



## Determination of corticosterone in mouse plasma by a sweeping technique using micellar electrokinetic chromatography

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### Abstract

The separation and on-line concentration of corticosterone in mouse blood was achieved by means of capillary electrophoresis/UV absorbance using sodium dodecyl sulfate (SDS) as a surfactant. The procedure involved the use of an on-line sample concentration method by sweeping-micellar electrokinetic chromatography (sweeping-MEKC). Optimal on-line concentration and separation conditions were determined. The detection limit for this method was 5 ng/ml ( $S/N=3$ ) and photodiode array detection at 247 nm was used for identification. For the analysis of actual samples, corticosterones from blood samples of a non-stressed and stressed mouse were determined. The results show that only a minor amount of corticosterone was produced by a non-stressed mouse, whereas a significant amount was present in the blood sample from a stressed mouse. The method developed here can be used to examine corticosterone levels as a marker of stress in test animals and may also be used for estimating the effect of stress-release medications.

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### 1. Introduction

In many physiological experiments, a change in hormone concentration can be regarded as an indicator of experimental treatment, such as the increased production of glucocorticoid or epinephrine in plasma in stressed animals [1,2]. A technique for quantifying the level of hormones in samples during these types of experimental treatment would be highly desirable. Glucocorticoids are adrenal steroid

hormones which are synthesized in the adrenal cortex. Stress increases the release of corticotropin-releasing factor (CRF) thus raising glucocorticoid levels [3]. Corticosterone is a glucocorticoid that is produced by rodents. Most previous studies have employed radioimmunoassay [4–7], fluorimetry [8–11], or liquid chromatography–electrospray ionization mass spectrometry [12–14] to measure corticosterone levels. Gas chromatography/negative ion chemical ionization mass spectrometry has also been used to determine endogenous steroids in bovine samples [15–17]. Mohammad et al. reported on the analysis of cortisol by solid-phase extraction (SPE)-CE in which detection of limit of free cortisol was 10

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$\mu\text{g/l}$  [18]. Each method has unique advantages and disadvantages with respect to sensitivity, precision and simplicity of use. However, in routine analysis, the detection of corticosterone continues to be difficult because of the extremely low concentration present (ppb level).

CE has become an accepted technique and is a very useful method in many fields because of its advantages in terms of speed, higher efficiency and resolution for separation, and the need for a smaller injection volume than is typically used for HPLC or GC. However, the small injection volume is sometimes a major drawback because of the poorer *S/N* ratio. To improve the limit of detection, laser-induced fluorescence is the most sensitive detection method. However, only a few molecules of interest fluoresce at available laser lines and derivatization steps are frequently required. In general, the most popular detection scheme used in CE is UV/Vis absorption. However, in this method, even for analytes with a molar absorptivity of  $\sim 10\,000\text{ l mol}^{-1}\text{ cm}^{-1}$ , the detection limits are typically  $\sim 5 \times 10^{-6}\text{ M}$ . Thus far, attempts have been made to increase the sensitivity of CE/UV detection, such as the use of a bubble cell, a Z-bend capillary or multiple reflection in a capillary. Recently, a series of reports appeared by Terabe et al., as well as other groups, concerning the so-called “sweeping” technique for on-line sample concentration [19–30]. Using this technique, an increase in sensitivity of a million-fold can be obtained by cation-selective exhaustive injection and sweeping [21]; an anion-selective exhaustive injection-sweep-MEKC provides 1000- to 6000-fold increases in the case of some aromatic carboxyl acids [22]. For neutral analytes, both sweeping or stacking techniques have also been proposed [23,24]. The concentration of corticosterone is very low in actual samples and its detection in body fluids poses a problem, especially when the UV absorption method is used. Therefore, a technique involving on-line concentration becomes increasingly important. In this study, we report on a simple and highly sensitive method, using sweeping-MEKC, for the detection of corticosterone in mouse blood. Several electrophoretic parameters such as buffer pH, SDS concentration, and the amount of organic solvent required for the separation were optimized.

