

Urinary high performance reverse phase chromatography cortisol and cortisone analyses before and at the end of a race in elite cyclists

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Received 14 February 2005; accepted 23 June 2005

Available online 27 July 2005

Abstract

A functional and basic method for the quantitative analysis of urine cortisol (*F*) and cortisone (*E*) using a Solid-Phase Extraction column and HPLC with ultraviolet detection is here described and validated to analyse urine samples. Urine specimens were analysed to study *F* and *E* relation and ratio in athletes and healthy sedentary subjects. The *F* and *E* concentrations in random urine specimens were significantly higher in the post exercise versus pre exercise condition in cyclists (*F*: 136 ± 93 nmol/l versus 67 ± 50 nmol/l ($p < 0.001$); *E*: 797 ± 400 nmol/l versus 408 ± 252 nmol/l ($p < 0.001$)). The *F/E* ratio was 0.18 ± 0.11 versus 0.16 ± 0.07 , respectively, and a significant difference was only demonstrated comparing sedentary (0.11 ± 0.07) and cyclist individuals at rest ($p < 0.05$).

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Keywords: Solid phase extraction; Exercise; 11 β -HSD

1. Introduction

The physiologically active steroid hormone cortisol (*F*) and its inactive metabolite cortisone (*E*) have different chemical structures. Two isoenzymes of 11 β -hydroxysteroid dehydrogenase (11 β -HSD), catalysing their interconversion within target cells, have been shown to modulate the action of the *F* at an autocrine level in several peripheral sites. The 11 β -HSD type 1 regenerates cortisol from cortisone, demonstrating its strongest enzyme activity in the liver, nevertheless it is widely distributed in the organism to maintain adequate activation of glucocorticoid receptors, where *F* has crucial metabolic functions [1]. The 11 β -HSD type 2 is mainly

localized in the kidney and acts selectively on intrinsically non-specific mineralcorticoid receptors by inactivating cortisol. The balance between the enzymatic activity of the types 1 and 2 enzymes maintains the available cortisol [2]. Alteration in the expression of these isoenzymes in peripheral tissue modifies corticosteroid action: loss of hepatic 11 β -HSD1 activity improves insulin sensitivity through a reduction in cortisol-induced gluconeogenesis and hepatic glucose output. Conversely, over-expression of 11 β -HSD1 in omental adipose tissue can stimulate glucocorticoid-induced adipocyte differentiation, which may lead to central obesity [3,4]. The ratio between cortisol and cortisone (*F/E*) in urine depends on 11 β -HSD type 2 activity and has been suggested as a marker of the activity of 11 β -HSD type 2 in the kidney. It might be useful to study this *F–E* interconversion. Both *F* and *E* are needed in several metabolic processes such as in adaptation to stress. In fact *F* and *E* increase when nervous tension occurs, e.g. during sport training or competition [5]. Urinary free *F* (UF) excretion has been shown to be a reliable single

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index of adrenal/cortical secretion and has been accepted as a measurement of the free fraction in plasma [6,7]. Recently, the 24-h urinary *F/E* ratio has been proposed as a useful indicator for monitoring the overreaching state in elite swimmers [8]. It is also known that a few athletes illicitly use corticoids to improve their performance and the *F/E* ratio in a doping test could be used for doping detection [9]. Furthermore, a combined test of *F* and *E* is potentially useful in evaluating patients with adrenal disorders and also in athletes in relation to physical exercise and training. Various immunoassays have been developed for the urine *F* (UF) measurement, but these analysis methods are susceptible to interferences from cortisone and/or other endogenous steroid metabolites and synthetic glucocorticoids [10]. Therefore a serious problem is the lack of specificity in the measurement of UF [11,12]. Chromatographic methods accurately measure UF, not only reducing the interference for the *F* quantification, but also allow quantification of the *E* [13–15]. Moreover, liquid chromatography–mass spectrometry (LC–MS), and gas chromatography–mass spectrometry (GC–MS) [15–17] have the disadvantage of being expensive and are not available in all laboratories. Liquid chromatography with ultraviolet detection (LC–UV) coupled with solid phase extraction (SPE), to eliminate interfering compounds, is a practical and cheaper technique [18,19]. The aim of this work has been to describe the development and validation of a HPLC UV method utilising a solid phase extraction with SPE cartridges for the simultaneous measurement of cortisol and cortisone in random urine specimen, to measure the *F/E* ratio in cyclists and to evaluate if this ratio changes before and at the end of a strenuous exercise.

