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Indirect detection of rHuEPO and synacthen administration in human urine

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Abstract

In this study, we hypothesized that a change in ADMA-DDAH-NOS[1] system could be an indirect marker of EPO administration as well as changes in F/E and 6β-OHF/F ratios could be an indirect markers of corticotrophin administration in doping control.

Introduction

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) and its accumulation has been associated with reducing NO bioavailability and increasing superoxide generation.

Scalera *et al*. [1] showed that Epoetin beta (EPO) and Darbepoetin alfa (NESP) can modify the levels of ADMA in endothelial cells. This work demonstrates that EPO increases the amount of ADMA in plasma. Administration of EPO at a dose of 6,000 U/week increases plasma ADMA by 16% in patients with renal failure. Authors showed [2] that this effect was associated with a reduced activity of DDAH, the enzyme that degrades ADMA. Furthermore, EPO- or NESP-induced accumulation of ADMA was accompanied by a significant reduction of NO synthesis and an increase in oxidative stress.

Therefore the aim of this work was to develop an indirect method that will result in a fast and cheap pre-screening method to recognize negative and probable positive samples before applying the direct r-HuEPO test, using biochemical markers such as ADMA, SDMA, L-arginine and Citrulline. Primary results were presented our laboratory in work [3].

Up to now there is no test that could detect Synacthen administration in human urine. Thevis et al. [4] presented a method of Synacthen detection in human plasma using immunoaffinity purification followed by liquid chromatography/tandem mass spectrometry.

[1] ***Abbreviation:*** ADMA - asymmetric dimethylarginine, SDMA - symmetric dimethylarginine DDAH – dimethylarginine dimethylaminohydrolase, NOS - nitric oxide synthase, F - cortisol, E - cortisone, 6β-OHF - 6β-hydroxycortisol, ACTH - adrenocorticotropic hormone, EPO - erythropoietin.

But nowadays doping control is mostly based on urinary analysis rather than blood.

Therefore the aim of the present work was to determine the values of F/E and 6β-OHF/F ratios in human urine collected at different time frames of a day before and after Synacthen administration.

Materials and Methods

HPLC-ESI/MS analysis of ADMA, SDMA, arginine and citrulline:

A Thermo Finnigan TSQ Quantum AM triple stage quadrupole mass spectrometer (Thermo Electron Corp, San Jose, CA) equipped with ESI source and a Surveyor auto-sampler plus and a MS-pump plus was used in this study. The analytical column was an Eclipse XDB-C18 (150 mm × 2.1 mm I.D., 5 µm, Zorbax, Agilent) connected to a BDS-Hypersil-C18 guard-cartridge (10 mm × 2.1 mm I.D., 3 µm, Thermo Scientific) in a guard-cartridge holder. Separations were obtained at 30°C. The mobile phase was methanol and 0.05% formic acid in water with 20 mM ammonium acetate pH 3. The flow rate was 0.2 mL/min. The solvent gradient program was as follows: initial methanol was 40%, linearly increased to 90% for 8 min, isocratic for 1 min, linearly decreased to 40% for 12 min, and finally maintained at 40% for 18 min. Total run time was 18 min, the injection volume was 15 µL.

For the ionization of positive electrospray ionization mode the spray voltage was set at 4000 V. For the analysis by LC-ESI-MS/MS with multiple reaction monitoring (MRM), the ion source was heated to 245°C and instrumental parameters were optimized for ADMA, SDMA, arginine, citrulline, and ISTD, monitoring the *m/z* 203 -> 46, *m/z* 203 -> 172, *m/z* 175 -> 70, *m/z* 176 -> 70, *m/z* 150 -> 135 MS/MS transitions, respectively. Collision gas was argon.