## 2. Experimental

### 2.1. Reagents

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

### 2.2. Apparatus

A Hewlett-Packard  $^{3\text{D}}$ CE system (Hewlett-Packard, Waldbronm, Germany) with a photodiode array detector was used for the determination of corticosterone in the samples. The wavelength used for the detection was 247 nm. UV spectra (200–300 nm) were collected from every peak for the purpose of identification. A 50- $\mu\text{m}$  I.D. fused-silica capillary column (J&W Scientific, CA, USA) was used for the separation (total length: 70 cm; effective length: 61 cm). The applied voltage used was  $-30\text{ kV}$  and temperature was  $25\text{ }^\circ\text{C}$  in capillary. Before use, the capillary was conditioned with  $0.1\text{ M NaOH}$  for 30 min, with purified water for 30 min, and then with the electrolyte solution for 10 min. The sample injection was carried out hydrodynamically with a pressure of 50 mbar for 0.6–900 s (depending on the specific situation).

### 2.3. Methodology: sweeping-MEKC

The background solution (BGS) consisted of 100 mM SDS and 30 mM  $\text{H}_3\text{PO}_4$  in a mixed methanol–water solution (30:70, v/v). pH\* was 2.15 (conductivity, 5.2 mS/cm). The analytes (corticosterone and 17-hydroxycorticosterone) were dissolved in a phosphate buffer (pH 1.8) resulting in a non-micelle buffer, and adjusted to the same conductivity as the BGS by the addition of  $1.0\text{ M H}_3\text{PO}_4$ . Under these conditions, the electrophoretic flow (EOF) was negligible. Hydrodynamic injection was achieved by a

pressure of 50 mbar. By using this procedure, ~5, 15, 25, 30, 35 and 40 cm column lengths (in times: 100, 300, 500, 600, 700 and 900 s) of solution were injected into the capillary. The total and effective lengths of the capillary were 70 and 61 cm, respectively, in these experiments. When the injection was completed, -30 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 back-

ground solution. As in the following step, the samples were separated by the MEKC mode.

#### 2.4. Blood samples

Inbred male mice of the BALB/c strain (10–12 weeks) were purchased from the 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. They received free access to both food and water throughout the experiment. All animal manipulations followed the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996). Mice were assigned to control (non-stress) and experimental (stress) experiments. Injections were carried out 2 h after the lights were turned on. For each injection, a total inoculum of 0.28 ml/mouse was administered intraperitoneally. The stressed-mouse received an injection composed of 2-DG (500 mg/kg body wt) where 2-DG was used as stressor because it stimulates by causing central neuroglucopenia and peripheral hyperglycemia. Under ether anesthesia, whole blood (1 ml) was collected from the heart of each animal. 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 -18 °C until used for assay.

#### 2.5. Extraction

Corticosterone (300 ng/ml) was added to the blood sample (25  $\mu$ l) from a non-stressed mouse, followed by 50  $\mu$ l of ethyl acetate. Following this, after shaking the mixture for 20 min, it was centrifuged for 3 min at 5000 rpm. The upper layer was collected, transferred to a clean tube and then removed in a vacuum chamber to dry out. The residue was acidified by the addition of 25  $\mu$ l of  $H_3PO_4$  (pH 2.15; conductivity: 4 mS/cm). By subtracting the original corticosterone in the blood sample and then comparing the corticosterone standard (300 ng/ml), the efficiency of this liquid–liquid extraction was determined to be ~58%.