2. Experimental

2.1. Chemicals and reagents

Cortisol (11- β , 17- α , 21-trihydroxy, Δ -4 pregnene 3,20-dione, hydrocortisone: *F*) PM 362.5, 98%, (Sigma Chem.), cortisone (17 α , 21-dihydroxy, Δ -4 pregnene 3,11,20 tri-one: *E*) PM 360.5, 98%, (Sigma Chem.) and 6- α methylprednisolone (internal standard, IS) PM 374.5, 98% (Sigma Chem.) were purchased from Sigma Chemical (Sigma–Aldrich, St. Louis, MO, USA).

Methanol HPLC grade (99.9%, Carlo Erba, Limite, Italy), Acetone (acetone HPLC pure 99.8%, LabScan Analytical Science, Dublin, Ireland), distilled water from an ELGA-STAT UHQ system (Elga, High Wycombe, UK), *n*-Hexane (98%, Carlo Erba, Limite, Italy) and diethyl ether (99.8%, Carlo Erba, Limite, Italy).

Standard stock solutions were prepared by dissolving in methanol 1 mg/ml of commercial preparations of cortisol and cortisone and IS. All solutions were stable for up to 3 months when stored at -20°C .

The SPE Discovery DSC 18 columns (endcapped—500 mg/3 ml) were used for sample clean-up. These SPE

columns were purchased from Supelco (Bellofonte, PA, USA).

A reversed-phase HPLC system with UV detector was used to separate and quantify the urinary steroids. The chromatographic equipment consisted of a solvent delivery system mod. 112 (Beckman Coulter, Fullerton, CA, USA), an injector mod. 7725 Rheodyne (Beckman Coulter, Fullerton, CA, USA), a column oven Gecko 2000 (Beckman Coulter, Fullerton, CA, USA). The analytical column was a stainless steel ODS column (Supelco Discovery DSC 18 150 mm \times 4.6 mm, i.d.; 3 μm particles, 120 \AA pore diameter) (Supelco, Bellofonte, PA, USA) equipped with an ODS guard column (Phenomenex C18 OSD 4 mm \times 3 mm, i.d.) (Phenomenex, Torrance, CA, USA). The detector was an UV–vis spectrophotometer (Beckman mod. 126, Beckman Coulter, Fullerton, CA, USA).

Urinary creatinine (crea) was measured by a capillary electrophoresis method [20] and the urinary total protein (uTp) concentration was measured by the Coomassie method using a commercial kit purchased from Sigma Diagnostics (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Chromatographic conditions

2.2.1. SPE urine treatment

Each urine or standard sample (3.0 ml), spiked with IS 133 nmol/l, was applied to a SPE column DSC 18 packed, which had previously been equilibrated with 10 ml of methanol followed by 5 ml of water. After sample injection the washing steps were: 1 ml of water followed by 5 ml of acetone water solution (1/5, v/v) and 1 ml of hexane. Two millilitres of diethyl ether was then added and the eluate was desiccated. Each dried sample was successively re-suspended with 200 μl of HPLC mobile phase.

2.2.2. Reversed-phase HPLC

Chromatography separation was carried out by isocratic elution, with methanol–water (63/37, v/v) at 30°C and the process of each chromatographic analysis ended in 11 min.