Sample preparation: Measurement of urinary ADMA, SDMA, arginine and citrulline was performed using the previously described method by Martens-Lobenhoffer *et al*. [5] with modifications. In brief, after thawing the frozen samples, 50 µL of urine was diluted with 840 µL of deionized water and 100 µL of acetonitrile. Synephrine (10 µL of 50 ng/mL) was added as an internal standard. Precipitated proteins were separated by centrifugation at 10,000 *g* for 5 min. The resulting supernatant was transferred to an autosampler vial.

Administration: Two healthy Caucasian volunteers (man №1 - 23 years old, out of sport; man №2 - 37 years old, long-distance runner) gave their informed consent to participate in the study. Volunteers had to take no drug for at least 10 days prior and during study. Urinary

ADMA, SDMA, arginine and citrullin levels were determined on complete 24 hrs collections for 13 consecutive days. The first 3 days urine samples were used as a control. Starting on day 4 the volunteers are given single intravenous Erythropoietin injection (2000 U/day, Epocrine®, St-Petersburg). Excreted urine fractions were collected for a period of 10 days after EPO administration. Urine samples were stored at -20°C until analysis.

HPLC-ESI/MS analysis of cortisol, cortisone and 6β-OH-cortisol:

The 1100 Series LC/MSD Ion Trap system from Agilent Technologies (Palo Alto, CA, USA) was equipped with autosampler and autoinjector. Zorbax Eclipse XDB-C18 (2.1 x 150 mm I.D., 5 μm) analytical column from Agilent Technologies (Palo Alto, CA, USA) was connected to a guard column cartridge 2.1 x 12.5 mm filled with the same packing material. The temperature of the column compartment was 30°C. The mobile phase was 0.2 mM ammonium acetate with 0.05% formic acid (A) – acetonitrile (B) at a flow rate of 0.2 ml/min. The solvent gradient program was as follows: 0 min – 15% B; 10 min – 60% B; 15 min – 75% B; 25 min – 85% B.

An Ion Trap mass spectrometer with atmospheric pressure electrospray ionization was used for quantification in a negative ionization mode. The operating conditions were as follows: dry temp (350°C), capillary voltage (-4000 V), nebulizer (30 psi), dry gas (helium, 9 l/min). **Administration:** Two healthy Caucasian volunteers gave their informed consent to participate in the study. Volunteers had to take no drug or grapefruit juice for at least 10 days prior and during study. Urinary cortisol, cortisone and 6β-hydroxycortisol levels were determined on complete 24 hrs collections for 6 consecutive days. The first 3 days urine samples were used as a controls. Starting on day 4 the volunteers are given, single, i.m. injections of 1 mg Synacthen Depot. Excreted urine fractions were collected for a period of 6 days. Urine samples were stored at -20°C until analysis.

Reference range for healthy population: For the reference interval evaluation, we collected urine sample from Caucasian healthy volunteers. Thirteen healthy volunteers aged 21–50 years; 6 males and 7 females, formed the reference population. To study the circadian rhythm, urine samples were collected from 06-10, 10-14, 14-18, 18-22 and 22-06 hrs.

Reference range for athletes: For the reference interval evaluation, we collected urine sample from three competing athletes (age 20-32 year, one female and 2 males). Excreted urine fractions were collected for a period of 3 days without training, 3 days with moderate training and 3 days after heavy training. Urine samples were stored at -20°C until analysis.

Urine extraction: To 5 ml of urine samples were added 5 μl of fluoxymesterone (ISTD, 100

µg/ml) and 0.1 g of solid buffer ($NaHCO_3/K_2CO_3$, 2:1 mixture) to adjust the pH to 9.0. Then, 5 g of ammonium sulfate were added and extracted with 5 ml of mixture diethyl ether-toluene (50:50, v/v). After shaking (2 minutes) and centrifugation (5 minutes, 1000 *g*), the organic layer was separated and evaporated to dryness under nitrogen at 40°C. The dried residue was reconstituted with 50 µl of methanol, and 5 µl of this solution was injected into the LC-MS Ion Trap.