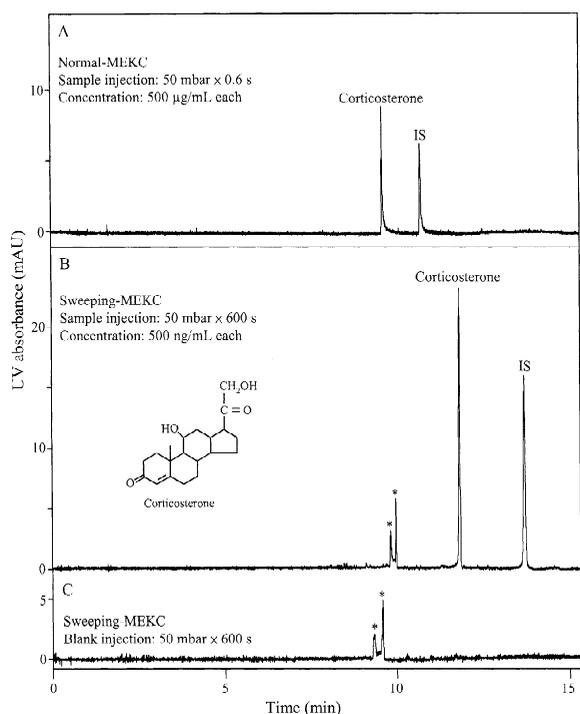


Fig. 1. (A) CE electropherogram of corticosterone and 17-hydroxycorticosterone (internal standard) by the normal MEKC mode. The concentrations of the analytes were 500  $\mu$ g/ml each and a 2.4-nl sample was injected (50 mbar $\times$ 0.6 s). (B) CE electropherogram of corticosterone and 17-hydroxycorticosterone (internal standard) by the sweeping-MEKC mode. The concentrations of the analytes were 500 ng/ml each and 2.4  $\mu$ l of sample was injected (50 mbar $\times$ 600 s). CE conditions: phosphate buffer (30 mM), SDS (100 mM) in a methanol–water solution (30:70, v/v).

### 3. Results and discussion

#### 3.1. On-line sample concentration

Fig. 1 shows typical CE electropherograms of corticosterone and 17-hydroxycorticosterone (internal standard) by normal MEKC (frame A) and sweeping-MEKC (frame B). The concentrations of the two analytes are 500  $\mu\text{g/ml}$  and 500  $\text{ng/ml}$  in frames A and B, respectively. In the normal MEKC mode, the sample injection volume was 2.4 nl (50 mbar $\times$ 0.6 s), whereas in the sweeping-MEKC mode, the sample injection volume was 2.4  $\mu\text{l}$  (50 mbar $\times$ 600 s). A complete, optimal separation of the two analytes was achieved using phosphate buffer (30 mM) containing SDS (100 mM) in a methanol–water solution (30:70, v/v). The peaks indicated with asterisks represent system peaks and are typical for sweeping-MEKC. Frame C shows the electropherogram of a blank injection. As a result, the system peaks also occur. In comparison with the two electropherograms in frames A and B, a  $\sim$ 2400-fold

improvement ( $S/N=3$ ) in detection sensitivity could be obtained. Basically the intensity of the signal should increase linearly when the injection length is longer. However, we found that when a large volume was injected, the micelle appeared be able to take up more analyte. As a result, a nonlinear improvement was obtained. Another possibility is that the phenomenon “stacking” occurred, as the sweeping progressed. Furthermore, the ratio of both sample compounds (corticosterone and the I.S.) differs with different injection lengths. Fig. 2 shows the relationship between the length of the sample injection and the corresponding peak areas in sweeping-MEKC. The peak area increases with longer injection times. The inset shows the relationship between the length of the sample injection and plate numbers. The plate numbers decreased when the injection length exceeded 30 cm. Thus,  $\sim$ 30 cm is a reasonable length for sample concentration by sweeping, and the next  $\sim$ 30 cm is necessary for separation in this case (effective length: 61 cm). For this reason, a 30-cm injection length was used in all subsequent experi-