The mobile phase flow rate was 0.8 ml/min. The sample injection volume was 20 μl . A System Gold (Beckman, V 8.1) software integration was employed for *F* and *E* quantitative analysis measuring the absorbance at 254 nm wavelength. The chromatographic conditions were chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution.

2.3. Urine specimen collection and subjects

Urine specimens were collected from a group of well-trained cyclists ($n=21$, age 17.3 ± 0.5 years, weight 66.8 ± 6.1 kg, height 174.6 ± 1.7 cm; average \pm standard deviation) and from an age-matched male sedentary control group ($n=10$, age 24.6 ± 3.6 years, weight 71.0 ± 10.3 kg, height 180.3 ± 6.5 cm). The cyclists were elite athletes in a

state of continuous training during their racing season. Each cyclist, questioned about the use of drugs, confirmed that no drug had been taken after eight p.m. the previous evening and that no special medical treatment was ongoing. All studied cyclists had a 1350–1500 kcal meal (65% carbohydrates, 25% lipids, 10% proteins) 120–180 min before the competition. The approval by the Ethics Committee of the University of Padua was obtained and all cyclists gave their informed consent.

All cyclists performed a competitive mountain bicycle race of about 102 km. The competition took place in the late morning, starting about midday and was concluded about 2.5 h later. At the end of the race the weight of each cyclist was decreased about 1.5 kg. During the race, the average fluid intake (sweetened weak tea) of each athlete was approximately 500–800 ml. The climatic temperature was about 15 °C and it was raining heavily. The urine samples were collected from each cyclist at rest, before exercise (10–15 min before competition), and at the end of exercise (no later than 10–15 min after his arrival). All collected specimens were stored at 4 °C until the return to the laboratory (about 02:00 p.m.) and placed in the refrigerator at –80 °C. The urine samples (7 ml specimens), collected from the sedentary controls during the late morning to match the time of the basal athlete urine collection, were stored in suitable aliquots in the refrigerator (–80 °C) within 1 h from the time of collection. All samples were stored in the refrigerator until assayed.

2.4. Statistical analysis

The data were analysed by using conventional statistical methods. The pre and post exercise comparison was calculated using t test for paired data. The comparison between different groups (athletes and sedentary) used unpaired t test. The data are reported as average \pm standard deviation.

3. Method validations and results

3.1. Recovery test

Aliquots of two urine samples were prepared for determining the accuracy and precision of the method. Endogenous *F* concentrations measured in these samples were 115 ± 9 and 116 ± 18 nmol/l (specimens 1 and 2, respectively), and *E* were 260 ± 36 and 481 ± 57 nmol/l. Known amounts of standard solution of *F* (345 and 138 nmol/l), *E* (277 and 693 nmol/l) and IS (133 nmol/l) were added to these sets of urine. The samples were then carried through the sample preparation procedure described above. The recoveries were calculated by comparing the *F* and *E* concentrations of these compounds between the expected and measured values after the extraction procedures. The expected values were calculated using the average obtained from repeated analyses on the same urine sample. A standard solution was added to a

Table 1
Analytical recovery test of cortisol added into two urine samples (A and B) prior SPE extraction

Endogenous (nmol/l)	Added (nmol/l)	Measured (nmol/l)	Recovery (%)
Cortisol (<i>F</i>)			
Urine sample A (<i>n</i> = 3)			
115 ± 9	138	262 ± 38	103 ± 14
	345	427 ± 43	93 ± 9
Urine sample B (<i>n</i> = 5)			
116 ± 18	138	240 ± 16	92 ± 1
	345	420 ± 26	92 ± 3

Table 2
Analytical recovery test of cortisone added into two urine samples (A and B) prior to SPE extraction

Endogenous (nmol/l)	Added (nmol/l)	Measured (nmol/l)	Recovery (%)
Cortisone (<i>E</i>)			
Urine sample A (<i>n</i> = 3)			
260 ± 36	277	503 ± 89	93 ± 13
	693	962 ± 87	101 ± 12
Urine sample B (<i>n</i> = 5)			
481 ± 57	277	708 ± 76	95 ± 4
	693	1142 ± 102	94 ± 5

sample of this urine to reach a known concentration, namely the expected concentration (Tables 1 and 2).