Results and Discussion

EPO analysis: The clinical scientific interest in ADMA and related compounds has increased quickly in the last years. Due to the potential regulatory role of methylated arginine analogs on NOS activity, special attention has been paid to analytical approaches to the quantitative determination of NMMA, ADMA and SDMA in biological fluids.

Table 1. Accuracy data for determination of ADMA, SDMA, arginine and citrulline.

Concentration added, µg/ml (n=5)	Concentration measured, (Mean±SD), µg/ml	Concentration calculated, µg/ml	Recovery, % (CV, %)
ADMA			
0	23,5 ± 0,5	-	-
10.0	33,1 ± 0,7	9,6	93,2 ± 14 (15,0)
30.0	54,7 ± 1,6	31,2	96,8 ± 8 (8,3)
100.0	127,1 ± 2,5	103,6	104,0 ± 5,4 (5,2)
200.0	224,1 ± 6,4	200,6	99,8 ± 2,2 (2,2)
SDMA			
0	24,2 ± 0,2	-	-
10.0	34,4 ± 0,9	10,2	96,7 ± 10 (10,3)
30.0	54,0 ± 1,2	29,8	102,0 ± 7 (6,9)
100.0	123,9 ± 2,7	99,7	98,7 ± 3,2 (3,2)
200.00	231,4 ± 7,2	207,2	99,9 ± 1,8 (1,8)
Arginine			
0	7,1 ± 0,05	-	-
2.0	8,8 ± 0,08	1,7	97,7 ± 14 (14,3)
8.0	14,7 ± 0,2	7,6	100,8 ± 8 (7,9)
20.0	26,6 ± 0,3	19,5	102,2 ± 5,4 (5,3)
60.0	65,5 ± 1,4	58,4	99,6 ± 2,2 (2,2)
Citrulline			
0	8,2 ± 0,08	-	-
2.0	10,3 ± 0,1	2,1	92,3 ± 9 (9,8)
8.0	22,5 ± 0,3	7,8	99,6 ± 8 (8,0)
20.0	29,4 ± 0,3	21,2	100,4 ± 4,0 (4,0)
60.0	68,9 ± 1,2	60,7	101,2 ± 1,5 (1,5)

Calibration curves (based on peak areas of selected extracted ions) were linear in the region 1–250 μg/mL (four points, five repeated measurements, 10, 30, 10 and 200 μg/mL of standards injected). The correlation coefficients were larger than 0.991 with a precision (expressed as coefficient of variation, CV) within 10%. Accuracy for spiked samples (recovery) fell within 92.3–104.0% (Table 1). Detection limits (LODs) (signal-to-noise ratio 3:1) of the signal of the extracted ions were ca. 25 ng/mL and refer to spiked samples. Ion suppression was < 12%.

The results of this study provide simple procedure for the detection of EPO abuse. Measurement of urine ADMA, SDMA, arginine, and citrulline concentrations has great potential value for assessing EPO abuse by athletes.

The variations and changes of the urinary levels of ADMA, SDMA, arginine, and citrulline were monitored at different time frames of a day for healthy population. The highest level occurs at about 8 to 12 am, and lowest level is at about 4-10 pm. Therefore ADMA, SDMA, arginine, and citrulline levels submit to circadian rhythm (Table 2).

Table 2. The circadian rhythm of urinary ADMA, SDMA, arginine and citrulline excretion in fifteen healthy volunteers (n=15)

	ADMA, μg/ml (CV,%)	**SDMA, μg/ml (CV,%)**	**Arginine, μg/ml (CV,%)**	**Citrullin, μg/ml (CV,%)**
06.00-10.00	35,6± 3,1 (9)	34,2±3,5 (10)	8,8±1,2 (15)	8,1±1,3 (16)
10.00-14.00	19,4± 2,6 (13)	20,1±2,8 (14)	4,9±0,8 (16)	4,4±0,7 (16)
14.00-18.00	17,1± 2,2 (13)	14,2±1,8 (13)	3,2±0,7 (22)	4,1±0,7 (17)
18.00-22.00	15,7± 1,9 (12)	12,0±1,5 (13)	2,9±0,5 (17)	3,4±0,6 (18)
22.00-06.00	25,4± 3,4 (13)	24,6±2,6 (11)	6,5±0,9 (14)	5,9±1,1 (19)

