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## **Analysis of Glucocorticosteroids by GC-MS in Doping Control**

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### **Introduction**

Glucocorticosteroids (CT) are on the current Prohibited List of Substances for In-Competition Testing<sup>1</sup>. Despite the fact that GC-MS is the most used technique for residue detection in doping control labs, LC-MS<sup>n</sup> has been suggested due to its greater sensitivity and to eliminate a derivatization step. Indeed, in animal residue analysis, where ultimate sensitivity is necessary, LC-MS<sup>n</sup> has been the technique of choice<sup>2</sup>. Recently, WADA established a minimum requirement performance limit (MRPL) of 30ng/mL<sup>4</sup>. There fore, in doping control scope, lower sensitivity (< 1pb) is not required for CT analysis. In addition, TMS-enol-TMS derivatives seem to be suitable for detection of some CT by GC-MS<sup>5,6</sup>. Another aspect not well studied in doping control is the analysis of the profile of endogenous CT as marker for abuse of exogenous and /or endogenous agents. Our goal was to evaluate a method to detect synthetic and endogenous glucocorticosteroids based on hydrolysis with *H. pomatia*, liquid-liquid extraction with TBME:*tert*-butanol (4:1, v:v), derivatization step with MSTFA:NH<sub>4</sub>I:2-mercaptoethanol and analysis by GC-MS in positive ionization mode (EI).

### **EXPERIMENTAL**

#### ***Sample Preparation***

In 2 mL of urine, 250 µL of acetate buffer 0.2 M and 100 µL of β-glucuronidase / aril-sulfatase were added. The mixture was incubated at 50°C for 1h. After that, the pH was adjusted to 7.0 with 500 µL of phosphate buffer 0.2M. The analytes were extracted with a mixture of 4 mL of TBME and 1 mL of t-butanol (shaker for 20 min and centrifuge for 5 min at 3000 rpm). After transfer the upper organic phase was evaporated under N<sub>2</sub> flow. The residue was kept in desiccators with KOH/P<sub>2</sub>O<sub>5</sub> for 30 min. The formation of TMS–Enol–

TMS derivatives was done using MSTFA–NH<sub>4</sub>I– 2 – mercaptoethanol (1000:2:6, v/w/v) reaction mixture at 60°C for 20 min.

### **GC-MS analysis**

Agilent 6890 Series GC System equipped with a 7683 automatic injector with electronic pressure control and interfaced to an Agilent 5973 mass selective detector. MS operating temperatures were as follows: transfer line, 280°C; ion source, 230°C; and quadrupole, 150°C. Detection was done by selected ion monitoring (SIM) with a dwell time of 20 ms. The ionisation was done by electron impact at 70eV. GC operation conditions were as follows: injector, 280 °C; column, 140°C (initial temperature) followed by a gradient of 40°C/min to 180 °C, followed by a gradient of 3.0 °C/min to 229 °C/min and 40 °C/min to a final temperature of 310 °C (held 3.0 min); total flow of He, 18.4 ml/min; pressure, 16.0 psi; average linear velocity, 38 cm/s; 1 µl samples were injected in the split mode (ratio 1:10). An HP-1 fused-silica capillary column (17.0 m x 0.2 mm x 0.11 µm film thickness) was used.

### ***Validation of the analytical procedure***

To validate the present method a pre-study validation routine has been performed, including sensitivity, specificity, linearity, accuracy, and repeatability.

### ***Endogenous CT profile***

The CT profile from athletes and sedentary volunteers were compared. The first urine of the morning was collected from sedentary volunteers (n = 42). Athletes' samples from Brazilian Soccer Championships (n = 95) were analyzed according to the doping control protocol. Rio de Janeiro Federal Hospital Ethics Committee of Clinical Investigation approved the clinical protocol (protocol number: 032/03).