3.2. Linearity

Three different urine samples were diluted 1:2, 1:4 and 1:6 to verify the possibility of diluting urine samples. These diluted and undiluted samples were analysed for cortisol and cortisone measurements. Figs. 1 and 2 show the correlation between the expected and measured concentrations of cortisol and cortisone, respectively. The original concentration of undiluted sample was defined as 100% and the successive dilutions were calculated as relative percent. The equations in figures were the final average of the three different dilutions of each sample.

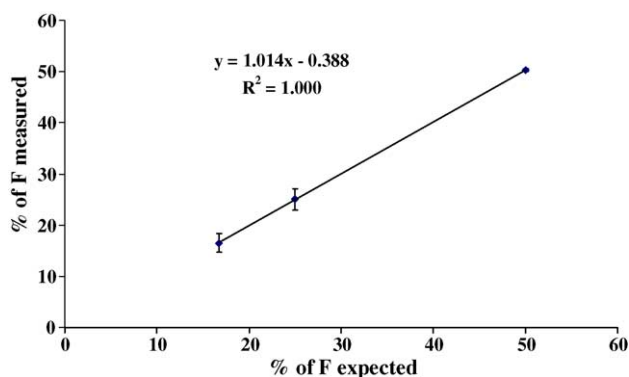


Fig. 1. Correlation between the expected and measured cortisol percent values. (◆): average of three urine samples.

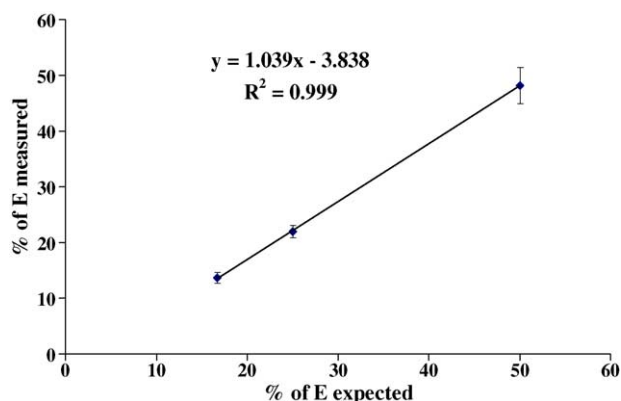


Fig. 2. Correlation between the expected and measured cortisone percent values. (◆): average of three urine samples.

3.3. Inter-assay and intra-assay variability

Inter-day precision was calculated from data obtained analysing, during 7 days, aliquots of the SPE urine sample (single extraction), stocked and stored at -20°C and the CV was 3.1% and 1.5%, and the concentrations measured for this sample were 354 ± 15 and 118 ± 2 nmol/l (*E* and *F*, respectively).

The inter-day precision was also calculated from data obtained analysing one urine specimen extracted in 7 different days (multiple extraction). The CV was 5.0% and 7.1% and the concentrations measured were 454 ± 23 and 108 ± 8 nmol/l (*E* and *F*, respectively). Intra-day precision was calculated from six repetitions of a single extraction of a urine sample (550 ± 12 and 124 ± 1 nmol/l *E* and *F*, respectively) and the CV was 2.2% and 0.9%, respectively.

3.4. Calibration graphs

To each of five standards, containing known concentrations of *F* (0, 69, 138, 345, 690 nmol/l) and *E* (0, 139, 277, 693, 1387 nmol/l) dissolved in phosphate buffer (sodium salt 70 mmol/l, pH 7.4), 133 nmol/l of IS was added. The samples were treated using the same procedure as a urine sample extraction for HPLC separation.