The study of variation of ADMA, SDMA, arginine, and citrulline levels before and after r-HuEPO administration was performed with two healthy male volunteers. Before EPO administration urinary concentrations of ADMA and SDMA were 10.0 – 40.0 μg/ml, arginine and citrulline were 0.5 – 10.0 μg/ml.

Table 3. Mean concentrations of ADMA, SDMA, arginine, and citrulline before and after EPO administration

		ADMA, μg/ml (CV,%)	**SDMA, μg/ml (CV,%)**	**Arginine, μg/ml CV,%)**	**Citrulline, μg/ml (CV,%)**
Men № 1	Before(n=18)	14,8± 6,2 (42)	14,2±5,8 (41)	4,8±1,9 (40)	4,1±1,7 (41)
	After (n=65)	**54,1**±15,1 (28)	**56,1**±14,7 (26)	**14,6**±3,2 (22)	**16,1**±4,4 (27)
Men № 2	Before(n=24)	28,7± 9,6 (33)	28,5± 10,5 (37)	5,6± 1,8 (32)	7,3± 2,3 (32)
	After (n=72)	**125,4**± 23,8 (19)	**120,1**± 20,4 (17)	**24,8**± 6,3 (25)	**37,2**± 9,2 (25)

A single dose injection of EPO cause the increase of ADMA, SDMA, arginine, and citrulline concentrations up to 40-270 μg/ml, 40-240 μg/ml, 10-60 μg/ml and 12-140 μg/ml, respectively (Table 3).

The results showed that concentration of ADMA, SDMA, arginine, and citrulline were nearly 4-fold increased after administration of single intramuscularly Erythropiethine (2000 U/day, r-HuEpo, Epoetin Alfa, Epocrine®, St-Petersburg) injection (Fig. 1).

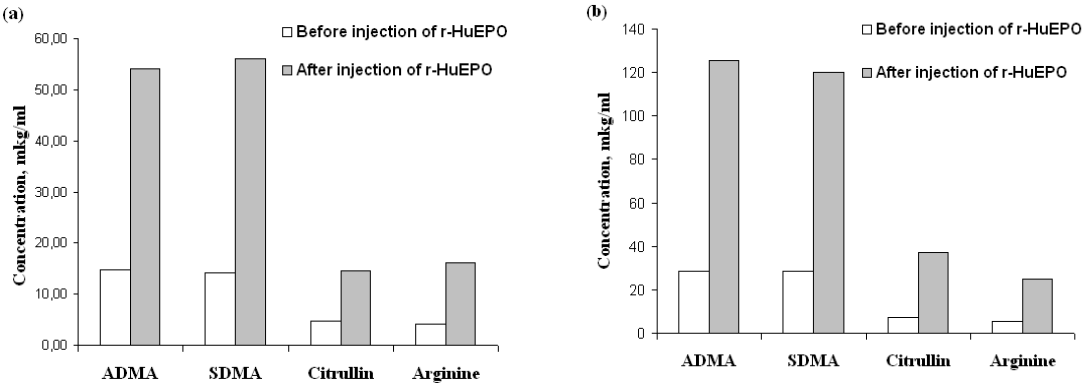

Figure 1. Comparison of mean concentrations of ADMA, SDMA, arginine, and citrulline before and after EPO administration. (**a**) male, 23 years old, out of sport; (**b**) male, 37 years old, long-distance runner