## **RESULTS AND DISCUSSION**

The method is very similar to the screening IV (it differs only in the hydrolysis and extraction steps), therefore it is applicable in any doping control lab. Even considering the observations of Fluri *et al*<sup>2</sup> and Deventer & Delbeke<sup>3</sup>, that in humans exogenous corticosteroids are excreted non-conjugated, the hydrolysis step was kept aiming the study of endogenous profile. As reported previously<sup>5</sup>, TMS-enol-TMS derivatives for CT show some chromatographic and spectrometric advantages in comparison with MO-TMS derivatives. Some CT, such as beclometasone and triamcinolone, were difficult to derivatize under the conditions used. This fact limits the use of the method for screening proposes. The use of microwave-assisted derivatization approach as suggested by Amendola *et al*.<sup>6</sup>, might

overcome some of these inconveniences. On the other hand, the GC-MS method using the traditional thermal incubation could be an alternative for confirmation analysis of a series of CT, e.g. as dexametasone, betametasone, prednisolone. With the TBME/t-butanol extraction procedure, the recovery of endogenous and exogenous CT is above 85%. The repeatability was better than 15% and the linearity was suitable ( $r^2 > 0.99$ ) for all compounds evaluated. The LOD for endogenous analytes as cortisol (F), cortisone (E), tetrahydrocortisol (THF), tetrahydrocortisone (THE) and 6 $\beta$ -hydroxycortisol (6 $\beta$ OH) was near 10 ng/mL. The method was used for the quantification of these analytes aiming at a CT profiling. Figure 1 shows the concentration ratios of CT from athletes and sedentary volunteers.

The athletes and sedentary individuals show a non-normal distribution profile (Kolmogorov-Smirnov test). So, a non-parametric statistic approach was used to confront the concentrations and ratios of endogenous CT. As expected, it was observed a great disparity considering the concentration of endogenous CT (data not shown). In fact, it's well known that the absolute concentrations of CT do not give consistent information because of their great variability due to the response of the hypothalamic-pituitary axis to stress. The ratios model proposed by Yap & Kazlauskas<sup>7</sup> seems to be the best approach available to future studies for profiling changes in endogenous CT due to ACTH or tetracosactide doping. The non-parametric Mann-Whitney test was used to compare the ratios of athlete and sedentary populations. The ratios THE/E, THE/F, THE/THF, E/F e 6 $\beta$ OH/F show no statistic difference in these populations. Further studies must be done to evaluate if these parameters are stable in order to be used as markers of change of CT profiles due to the abuse of exogenous or endogenous agents.

The method was also evaluated for the detection of exogenous CT. Different aliquots of a pool of urine (BU) was spiked at concentrations of 50 ng/mL, 30 ng/mL, 20 ng/mL, 10 ng/mL and 5 ng/mL. Figure 2 shows the fragmentogram of betamethasone at concentrations of 30 ng/mL and 10 ng/mL and BU. The results indicate that the method is suitable for the detection of betamethasone in concentration of 30 ng/mL. Despite of the dirty extract due the use of *H. pomatia* (aiming endogenous profile study) the LOQ for the betametasone is near of 10 ng/mL (S/N > 5). For the MRPL (30 ng/mL) the repeatability is 11%. The comparison between the spiked samples and the BU shows no coelution of betamethasone and endogenous compounds, demonstrating a suitable specificity. The coelution with others exogenous was previously evaluated and just dexametasone show poor specificity<sup>5</sup>. The extrapolation to other glucocorticosteroids awaits on a validation.

## CONCLUSION

Being similar to the screening IV the method is applicable in any doping control lab. The solvent extraction mixture shows good recovery for all CT evaluated. Despite of low yield for beclometasone and triamcinolone, the derivatization step is not time consuming for most CT as dexametasone, betametasone and prednisolone, an advantage if compared other derivatization strategies in the literature. The validation study for endogenous CT shows good repeatability and sensitivity. So, considering WADA's MRPL the method seems suitable to confirm several synthetic glucocorticosteroids and evaluate the endogenous CT profile in athletes' urine.