The calibration graphs were obtained by an un-weighted least-squared linear fitting of the peak-area ratios versus the concentrations of *F* and *E* on each analysis of the standard mixtures. The calibrations curves of the two metabolites are shown in Figs. 3 and 4: the slopes, the intercepts and the correlation coefficients are reported. By examining the curves it can be seen that the relationship between peak area ratio and concentration was linear within the studied concentration ranges. Typical HPLC chromatogram after urine SPE extraction is shown in Fig. 5. A single peak of *F* was obtained at the retention time of 5.9 min and *E* at 4.8 min, respectively. A single peak of the IS was also obtained at 8.6 min.

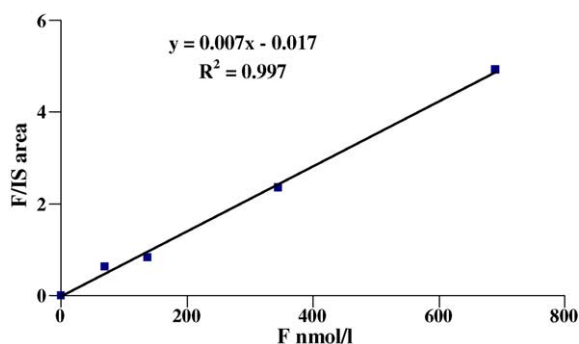


Fig. 3. Cortisol (*F*) calibration curve for urine analysis.

3.5. Limit of detection (LOD)

The LOD was expressed by measuring the noise of the chromatography signal in the zone (1–2 min interval) after the *E*, *F* and before IS peaks using urine extracted samples ($n = 10$). The average of the various peaks in these zones ± 3 standard deviations represented the LOD. The LOD for *F* and *E* in urine were therefore calculated to be 9 and 82 nmol/l, respectively.

3.6. Cortisol and cortisone urine profile of athletes and sedentary subjects

The present method was applied to analyse pre and post competition random urine specimens collected from cyclists and basal random urine specimen collected from sedentary subjects.

Table 3 shows the cortisol and cortisone pre and post competition urine concentrations and their relation with creatinine urine concentration in cyclists. A significant increase of urine *F* and *E* concentrations in post exercise was observed ($p < 0.001$). After the normalization by creatinine, in both metabolites, a post exercise increase was not present. The *F/E* ratio value pre versus post competition was not significantly different. On the contrary the pre competition *F/E* ratio in athletes was significantly different ($p < 0.05$) from the basal value in sedentary individuals (Table 3). A significant correlation was found between *F* and creatinine and *E* and creatinine in pre exercise ($p < 0.05$ and $p < 0.001$; $r = 0.529$

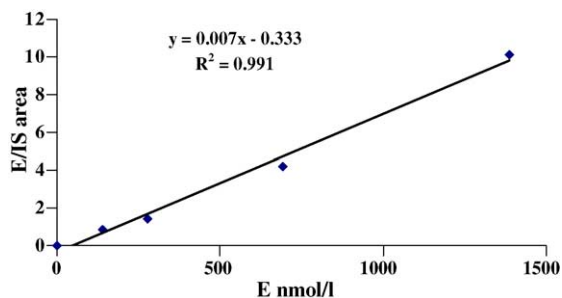


Fig. 4. Cortisone (*E*) calibration curve for urine analysis.