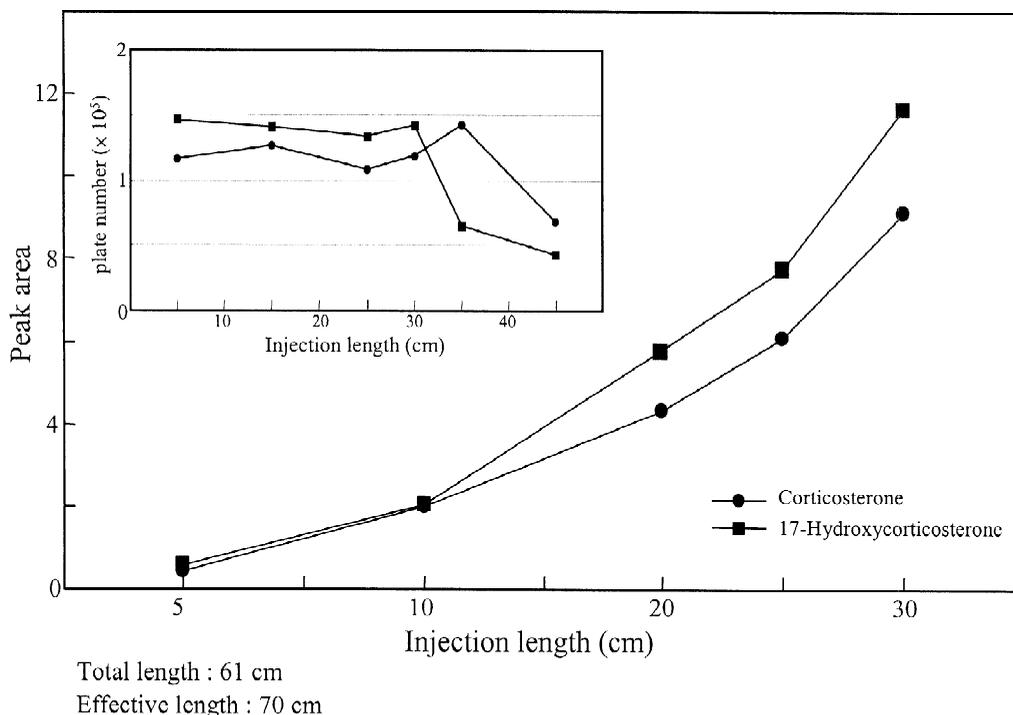


Fig. 2. Relationship between sample injection length (mbar $\times$ s) and related absorbance and plate numbers (inset) in sweeping-MEKC.

ments. Using the conditions described in Fig. 1, limit of detection (LOD) values, relative standard deviations (RSD%) of peak areas and migration times were calculated and these data are summarized in Table 1. The tested concentration ranges were 10–1000 ng/ml. For repeatability (RSDs) tests, the concentration was 500 ng/ml. Fig. 3, frames A and B show the optimized SDS concentrations and ratios of methanol in the aqueous CE buffer, respectively. In frame A, the CE buffers were methanol–water solutions (30:70, v/v), containing 30 mM H<sub>3</sub>PO<sub>4</sub> and different concentrations of SDS (electropherograms a–d: 25, 50, 750 and 100 mM). The sample concentration was 500 ng/ml in each run. Peaks 1 and 2 correspond to corticosterone and internal standard, respectively. As a result, the optimum concentration of SDS for sweeping was 100 mM. Furthermore, it is tricky to avoid a migration time shift during the sweeping experiments. Therefore, the migration-time-corrected peak area was used for quantitative purposes in order to avoid over-estimation of the peak area. In order to investigate the effects of organic solvent, under exactly the same

experimental conditions, the methanol–water solutions (20:80, 25:75, and 30:70 in electropherograms e, f and g) were used on actual plasma samples, extracted from a non-stressed mouse, as shown in frame B (spiked concentration was 500 ng/ml for each). The findings show that the optimum ratio of methanol was 30% and this is very effective in isolating corticosterone from the other compounds because for a non-selective extraction procedure it would be expected that additional compounds would appear in the chromatogram. Thus, the complete, optimal separation of corticosterone and its internal standard from plasma samples could be achieved with phosphate buffer (30 mM) containing SDS (100 mM) in a methanol–water solution (30:70, v/v).

### 3.2. Analysis of actual samples

Fig. 4 shows a typical CE electropherogram of a blood sample extract from the non-stressed (frame A) and stressed mouse (frame B) by applying the sweeping-MEKC technique. These extracts were spiked with 500 ng/ml 17-hydroxycorticosterone as

Table 1  
Limit of detection (LOD) and RSD for corticosterone and 17-hydroxycorticosterone by sweeping-MEKC/normal-MEKC

