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Short communication

Simultaneous measurement of aldosterone and cortisol by high-performance liquid chromatography–tandem mass spectrometry: Application to dehydration–rehydration studies

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ABSTRACT

Aldosterone and cortisol are useful biomarkers of dehydration and stress, respectively. The aim of this study was to develop an HPLC–tandem mass spectrometric method for the simultaneous measurement of aldosterone and cortisol in human plasma that could be applied to the study of athletes undergoing exercise and rehydration. Samples were prepared and analysed using an on-line sample preparation/HPLC system coupled to a triple quadrupole tandem-mass spectrometer. Samples (200 μL) were pre-treated and extracted on Hysphere C18 HD cartridges (7 μm, Spark Holland). Chromatography was performed on a Sunfire C18 analytical column (50 mm × 3.0 mm, 3 μm, Waters) under isocratic conditions at a flow rate of 0.3 mL/min. The mobile phase consisted of 35% acetonitrile/water. Mass spectrometric detection was by selected reaction monitoring using negative electrospray ionization conditions. The assay had an analytical range of 25–500 pg/mL and 25–500 ng/mL for aldosterone and cortisol, respectively ($r^2 > 0.992$, $n = 22$). Inter-day accuracy and imprecision for quality control samples was 99.4–106% and <16%, respectively ($n = 10$). In a study of nine human subjects, both aldosterone and cortisol concentrations reflected the expected physiological responses to dehydration, rehydration and exercise when measured by this method. The reported method is suitable to facilitate the study of athletes undergoing dehydration and rehydration protocols.

1. Introduction

Dehydration induces physiological stress by altering cardiovascular function and thermoregulation. Exercise capacity and performance may ultimately be reduced when fluid losses exceed 2% body weight [1]. Rehydration is thus an integral component of recovery between exercise sessions. Recently, athletes in numerous sports have chosen to rehydrate with intravenous (IV) fluid because it rapidly enhances circulating blood volume since it bypasses the absorption delays associated with oral rehydration (gastric emptying, intestinal absorption). However, IV fluid has also been associated with a rapid diuresis. Thus, it is important to monitor both fluid-regulating and stress hormone responses following IV rehydration.

With dehydration, the reduction in arterial blood pressure, from the drop in extracellular fluid volume, stimulates the renin–angiotensin–aldosterone system. Primary factors affecting aldosterone secretion include plasma volume [2–4], plasma sodium and potassium concentrations [4,5], and plasma adrenocorticotropic hormone (ACTH) concentration [3,6]. Thus, aldosterone increases during periods of dehydration and decreases with rehydration. Cortisol secretion is almost entirely controlled by ACTH [7]. In turn, ACTH is influenced by thermosensitive control centers and plasma volume [4,8]. Cortisol fluctuations during exercise mirror the changes in aldosterone and reflect the increased physiological strain resulting from dehydration [2,8] and from increased core temperature and heart rate, and reduced sweat rate [9–11]. Therefore, cortisol has been shown to be a very sensitive measure of heat stress and increases are accompanied by signs of subjective discomfort [6,10].

The endogenous steroids, aldosterone and cortisol, are routinely measured in the clinical setting by immunoassay [12–14]. These antibody-based methods can suffer from interferences such as the non-specific binding of other circulating steroids causing an over-

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estimation in result [15,16]. Chromatographic methods, such as gas chromatography and liquid chromatography with mass spectrometric detection, offer a potentially more specific methodology and have been used as reference methods [17–22]. The aim of this study was to develop an HPLC–tandem mass spectrometric method (HPLC–MS/MS), based on our previous assay for aldosterone [23], for the simultaneous measurement of aldosterone and cortisol in human plasma and apply this method to the study of rehydration protocols in athletes.