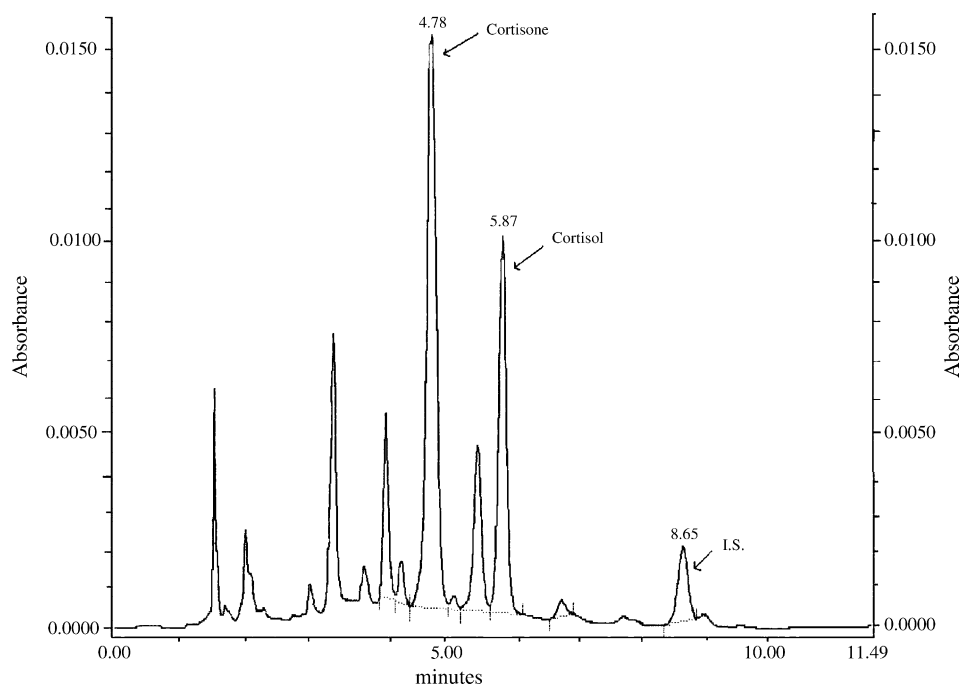


Fig. 5. HPLC separation of an urine sample. The HPLC conditions were as follows: Supelco Discovery HSC₁₈ (150 mm × 4.6 mm, i.d.; 3 μm, 30 °C) column; mobile phase: methanol–water (63/37, v/v); flow rate: 0.8 ml/min. Marked peaks: cortisone (*E*), cortisol (*F*), internal standard (IS).

Table 3

Urine concentrations of cortisol (*F*), cortisone (*E*), creatinine (crea), *F*/crea, *E*/crea and *F*/*E* ratios in the sedentary subjects and cyclists

	Sedentary subjects, <i>n</i> = 10 (mean ± S.D.)	Cyclists pre-competition, <i>n</i> = 21 (mean ± S.D.)	Cyclists post-competition, <i>n</i> = 21 (mean ± S.D.)
<i>F</i> (nmol/l)	51 ± 25	67 ± 50	136 ± 93*
<i>E</i> (nmol/l)	555 ± 318	408 ± 252	797 ± 400*
Crea (mmol/l)	14 ± 6 [§]	6 ± 4	10 ± 4*
<i>F</i> /crea (nmol/mmol)	5 ± 3 [°]	16 ± 18	16 ± 13
<i>E</i> /crea (nmol/mmol)	53 ± 37	101 ± 104	92 ± 56
<i>F</i> / <i>E</i>	0.11 ± 0.07 [°]	0.16 ± 0.07	0.18 ± 0.11

Pre-competition vs. post-competition: **p* < 0.001; sedentary subjects vs. pre-competition cyclists: [°]*p* < 0.05; [§]*p* < 0.001.

and *r* = 0.721, respectively). Post competition urinary total proteins were significantly increased compared to the pre competition (27 ± 30 mg/l versus 32 ± 42 mg/l, *p* < 0.05) and this significant increase was also present when the ratio with the creatinine was considered (5 ± 7 mg/mmol versus 32 ± 108 mg/mmol, *p* < 0.05).

4. Discussion

The present results demonstrated that this analysis method is suitable for urinary *F* and *E* analysis. The use of SPE and reverse phase chromatography with UV detection demonstrate a calibration range from 0 to 690 and 0 to 1387 nmol/l for *F* and *E*, respectively. The dilution tests demonstrated linearity for the two metabolites as shown in Figs. 1 and 2. The recovery tests demonstrated an average recovery of 95 ± 7% and 96 ± 8% for *F* and *E*, respectively. The intra-day precision demonstrated a CV: of 0.9% and 2.2% for *F* and *E*, respectively and an inter-day precision of 1.5% and 3.1%

and of 7.1% and 5.0% for a single and multiple extraction of *F* and *E*, respectively.