	Corticosterone	17-Hydroxycorticosterone
<i>A. Sweeping-MEKC</i>		
Equation of the line	$y = 2.21 + 0.06x$	$y = 3.83 + 0.07x$
Coefficient of variation	0.9956	0.9963
LOD ( $S/N=3$ )	5 ng/ml ( $1.4 \times 10^{-8}$ M)	4 ng/ml ( $1.1 \times 10^{-8}$ M)
RSD		
(a) Migration time ( $n=5$ )%		
Intra-day	0.63	0.76
Inter-day	1.93	1.75
(b) Peak area ( $n=5$ )%		
Intra-day	1.71	4.63
Inter-day	3.32	8.96
<i>B. Normal-MEKC</i>		
Equation of the line	$y = 2.88 + 0.17x$	$y = 1.63 + 0.12x$
Coefficient of variation	0.9959	0.9929
LOD ( $S/N=3$ )	12 µg/ml ( $3.5 \times 10^{-5}$ M)	8 µg/ml ( $2.2 \times 10^{-5}$ M)
RSD		
(a) Migration time ( $n=5$ )%		
Intra-day	0.71	0.81
Inter-day	1.41	1.52
(b) Peak area ( $n=5$ )%		
Intra-day	3.18	5.12
Inter-day	4.74	6.84

UV absorption at  $247 \pm 3$  nm.

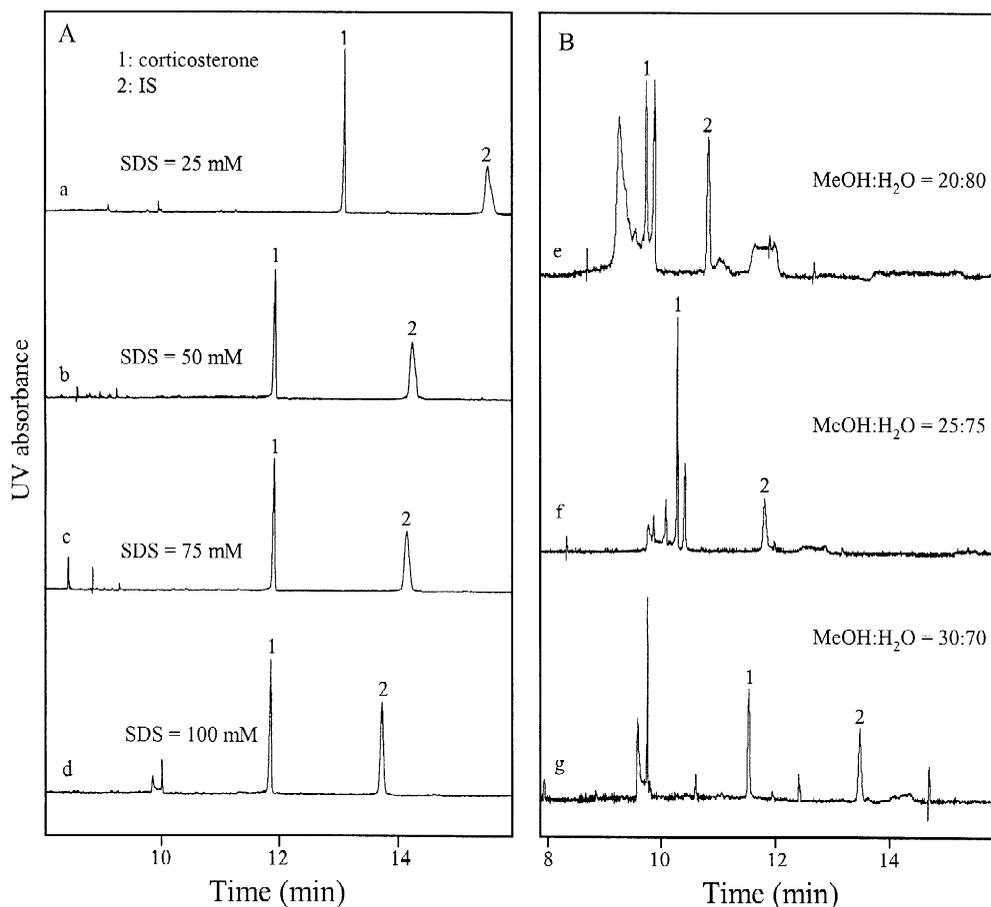


Fig. 3. Effects of different parameters on CE separation. Frame A (sample: standards), conditions: capillary, 71 cm (61 cm to the detector), 50  $\mu$ m I.D.; applied voltage,  $-30$  kV;  $\lambda_{\text{ab}}=247$  nm. Sample concentration: 500 ng/ml for each (peak 1: corticosterone; peak 2: internal standard). Buffers: 30 mM H<sub>3</sub>PO<sub>4</sub> in methanol–water solutions (30:70, v/v); SDS concentrations: 25, 50, 75 and 100 mM (electropherograms a–d). Frame B (sample: plasma extraction), 100 mM SDS and 30 mM H<sub>3</sub>PO<sub>4</sub> in different solutions (methanol–water): 20:80, 25:75, 30:70, v/v (electropherograms e–g).