2. Experimental

2.1. Chemicals and reagents

Aldosterone and cortisol were purchased from Sigma–Aldrich. The internal standards, d_7-aldosterone (internal standard for aldosterone) and d_4-cortisol (internal standard for cortisol), were purchased from IsoSciences (King of Prussia, PA, USA) and CDN Isotopes (Quebec, Canada), respectively. Stock solutions of aldosterone, cortisol and the two internal standards were prepared in methanol. A precipitation reagent was prepared with 0.3 M zinc sulphate: methanol (1:5, vol:vol) that contained both d_7-aldosterone (1000 pg/mL) and d_4-cortisol (200 ng/mL).

The calibrators and quality controls were prepared in water. The final calibrator concentrations were 25, 50, 100, 250 and 500 pg/mL for aldosterone and 25, 50, 100, 250 and 500 ng/mL for cortisol. The nominal concentrations of the quality controls were 25, 60, 200, 400, and 500 pg/mL for aldosterone and 25, 60, 200, 400 and 500 ng/mL for cortisol.

2.2. Sample preparation

Plasma standards, quality controls and patient samples (200 μL) were pre-treated with precipitation reagent (200 μL), mixed and centrifuged. The supernatant (approximately 300 μL) was transferred to a Symbiosis HPLC-online solid phase extraction system (Spark Holland, Emmen, The Netherlands). A Hysphere C18 HD extraction cartridge (10 mm × 2 mm, 7 μm) was preconditioned with acetonitrile (1 mL) followed by water (1 mL) and loaded with the supernatant (250 μL) using water (1 mL). The cartridge was sequentially washed with 10% acetonitrile in 0.1% ammonium hydroxide (1 mL), 10% acetonitrile in 0.1% formic acid (1 mL) and 10% acetonitrile in water (1 mL). The analytes were eluted from the cartridge under the initial chromatographic conditions (shown below) for 60 s before the cartridge was taken off-line. The processing of samples was performed in parallel (i.e. while one sample is being extracted the previous is eluted).

2.3. Equipment

Chromatography was performed on a Waters Sunfire C18 analytical column (50 mm × 3.0 mm, 3 μm) at ambient temperature, under isocratic conditions (35% acetonitrile/water) with a flow rate of 0.3 mL/min. For the first minute of the analysis the eluate was diverted to waste. At 2.5 min after injection, a column wash with 100% acetonitrile at 1 mL/min was performed for 2 min. The column was then re-equilibrated at starting conditions for 2.5 min giving a total chromatographic analysis time of 7 min.

Under negative electrospray ionization conditions (−2500 V), the analytes were predominantly in the de-protonated form, $[M-H]^-$. The compound specific operating parameters of cone voltage and collision energy were −30 V for all analytes and −17 and −12 eV for aldosterone and cortisol and their respective internal standards. Mass spectrometric detection was undertaken by selected reaction monitoring (aldosterone *m/z* 358.9 → 330.9; d_7-aldosterone *m/z* 365.9 → 337.9; cortisol *m/z* 361.0 → 331.1; d_4-cortisol: *m/z* 365.0 → 335.1) on a Quattro Premier triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA). The dwell time for aldosterone, d_7-aldosterone, cortisol and d_4-cortisol were 400, 200, 10 and 10 ms, respectively. The HPLC/MS/MS including the Symbiosis was controlled and data processed using MassLynx version 4.1 (Waters).

2.4. Validation

Linearity was assessed during validation and the analysis of study samples ($n = 22$). In-house calibration standards (5 concentrations over the range 25–500 pg/mL for aldosterone; 25–500 ng/mL for cortisol), quality control samples (5 across each analytical range) and two lots of pooled patient plasma (pool 1 and pool 2) with nominal low and high concentrations were analysed on each of 10 days. The pooled patient plasma was also analysed in a replicate of 10 on 1 day. For each batch analysed, a 5-point standard curve was constructed using weighted $1/x$ linear regression. The methods accuracy and imprecision was determined using the quality control results. The patient pools were used to determine both inter- and intra-day assay imprecision. Analytical recovery and the influence of matrix effects, in terms of inter-patient variability, were determined by measuring patient samples ($n = 5$) pre- and post-addition of aldosterone (350 pg/mL) and cortisol (350 ng/mL).