In conclusion, the analytical performance was proved to be adequate for measuring the random urine specimen concentrations of cortisol and cortisone. This enables us to detect changes of endogenous levels of these corticosteroids in random urine on the occasion of a cycle race.

The measurements carried out in athletes and sedentary subjects demonstrated that the urinary *F* and *E* levels in cyclists significantly increased after a stressful situation, such as competition. This difference was annulled when the ratio with urine creatinine was measured. The significant correlation, found between *F* and creatinine and *E* and creatinine in pre exercise values, suggests that the urinary excretion of *F* and *E* might follow the same profile as creatinine [21,22]. This correlation was not detected after exercise in this study and needs further investigation.

This fact might be due to the same outcome of physical exercise causing renal excretion variation [23]. Furthermore, the *F* and *E* ratio was not varied comparing before with end

exercise urine concentration. On the contrary, this ratio was significantly different when the basal levels of cyclists were compared with sedentary individuals. The measurement of this *F/E* ratio in the basal condition (random urine specimen) prompted a more profound study to evaluate if any relation existed in an acute (measurements carried out immediately after a strenuous physical exercise) and/or in a chronic condition (in a trained state, when physical exercise is carried out systematically). The strenuous exercise in fact did not demonstrate any difference between the pre and post competition *F/E* ratio; instead a significant difference comparing the basal *F/E* values of the sedentary subjects with the pre-competition cyclists, was demonstrated. This investigation develops Atlaoui's data and agrees with their suggestion that this ratio might be suitable for training state investigation [8]. Cortisol action on target cells is determined by 11 β -HSD and in acute condition the activity of type 1 enzyme probably does not change. On the contrary a chronic condition, like training, might modify the enzyme activity, which in turn would explain the different basal *F/E* ratio between sedentary subjects and cyclists.

The present analysis method can also be proposed for clinical application. In fact a defect of 11 β -HSD2 activity in the kidney results in cortisol-induced mineralocorticoid excess, leading to the so-called apparent mineralocorticoid excess syndrome (AME), a pseudo-hyperaldosteronism with hypertension and hypokalemia. Cortisol is elevated in both variants of the syndrome and can discriminate patients with AME [24–26]. Dietary flavonoids can inhibit this enzyme and, at high doses, may cause an apparent mineralocorticoid effect [27]. Glycyrrhetic acid, a compound of liquorice, also leads to pseudo-hyperaldosteronism by inhibiting the 11 β -hydroxysteroid dehydrogenase 2 [28].

Acknowledgements

We are indebted to Dr. Giuseppe Soldà for the cooperation and assistance with the collection of specimens from the athlete cyclists; to Dr. Silvia Chiappin and Chiara Bedin for their assistance in the analyses; to Mrs. Julia Stevens for her excellent and patient help with the English grammar; to the cyclist teams for their co-operation. This work was supported in part by a grant from Federazione Ciclistica Italiana (CONI-Roma – I), from Ministero della Salute (Roma – I) and from Ministero dell'Università e Ricerca Scientifica grant prot. no. 2003060354.003.