an internal standard prior to extraction. Corticosterones (arrows) appear to the left of the internal standard. The on-line UV spectra (dashed line: arrow marked peak; solid line: corticosterone) are shown in the insets, and were superimposable with spectra of known standards. We assigned these peaks (marked with arrows in frames A and B) to corticosterone and their concentrations were determined to be 43 and 182 ng/ml for the non-stressed and stressed mouse, respectively. Using the same procedure, we examined 10 animals, both stressed and non-stressed (mice and rats) and the results are shown in Fig. 5. The chronic stressed animals show a higher con-

centration ( $280 \pm 40$  ng/ml) and acute stressed animals show an intermediate concentration ( $200 \pm 39$  ng/ml). Control (normal) animals show a very low concentration of corticosterone ( $88 \pm 34$  ng/ml). Thus, we conclude that the CE/UV-sweeping-MEKC method was successful in detecting trace amounts of corticosterone in the plasma samples from stressed and non-stressed animals (rats and mice). Table 2 summarizes some analytical data for corticosterone in different biosamples and their detection methods. This represents the first successful detection of corticosterone in blood by CE/UV-sweeping-MEKC. This approach should be of interest to investigators

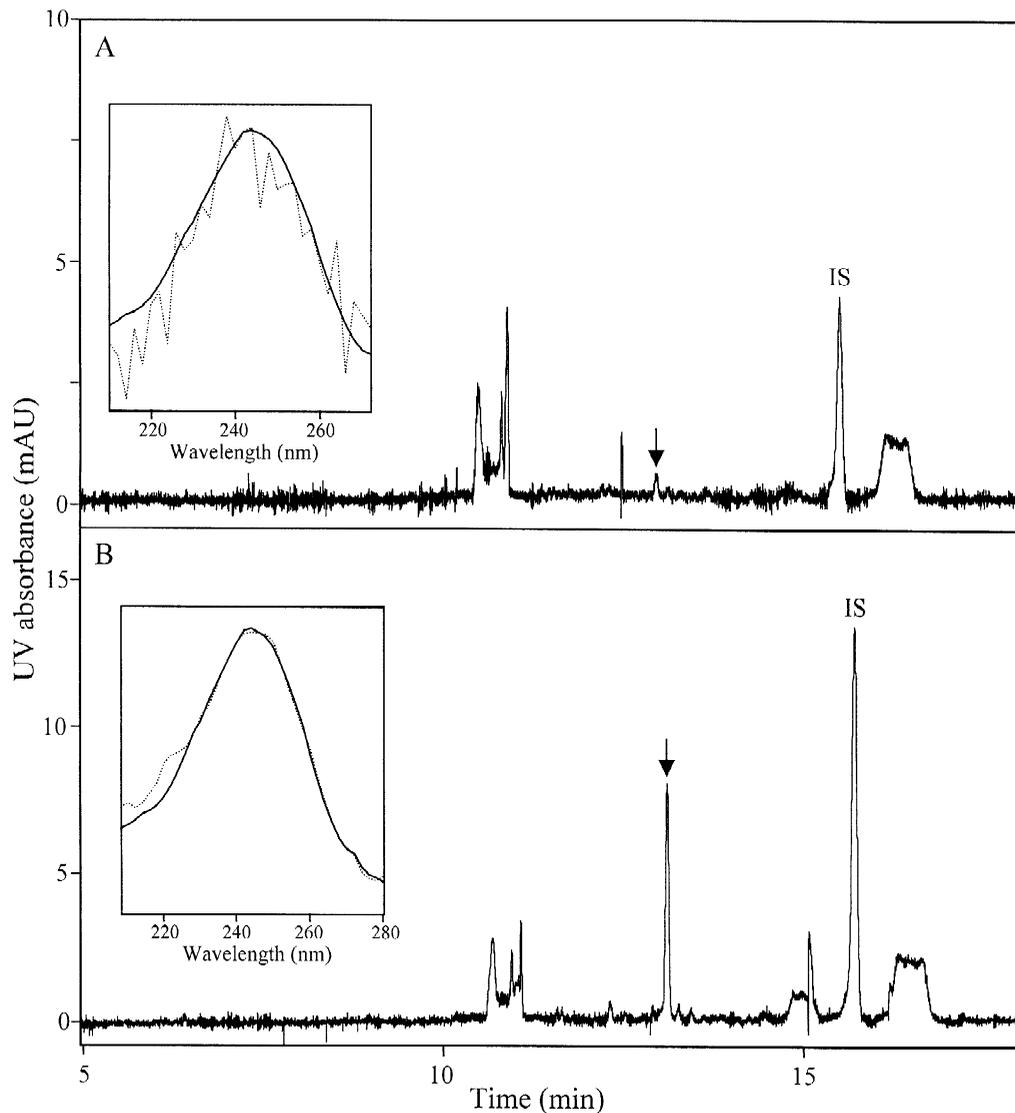


Fig. 4. CE electropherogram of blood sample extracts (frame A, non-stressed mouse; frame B, stressed mouse), spiked with 500 ng/ml of 17-hydroxycorticosterone as an internal standard. The inset shows the on-line UV spectra (dashed line: arrow marked peak; solid line: corticosterone standard).