2.5. Dehydration–rehydration study

Subjects entered an environment chamber, at 35 °C and 70% relative humidity (RH), and rested supine for 10 min to equilibrate to the environment and to minimise the effect of postural changes on body fluid distribution. Subjects then cycled on their own road bike mounted on a stationary windtrainer (Cyclosimulator CS-1000; Cateye Co. Ltd., Osaka, Japan) to induce dehydration. The dehydration protocol was broken down into 30 min blocks. The first 23 min involved continuous cycling at a moderate intensity (12–13 on the Borg RPE scale), after which they dismounted, toweled dry and urinated if needed. Body weight was then recorded before they lay supine on a massage table for 7 min, after which a 4 mL blood sample was collected. This procedure was repeated so that subjects had lost 4% of their pre-exercise body weight. Maximum dehydration duration was 2 h (4 exercise blocks). Over the next 2 h, subjects sat passively in a thermoneutral environment (22 °C) while they were rehydrated with a total of 150% of the fluid lost during dehydration. Half of the fluid was given orally as a combination of water and commercial sports drink (Gatorade®). The other half was infused intravenously (0.9% NaCl). Blood samples (4 mL) and body weight were collected at 60, 90 and 120 min. Following rehydration, the subjects remained in a thermoneutral environment for 60 min equilibration before reentering the environment chamber and undertaking a 90 min cycling bout in the heat (34 °C, 60% RH) without additional rehydration. Blood samples were collected at 30 and 60 min during equilibration and at performance check points that were approximately 30 min apart (Fig. 2).

In addition to aldosterone and cortisol, blood was analysed for haemoglobin (Hb), haematocrit (Hct), osmolality and electrolytes (Na^+ and K^+). Hb was measured from whole blood using the cyanmethemoglobin method (ATI Unicam 5625 UV/VIS Spectrometer set at 540 nm). Hct was measured from whole blood using 100 μL heparinised capillary tubes which were centrifuged at 14 000 × *g* for 5 min (Jouan HEMA-C micro-haematocrit centrifuge, Sussex, England). A Mikro–Hamatocrit chart was used to read the Hct reading. Percent change in plasma volume was calculated using the equations of Dill and Costill [24]. Plasma osmolality was measured via a vapor pressure osmometer (Wescor 5500 vapor pressure osmometer, Wescor Inc., Utah, USA) and plasma Na^+ and

Table 1
Accuracy and imprecision of the aldosterone and cortisol HPLC–MS/MS method. These data are based on single measurements of each quality control on each of 10 days.

Parameter	25	60	200	400	500
Aldosterone concentration (pg/mL)					
Accuracy (%)	105	102	106	101	100
Imprecision (%)	15.9	6.6	3.7	5.8	3.1
Cortisol concentration (ng/mL)					
Accuracy (%)	99.4	102	106	100	100
Imprecision (%)	6.1	2.3	2.8	3.4	2.7

K^+ via automated analysis (Cobas Mira, Roche Diagnostic Systems, Switzerland). All of these parameters were measured in duplicate and third measures were taken if the variation was greater than 3%. Tympanic temperature was measured using a tympanic thermometer featuring inbuilt equations that correct to core temperature (FirstTemp Genius® Infrared thermometer, model 3000A, Sherwood Medical, St Louis, USA).

3. Results and discussion

3.1. Validation

The assay was found to have acceptable linearity over the analytical range of 25–500 pg/mL and 25–500 ng/mL for aldosterone and cortisol, respectively ($r^2 > 0.992$, $n = 22$). The accuracy and precision using the back-calculated results for calibration standards was 96.1–104.0% and <7.2%, respectively (data not shown). We had previously reported an analytical range for aldosterone of 25–2000 pg/mL [23]. In this study, we adapted the previous method to incorporate cortisol. In doing so we decided to limit the number of calibration standards to five and the upper end of the analytical range for aldosterone to 500 ng/mL. It was expected that this analytical range would meet the requirements for the dehydration–rehydration study. Similarly, the analytical range for cortisol (25–500 ng/mL) was found to be suited to our current study. One problem that was encountered, at the upper end of the analytical range for cortisol, was saturation of the mass spectrometer detector due to excessive ion count. To overcome this problem, the collision energy used for cortisol (−12 eV) was not optimum voltage for maximum signal (i.e. detuned).

