeping-MEKC is a simple, highly sensitive and specific method for the qualitative and quantitative analysis of corticosterone in plasma and urine samples and it should be of interest to investigators who are interested in studying stress in experimental animals or humans as this relates to therapeutic strategies for releasing stress.

4. Conclusions

We demonstrate here that capillary electrophoresis/UV absorbance after applying the sweeping-MEKC technique can be successfully used for the online sample concentration and separation of corticosterones in mouse blood serum and human urine samples. For the analysis of 39 mouse serum and 7 human urine samples, the concentration of corticosterones within the non-stressed, chronic stressed and acute stressed animals shows a reasonable tendency. Furthermore, this method requires only small amounts (25 μl) of plasma and could be used as a routine tool in pharmacological studies dealing with different types of drugs, whether derived from natural herbs or by synthesis.

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