A.C.A. Silva[1)], H. M. G. Pereira[1)], F. R. Aquino Neto[1)].

Evidences of Interindividual Variability in Phase II Urinary Excretion Profiles of Endogenous Glucocorticosteroids

[1)] LABDOP-LADETEC, Institute of Chemistry, Federal University of Rio de Janeiro, Brazil

Introduction

Endogenous glucocorticosteroids are recognized as an important parameter for endocrine diseases diagnosis. Potentially, in doping control, those hormones can act as: i) biomarkers for abuse of exogenous glucocorticosteroids (negative feedback, decreasing the secretion of endogenous hormones), ii) biomarkers for the abuse of ACTH (in order to promote the hypertrophy of the adrenal gland and thus increase the endogenous production of testosterone and finally, iii) the abuse of endogenous glucocorticosteroids (cortisol and cortisone). Nevertheless, in contrast with the great knowledge accumulated regarding androgenic hormones, little information is available to the urinary excretion profile of glucocorticosteroids. Stress dependence and circadian cycle are the most well established characteristics. Indeed, even information related to the phase II metabolism is controversial and few reports in the literature are dedicated to this topic. Such information is essential to the development of analytical methods and evaluation of the urinary profile. Just after a better comprehension of this profile, the quantification of the urinary levels of endogenous glucocorticosteroids can be evaluated as a tool in doping analysis. LC-MS/MS is the technique of choice for the analysis of the endogenous glucocorticosteroids in urine due to its high sensitivity, selectivity and not requiring previous derivatization before analysis.

The aim of this work was to evaluate the excretion profile of endogenous glucocorticosteroids phase II metabolites in human urine.

Materials and Methods

The ethical committee from the Clementino Fraga Filho University Hospital supports the study (protocol n 168/02). Urine from volunteers were analyzed and compared with urines from professional athletes. Briefly, three aliquots of 2 mL for each sample were processed by liquid-liquid extraction (LLE) using TBME: ethyl acetate (3:1) as extraction solvent. In order to assess information about the phase II metabolites from the samples, a previous step of

enzymatic hydrolysis using an enzyme with β-glucuronidase activity (aliquot # 1) and other enzyme with β-glucuronidase / arylsulfatase activity (aliquot # 2) was included. The aliquot # 3 was processed without hydrolysis. Flumethasone 500 ng/mL was used as internal standard. The residues were dissolved in 200 µL of methanol:H_2O (1:1). 20 µL of the sample were injected into the LC-MS/MS system. The analyses were performed in the LC-MS/MS system (Varian 1200L Triple Quadrupole), Prostar 210 pumps, automatic injector Prostar 410, on line degasser and electrospray interface (ESI). The chromatographic conditions were: column eclipses (Agilent®) C-18 of reverse phase (150 mm x 4.6 mm, 5 µm), elution solvent: Methanol (B) and 0.1% (v/v) formic acid + 5 mM of ammonium formate in H_2O (A). MRM acquisition for cortisol, cortisone and flumethasone (IS) are described in Table 1. The validation was performed to agree with WADA rules (WADA, 2010).

Table 1: Structure of studied glucocorticosteroids, precursor ion, product ions and collision energy.

Analyte	Precursor ion (*m/z*) $[M+H]^+$	Product ion (*m/z*) and Collision energy (V)
Cortisone	361	135(-22), 145(-26), 163(-22)
Cortisol	363	121(-22), 309(-14), 327(-14)
Flumethasone	411	253(-12), 371(-8), 391(-6)

The reference population was composed of 65 non-athlete volunteers with ages between 18-40 years. For this group, the first urine in the morning was collected. The second population was composed of 80 athletes of different sporting modalities. The samples were collected after competition.

Results and Discussion

The developed methods allowed the identification and quantification of the analytes cortisol and cortisone in human urine by the technique of LC-MS/MS with ESI+ interface and acquisition mode by MRM. The validation data (not described) indicate the approaches are fit-to-purpose concerning the aim of the study. As expected, the nominal hormone concentration in urine is higher in the athletes' population, since effect of stress surpasses the circadian rhythm. Through the results of the procedures with and without hydrolysis it was possible to estimate for cortisol and cortisone the proportion of the excreted free, glucuconjugated and sulfoconjugated fractions. Evaluating the results related to the average of the cortisol and cortisone fractions excreted in urine (Figures 1A and 1B), it was possible to observe the difference in the phase II excretion profile between the athletes and non-athlete populations; cortisol was mainly excreted conjugated in both populations studied.