Study of the relation between urinary concentration of ADMA and different biochemical parameters of ADMA-DDAH-NOS system demonstrated a significant correlation ($r = 0.79$, $P < 0.001$) between the ADMA, SDMA, arginine, and citrulline. In this way we suppose that the analytical sample's ADMA, SDMA, arginine, and citrulline levels may be reported by the laboratory in normal concentration range or high range. The indirect HPLC-MS/MS analysis is used to eliminate negative samples from positive ones (atypical results). Due to the negative analysis results we can take into consideration a sample as no EPO drug abuse. High levels of ADMA, SDMA, arginine, and citrulline are interpreted as atypical results (e.g. to create target high-risk group for future test distribution plan), caused by the intrinsic stimulation effects, endocrine disorders or external synthetic r-HuEPO-like drug abuse. The results of atypical samples must be confirmed by direct EPO analysis.

Conclusion: The method will allow to cover a significant amount of urine samples in order to detect suspicious for further direct EPO analysis or follow-up those subjects who showed abnormal results (e.g. to create target high-risk group for future test distribution plan). Indirect HPLC-MS/MS method of r-HuEPO abuse detection based on urinary levels of ADMA, SDMA, arginine and citrulline variations is fast screening with minimal sample preparation. Important, that samples might be stored and analysed in a large group before

disposal since monitored compounds are not prone to biodegradation. In general it could be used as a screening in order to decrease the number of sample with low probability of EPO abuse and save costs and human workload because direct analysis (IEF+DB+ChD) of r-HuEPO is a very long, complex and expensive. Moreover, with regard to novel biosimilar r-HuEPOs, PEGylated r-HuEPO abuses and others anaemia drugs which may be not detectable via the electrophoresis test (IEF+DB+ChD), our urinary indirect biochemical markers will be of indispensable help in order to prove possible administration of those new pharmaceuticals.

Since the medical risks associated with polypeptide doping are still considerable we propose simple and cheap screening method which will be effective as for detection of r-HuEPO abuse for doping control purposes and as well for early recognition of cardiovascular risk that is a very important for healthy athletes.

Synacthen analysis: Cortisol and cortisone are steroid hormones synthesized and liberated in the suprarenal cortex. Natural cortisol level changes during daytime. Highest levels occur at 6 to 8 am, and lowest level is around midnight. It is known that cortisol and cortisone are released in humans for the adaptation to stress, and the concentrations of cortisol and cortisone change during emotional events like games or competitions. Episodic secretion of cortisol is caused by the intermittent transformation of cortisol from its precursors in the adrenal cortex stimulated by ACTH. And the ratio of urinary 6β-OHF/F in considered as a non-invasive marker of human *in vivo* CYP3A4 activity. Therefore it looks preferable to measure the F/E and 6β-OHF/F ratios in the doping test to evaluate possible abuse of ACTH stimulation drugs.

Table 4. Recovery of 6βOHF, F and E, spiked to human urine (n=5)

Added (ng/ml)	Found (ng/ml)	Recovered (ng/ml)	Recovery (%)
Cortisol (F)			
0	17.24	-	-
20.00	35.44	18.2	91
60.00	74.54	57.3	95.5
Cortisone (E)			
0	34.20	-	-
20.00	53.90	19.7	98.5
60.00	92.80	58.6	97.7
6β-OH-cortisol (6βOHF)			
0	256.8	-	-
100	352.0	95.2	95.2
300	562.2	305.4	101.8

To check the recovery, urine samples were spiked with two different concentration of cortisol, cortisone and 6β-OH-cortisol. The results are shown in Table 4. The lowest limits of

detection for cortisol, cortisone and 6β-OH-cortisol were 0.5, 1.2 and 5 ng/ml respectively, at a signal-noise ratio of 3. The reproducibility of the method was assessed by repeat analysis of urine samples containing two different concentrations of F, E and 6β-OHF. The within-day CV was below 4.3 %, while the between-day CV was found to be below 5.8 %.