who are interested in studying stress in experimental animals or human as this relates to adequate medication for releasing stress.

#### 4. Conclusions

We demonstrate here that capillary electropho-

resis/UV absorbance after applying the technique of sweeping-MEKC can be successfully used for the separation and on-line concentration of corticosterone in mouse blood samples. The optimum CE conditions for the analysis of the analyte were achieved using a methanol–water solution (30:70, v/v) containing phosphate (30 mM) and SDS (100 mM). In the sweeping-MEKC mode, for a 70 cm

Table 2  
Analysis of corticosterone in different biosamples and the detection methods used

Specimen	Corticosterone (ng/ml)		Method	LOD (ng/ml)	Ref.
	Non-stressed	Stressed			
Mouse blood	~88	200–280	CE/UV-sweeping-MEKC	5	This study
Mouse blood	~20	112–237	GC-MS	0.1	[36]
Mouse blood	8	176	LC-ESI-MS	0.3	[13]
Human urine	–	–	HPLC/ESI-MS	178	[14]
Human blood	–	–	GC/NICI/MS	425	[16]
Rat plasma	–	–	HPLC-UV	96	[31]
Rat plasma	56	100	RIA	2	[32]
Human urine	–	–	HPLC/UV	37	[33]
Rat urine	3–18	–	HPLC/SPE/UV	3.8	[34]
Mouse blood	35	261	HPLC-UV	–	[35]

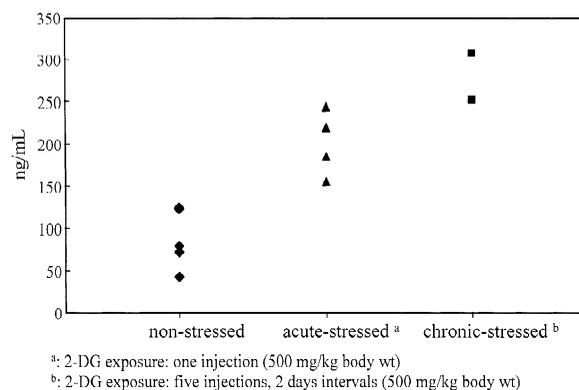


Fig. 5. Different concentration ranges of corticosterone from non-, chronic- and acute-stressed rats.

capillary (effective length: 61 cm) the optimum injection length is ~30 cm. In comparison with the normal injection used in the CE separation by the MEKC mode, a ~2400-fold improvement ( $S/N=3$ ) in detection sensitivity was achieved. Furthermore, this method requires only small amounts of plasma (25  $\mu$ l) and can be used as a routine tool for pharmacological studies dealing with different types of drugs, whether derived from natural herbs or by synthesis.

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