However, cortisone was mainly excreted free in the non-athlete population. From the analysis of the athlete's population, the glucuconjugated and sulfoconjugated fractions (G +S) seem to be more important after physical stress.

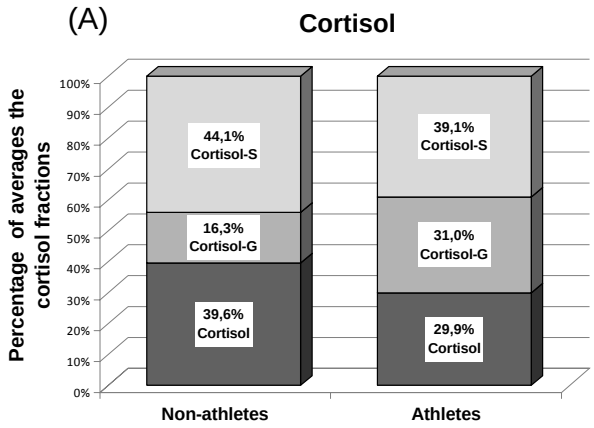

Figure 1A: Proportion averages of cortisol fractions excreted in the athletes and non-athletes populations.

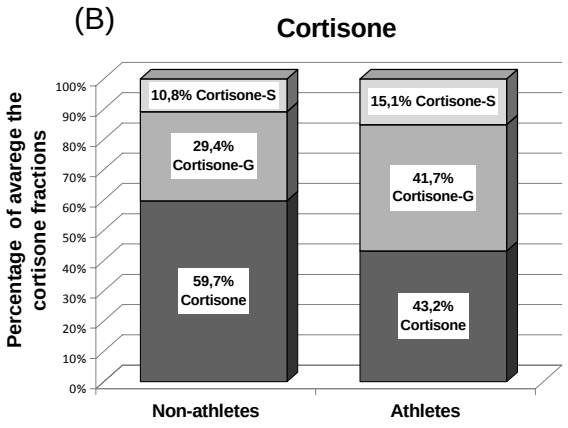

Figure 1B: Proportion averages of cortisone fractions excreted in the athletes and non-athletes populations.

The mean values results obtained for the phase II metabolites excretion corroborate the data previously described by Palermo *et al*. (1996) and Shibasaki *et al*. (1992). Nevertheless, clearly the approach considering only the averaged data does not evidence the complexity of the profile.

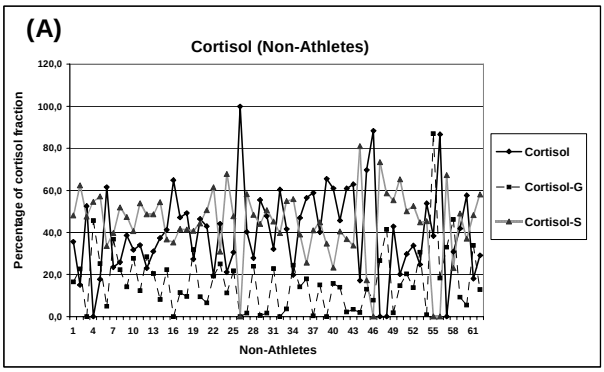

Figure 2A: Percentage of cortisol fraction in non-athletes' population.

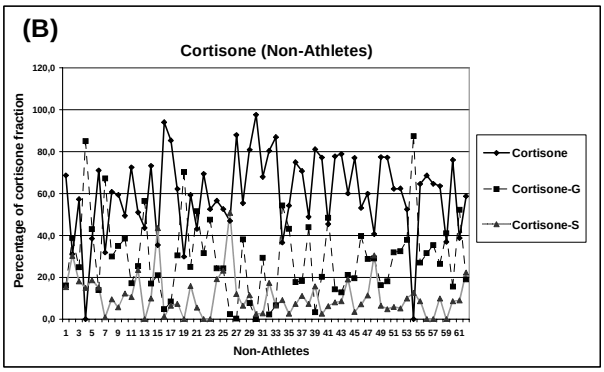

Figure 2B: Percentage of cortisone fraction in non-athletes' population.