Reference range for healthy population and athletes: The excretion of cortisol, cortisone and 6β-OH-cortisol showed strong circadian rhythm with a wide inter-individual variability as indicated by variation coefficients (Table 5). The variations in urinary concentration of cortisol, cortisone and 6β-OH-cortisol were relatively parallel to each other. Therefore changes in the urinary rations of F/E and 6β-OHF/F were not statistically significant during the day. The mean values of F/E and 6β-OHF/F ratios were about 0.4 and 16.5 respectively for healthy population. Therefore F/E ratios for healthy population are within 0.1-0.9 range and 6β-OHF/F ratios are within 4-28 range.

Table 5. The circadian rhythm of urinary 6βOHF, F and E excretion in thirteen healthy volunteers (n=13)

Collection period	F, ng/ml CV (%)	E, ng/ml CV (%)	6βOHF, ng/ml CV (%)	F/E CV (%)	6βOHF/F CV (%)
06.00-10.00	8.5±2.9 34	24.6±7.6 31	141.5±22.5 16	0.34±0.14 41	16.2±4.8 30
10.00-14.00	6.7±3.2 48	22.5±8.2 36	95.3±19.6 21	0.28±0.25 46	14.3±3.5 24
14.00-18.00	5.9±3.5 59	15.9±5.6 35	90.3±33.6 37	0.44±0.09 20	19.1±8.5 45
18.00-22.00	2.6±1.3 50	11.2±6.7 60	53.6±19.3 36	0.39±0.23 59	16.8±7.2 43
22.00-06.00	2.9±1.6 55	7.4±4.1 55	43.9±21.2 48	0.49±0.35 71	15.9±5.2 33

Further data variations and changes of F/E ratio were monitored for three competing athletes (age 20-32 year, female and 2 males), to obtain individual information about their F, E and 6β-OHF urinary excretion. The mean values for F/E ratio were about 0.6. After moderate training session the mean values were about 0.3, and after heavy training workload associated with stress the mean values were about 0.8. These results show that moderate trainings decrease F/E ratio, while competition do increase F/E ratio (Table 6). No significant differences were observed in the 6β-OHF/F ratios after moderate and heavy training. This may indicate that the level of hydroxylation of cortisol to 6β-OH-cortisol was unaltered by the training programme. F/E Ratios for athletes without and during training are within 0.2-1.4 range and 6β-OHF/F ratios are within 10-30 range.

Table 6. Change of F/E and 6βOHF/F ratios without training, after moderate and heavy training (n=3)

Collection period	Athletes without training and any stress situation		Athletes after moderate training		Athletes with heavy training	
	F/E (CV, %)	**6βOHF/F (CV, %)**	**F/E (CV, %)**	**6βOHF/F (CV, %)**	**F/E (CV, %)**	**6βOHF/F (CV, %)**
1 day	0.75±0.24 (32)	14.9±4.2 (28)	0.41±0.18 (44)	16.2±6.2 (38)	0.68±0.22 (32)	19.3±9.2 (48)
2 day	0.48±0.19 (40)	16.5±5.1 (31)	0.21±0.08 (38)	14.3±4.7 (33)	0.97±0.32 (33)	15.4±6.9 (45)
3 day	0.56±0.20 (36)	14.4±3.9 (27)	0.28±0.10 (36)	17.1±7.2 (42)	0.77±0.27 (35)	18.8±8.1 (43)

Therefore, possible variations of F/E and 6β-OHF/F ratios for healthy population and athletes are within 0.1–1.4 range and 4.0-30.0 range, respectively, which might be considered as normal.

Synacthen administration: As we have found free fraction F/E ratio was higher in healthy volunteers after ACTH stimulation (2.0–5.5) than in untreated controls (0.1–1.4). The ratios of free 6β-OH-F/F were 4.0-30.0 in healthy volunteers and 0.9-3.8 after Synacthen administration (Table 7). Therefore Synacthen decreases 6β-OHF excretion in human by 4-6 times.