## REFERENCE

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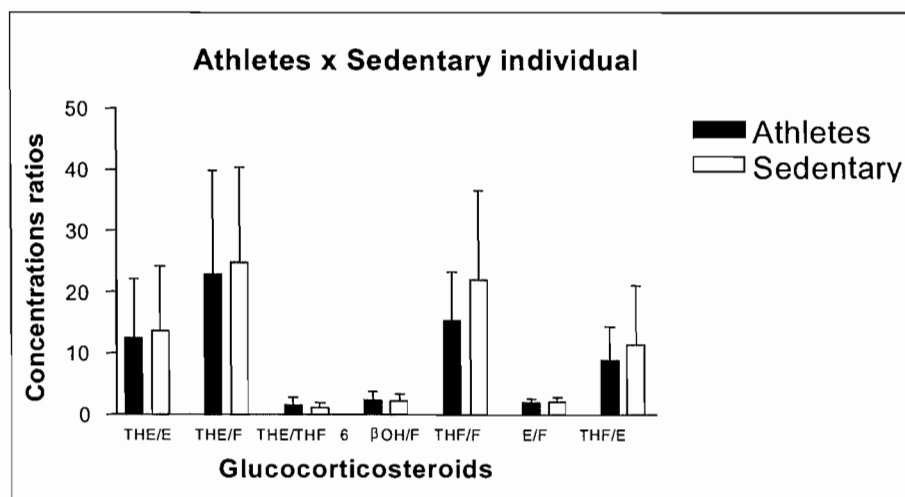


Figure 1. Comparison between concentration ratios of endogenous CT from athletes and sedentary volunteers.

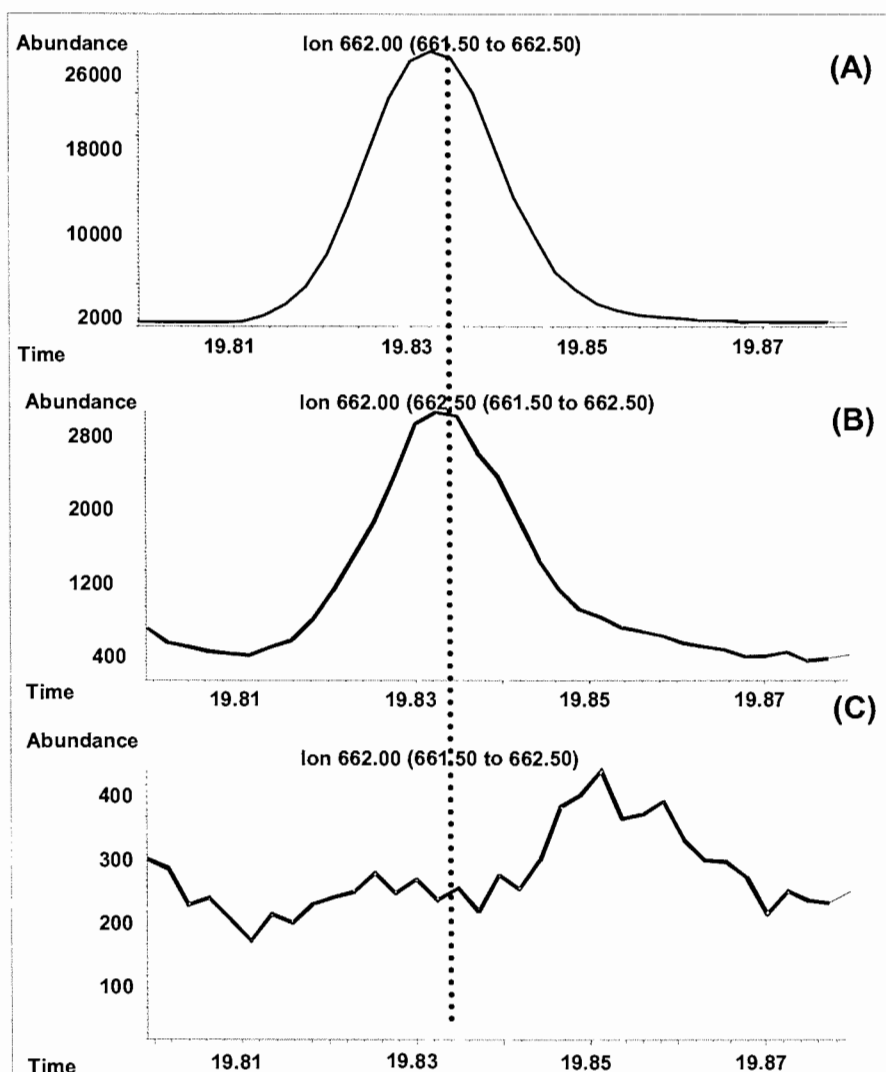


Figure 2. GC-MSD fragmentograms of betamethasone spiked urine. (A) 30 ng/mL, (B) 10 ng/mL and (C) urine blank (BU).