References

- [1] J.W. Tomlinson, E.A. Walker, I.J. Bujalska, N. Draper, G.G. Lavery, M.S. Cooper, M. Hewison, P.M. Stewart, *Endocr. Rev.* 25 (2004) 831.
- [2] M. Quinkler, P.M. Stewart, *J. Clin. Endocrinol. Metab.* 88 (2003) 2384.
- [3] P.M. Stewart, A.A. Toogood, J.W. Tomlinson, *Horm. Res.* 56 (S1) (2001) 1.
- [4] D.J. Wake, B.R. Walker, *Mol. Cell. Endocrinol.* 215 (2004) 45.
- [5] K.T. Kivlighan, D.A. Granger, A. Booth, *Psyendocr.* 30 (2005) 58.
- [6] C.L. Lin, T.J. Wu, D.A. Machacek, N.S. Jiang, P.C. Kao, *J. Clin. Endocrinol. Metab.* 82 (1997) 151.
- [7] T. Imaki, M. Naruse, K. Takano, *Endocr. J.* 51 (1) (2004) 95.
- [8] D. Atlaoui, M. Duclos, C. Gouarne, L. Lacoste, F. Barale, J.C. Chatard, *Med. Sci. Sports Exerc.* 36 (2) (2004) 218.
- [9] A. Santos-Montes, R. Gonzalo-Lumbreras, R. Izquierdo-Hornillos, *J. Chromatogr. B Biomed. Appl.* 673 (1) (1995) 27.
- [10] M. Schoneshofer, A. Fenner, H.J. Dulce, *Clin. Chim. Acta* 106 (1980) 63.
- [11] B.E. Murphy, *Clin. Chem.* 46 (6 Pt 1) (2000) 793.
- [12] B.E.P. Murphy, *J. Clin. Endocrinol. Metab.* 84 (1999) 2258.
- [13] M. Hay, P. Mormede, *J. Chromatogr. Biomed. Sci. Appl.* 702 (1997) 33.
- [14] C.L. Lin, T.J. Wu, D.A. Machacek, N.S. Jiang, P.C. Kao, *J. Clin. Endocrinol. Metab.* 82 (1997) 151.
- [15] R.L. Taylor, D. Machacek, R.J. Singh, *Clin. Chem.* 48 (9) (2002) 1511.
- [16] U. Turpeinen, U.H. Stenman, *Scand. J. Clin. Lab. Invest.* 63 (2) (2003) 143.
- [17] A.E. Nassar, N. Varshney, T. Getek, L. Cheng, *J. Chromatogr. Sci.* 39 (2) (2001) 59.
- [18] U. Turpeinen, H. Markkanen, M. Valimaki, U.H. Stenman, *Clin. Chem.* 43 (8 Pt 1) (1997) 1386.
- [19] S. AbuRuz, J. Millership, L. Heaney, J. McElnay, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 798 (2) (2003) 193.
- [20] R. Gatti, V. Lazzarotto, C.B. De Palo, E. Cappellin, P. Spinella, E.F. De Palo, *Electrophoresis* 20 (1999) 17.
- [21] E.F. De Palo, R. Gatti, F. Lancerin, E. Cappellin, C.B. De Palo, P. Spinella, *Clin. Chim. Acta* 351 (2005) 73.
- [22] K.C.A. Chan, L.C.W. Lit, E.L.K. Law, M.H.L. Tai, C.U. Yung, M.H.M. Chan, C.W.K. Lan, *Clin. Chem.* 50 (4) (2004) 757.
- [23] E.F. De Palo, R. Gatti, F. Lancerin, C.B. De Palo, E. Cappellin, G. Solda, P. Spinella, *Clin. Chem. Lab. Med.* 41 (10) (2003) 1308.
- [24] F. Mantero, M. Palermo, M.D. Petrelli, R. Tedde, P.M. Stewart, C.H. Shackleton, *Steroids* 61 (4) (1996) 193.
- [25] P.C. White, *Am. J. Med. Sci.* 322 (6) (2001) 308.
- [26] M. Palermo, G. Delitala, F. Mantero, P.M. Stewart, C.H. Shackleton, *J. Endocrinol. Invest.* 24 (1) (2001) 17.
- [27] Y.S. Lee, B.J. Lorenzo, T. Koufif, M.M. Reidenberg, *Clin. Pharmacol. Ther.* 59 (1) (1996) 62.
- [28] P. Heilmann, J. Heide, S. Hundertmark, M. Schoneshofer, *Exp. Clin. Endocrinol. Diab.* 107 (6) (1999) 370.