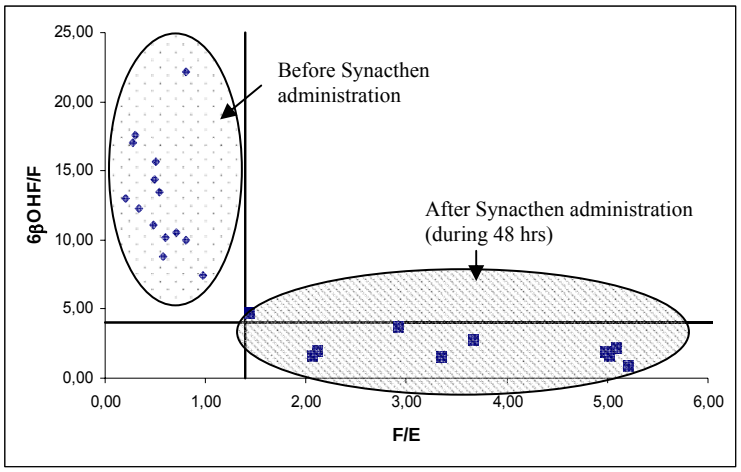

Figure 2. Variation in basal and after-Cosyntropin administration (Synacthen®, 1 mg intramuscularly) F/E and 6β-OHF/F ratios

Comparative analysis of F/E and 6β-OHF/F ratios before and after Synacthen administration showed changes that are observed during 30 hrs after ACTH stimulation (Fig. 2).

Table 7. Change of F/E and 6βOHF/F ratios before and after Synacten administration (two

healthy volunteers, i.m. 1 mg) (n=2)

Collection period	Before				After			
	F/E		6βOHF/F		F/E		6βOHF/F	
	Mean ±SD	CV (%)	Mean ±SD	CV (%)	Mean ±SD	CV (%)	Mean ±SD	CV (%)
1 day	0.42 ±0.12	29	17.1 ±5.6	33	4.28 ±1.29	30	1.67 ±0.42	25
2 day	0.50 ±0.30	60	13.3 ±8.5	64	2.03 ±1.20	59	3.45 ±2.08	60
3 day	0.45 ±0.23	51	15.2 ±6.7	44	0.53 ±0.21	40	12.90 ±4.85	38

Conclusions: Increase of F/E ratio appears after administration of corticotrophins (2-5 times). Therefore F/E ratio monitoring might be used as a screening procedure to select samples for the further confirmatory analysis to be developed in the near future. Our monitoring protocol does not require any changes in routine doping control operation procedures, and is recommended as a helpful tool for gathering important analytical information. The inhibition activity of Synacthen on liver microsamal enzyme 3A4 was evident in the present study through the observed significant decrease in 6β-OH-F/F rations among volunteers who received Synacthen compares to healthy control subjects.

References

Scalera F, Kielstein JT, Martens-Lobenhoffer J, Postel SC, Täger M, Bode-Böger SM. (2005) Erythropoietin increases asymmetric dimethylarginine in endothelial cells: role of dimethylarginine dimethylaminohydrolase. J Am Soc Nephrol 16, 892-898.

Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, Imaizumi T. (1999) Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. Circulation 99, 1141-1146.

Appolonova S, Dikunets M, Rodchenkov G. (2008) Possible indirect detection of rHuEPO administration in human urine by high- performance liquid chromatography tandem mass spectrometry. Eur J Mass Spectrom, 14, 201–209.

Thevis M, Bredehöft M, Geyer H, Kamber M, Delahaut P, Schänzer W. (2006) Determination of Synacthen in human plasma using immunoaffinity purification and liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 20, 3551-3556.

Martens-Lobenhoffer J, Krug O, Bode-Boger SM. (2004) Determination of arginine and asymmetric dimethylarginine (ADMA) in human plasma by liquid chromatography/mass spectrometry with the isotope dilution technique. J Mass Spectrom 39, 1287-1294.