The inter-day accuracy and imprecision, based on quality control samples, was satisfactory across the linear range (Table 1). At the lower limit of quantification for aldosterone and cortisol (25 pg/mL and 25 ng/mL, respectively) the accuracy and imprecision was 99.4–106% and <16%. The imprecision for aldosterone at the lower limit of quantification was 15.9%. This value was higher than that reported in our previous method of 9.4% [23].

The mean aldosterone concentration for pooled plasmas 1 and 2 were 87.1 and 477 pg/mL. The mean cortisol concentration for pooled plasmas 1 and 2 were 55.5 and 462 ng/mL. The inter- and intra-day imprecision of the method based on these pooled samples was acceptable (<9.4%) and is shown in Table 2. These data were comparable to results for quality controls suggesting matrix differences (i.e. water versus plasma) are not influencing results. The mean analytical recovery for aldosterone and cortisol was 104 and 112%, respectively. The inter-patient variability was 2.7% for aldosterone and 4.8% for cortisol ($n = 5$). These data suggest that matrix effects do not have a significant influence on assay performance [25].

Table 2
Inter- and intra-day imprecision of the aldosterone and cortisol HPLC–MS/MS method based on pooled plasma ($n = 10$).

Parameter	Pooled plasma 1	Pooled plasma 2
Aldosterone		
Inter-day imprecision (%)	9.3	6.0
Intra-day imprecision (%)	7.3	4.3
Cortisol		
Inter-day imprecision (%)	2.1	1.8
Intra-day imprecision (%)	3.7	1.6

This is not the first method to simultaneously measure aldosterone and cortisol by on-line solid phase extraction coupled to HPLC–MS/MS. Ceglarek et al. have reported the simultaneous measurement of 13 steroids, including aldosterone and cortisol [26]. The analytical specifications for cortisol using their method are similar to our current method. But for aldosterone, their method was significantly inferior with a lower limit of quantification of 400 pg/mL compared to 25 pg/mL for our reported method. The method of Ceglarek and co-workers would appear to be unsuitable for the measurement of aldosterone concentrations in exercise studies.

3.2. Dehydration–rehydration study

Representative chromatograms of a sample taken from a subject undergoing the 3 stage dehydration–rehydration–performance protocol are shown in Fig. 1. Fig. 2 shows the aldosterone and cortisol concentration changes over 13 time points during dehydration–rehydration–performance in a group of 9 subjects. As expected, both aldosterone and cortisol rose during the two exer-