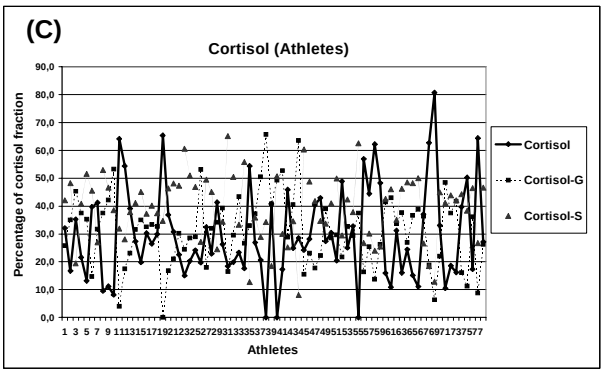

Figure 2C: Percentage of cortisol fraction in athletes' population.

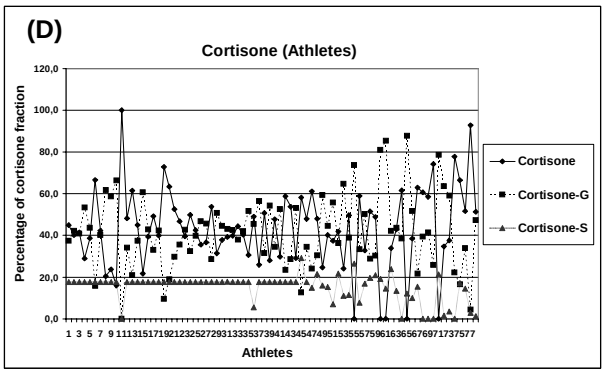

Figure 2D: Percentage of cortisone fraction in athletes' population.

Indeed, a great interindividual variability was observed for the profile of phase II excretion of cortisol and cortisone in both populations, as can be observed in the figures 2A, 2B, 2C e 2D. The large interindividual variability observed in the excretion profile of phase II metabolites of cortisol and cortisone can be explained by the large interindividual variability in the levels of hepatic UGT (UDP-glucuronosyltransferases). Two main families, UGT1 and UGT2 are predominantly involved in glucuronidation (Ishi *et al*. 2010). Several factors are recognized as having influence in UGT activity, including genetic polymorphism. Some studies also demonstrated that UGT2B7 is the major enzyme responsible for glucuronidation of several steroid hormones, such as glucocorticosteroids (Bock, 2010). The results of this study highlight the larger variability in phase II metabolism of glucocorticosteroids when compared with androgen hormones. More data are necessary to evaluate the source of this kind of difference. However, considering the possible interindividual instability, it should be considered to use the total fraction (F+G+S) in the evaluation of the endogenous glucocorticosteroids profile. Otherwise, the evaluation of this profile could be jeopardized due to the phase II metabolism influence.

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References

Bock, K. W. (2010) Functions and transcriptional regulation of adult human hepatic UDP-glucuronosyl-transferases (UGTs): Mechanisms responsible for interindividual variation of UGT levels. Biochem. Pharmacology, 80, 771-777.

Ishi, Y.; Nurrochmad, A.; Yamada, H. (2010) Modulation of UDP-Glucuronosyltransferase activity by endogenous compounds. Drug Metabolic Pharmacokinetic 25(2), 134-148.

Palermo, M.; Gomez-Sanchez, C.; Rotman, E.; Shackleton, C. H. L. (1996) Quantitation of cortisol and related 3-oxo-4-ene steroids in urine using gas chromatography/mass spectrometry with stable isotope-labeled internal standards. Steroids, 61, 583-589.

Shibasaki, H.; Furuta, T.; Kasuya, Y. (1992) Stable isotope diluition mass spectrometry for the simultaneous determination of cortisol, cortisone, prednisolone and prednisone in plasma. Journal of Chromatography, 579, 193-202.

World Anti-Doping Agency. The 2010 Prohibited List. International Standard, Montreal (2010) http://www.wada-ama.org/rtecontent/document/2010_List_En.pdf (access date 25.04.2010).