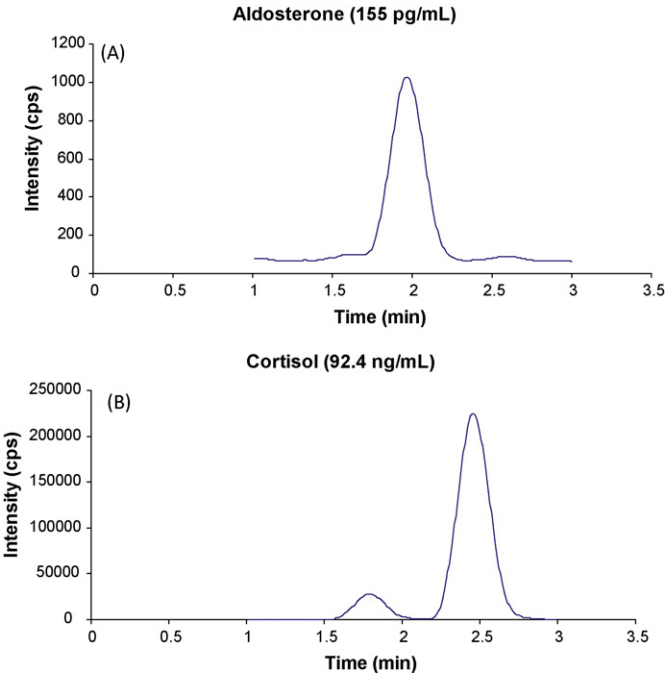

Fig. 1. Representative chromatograms of a blood sample taken from a subject undertaking the dehydration–rehydration protocol.

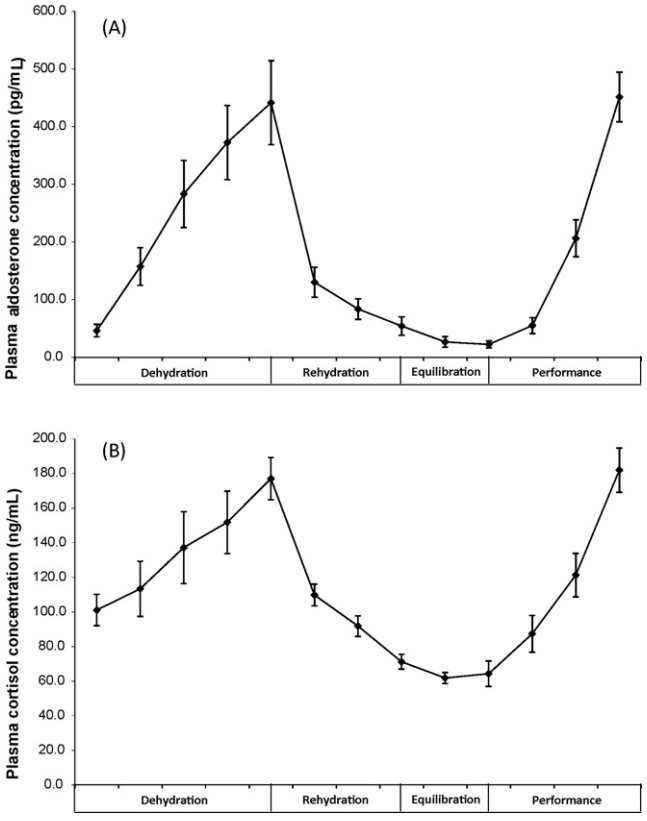

Fig. 2. Change in aldosterone (A) and cortisol (B) concentration during exercise-induced dehydration, rehydration with 50% oral and 50% IV fluid, passive equilibration and an exercise performance test. See Section 2.5 for detail of the experimental procedures. Data are presented as mean ± SEM ($n = 9$).

cise periods ("dehydration" and "performance") and fell during the rest/rehydration period.

In the present exercise heat stress study, the correlation between plasma aldosterone and cortisol concentrations across all 13 time points was $r = 0.97$ ($p < 0.001$). Aldosterone was well correlated with variables associated with hydration balance including change in plasma volume ($r = -0.89$; $p < 0.001$), electrolytes (Na^+, $r = 0.82$; $p < 0.001$ and K^+, $r = 0.75$; $p < 0.01$) and osmolality ($r = 0.77$; $p < 0.01$). Cortisol was highly correlated with variables of exercise heat stress including change in plasma volume ($r = -0.88$; $p < 0.001$) and tympanic temperature ($r = 0.76$; $p < 0.01$).

4. Conclusions

The reported simultaneous aldosterone and cortisol HPLC–MS/MS method has excellent analytical performance over the studied concentration range. The combination of minimal sample handling and simultaneous measurement of aldosterone and cortisol within a 7 min chromatographic analysis time makes this method cost effective. This method was shown to be suitable for the study of aldosterone (state of hydration) and cortisol (level of stress) in athletes undergoing exercise regimens.

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