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Quantification of Epitestosterone and Testosterone in Urine by Gas Chromatography-Mass Spectrometry

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## **Quantification of Epitestosterone and Testosterone in Urine by Gas Chromatography-Mass Spectrometry**

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### **INTRODUCTION**

The International Olympic Committee Medical Commission established that an epitestosterone (E) concentration in urine greater than 200 ng/ml must be investigated because this steroid can be used as a masking agent to provoke “false negative” results for testosterone (T), due to modification of the T/E ratio. Different HPLC methods, including HPLC purification assays for subsequent spectroscopic analysis, have been reported for anabolic steroids. More recently, an HPLC method for the determination of epitestosterone and testosterone in urine has been published [1].

The most popular technique employed for the determination of epitestosterone and testosterone is gas chromatography-mass spectrometry (GC-MS) which makes use of an internal standard (I.S.). The use of [16,16,17-<sup>2</sup>H<sub>3</sub>]-testosterone and [16,16,17-<sup>2</sup>H<sub>3</sub>]-epitestosterone as internal standards [2] is widely accepted. These standards are sold in solution as a mixture in a ratio 6/1. The cost of this mixture, and the fact that it is not well suited for quantification of epitestosterone levels above 260 ng/ml [3], prompted us to develop an alternative method of quantification. This work describes a reliable GC-MS method for the simultaneous quantification of epitestosterone and testosterone, using 17 $\alpha$ -methyl-testosterone as I.S. The method is selective, repeatable, and sensitive.

## EXPERIMENTAL

**Calibration standards.** Epitestosterone and testosterone-free urine was obtained from a five years old child. This urine provides the matrix for spiking with known amounts of testosterone and epitestosterone in the range 10-600 ng/ml, allowing the construction of calibration graphs. Quantification was based on peak-area ratios of selected ions of analyte to I.S. versus concentration.

**Sample preparation.** Urine (4.0 ml) was treated according to the Cologne protocol for follow up of high T/E ratios [4].

**Chromatographic and mass spectrometric conditions.** The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) 5890 Series II Plus gas chromatograph, equipped with an autosampler/autoinjector, coupled to a Hewlett-Packard 5972 Mass Selective Detector. The column used was an HP Ultra-1 (25 m x 0.2 mm x 0.11  $\mu$ m) operated at a constant head pressure of 12 psi. Helium flow was 0.38 ml/min. Injections were performed in the split mode and the split ratio was 10:1. The injector was maintained at 280 °C, and the interface at 310 °C. The temperature program used was:  $T_1$ , 190 °C; rate<sub>1</sub>, 2 °C/min;  $t_1$ , 0 min;  $T_2$ , 235 °C; rate<sub>2</sub>, 25 °C/min;  $t_2$ , 0 min;  $T_3$ , 310 °C;  $t_3$ , 5 min. The mass spectrometer worked under electron ionisation conditions with an ionisation energy of 70 eV and a repeller voltage of 30 V. Data were acquired in the SIM mode. Monitored ions were  $m/z = 446$  (I.S.), 434 (androsterone and ethiocolanolone), 432 (epitestosterone and testosterone) and 435 ([16,16,17-<sup>2</sup>H<sub>3</sub>]-testosterone). Dwell times were 100 ms. Ions  $m/z = 446$  and 432 were extracted from each chromatogram and integrated with the RTE Integrator option provided by the data system.

## RESULTS AND DISCUSSION

Child urine was used as biological matrix for the construction of the calibration curves for testosterone and epitestosterone, because this “real urine” is more easily available and has no cost compared to “artificial urine”[5]. Our preliminary results showed that the concentration of testosterone and epitestosterone in urine from children up to five years old did not exceed the limit of detection of our procedure.

### Calibration and linearity.

The linearity of the method was evaluated over the range of concentrations 10-600 ng/ml using duplicate samples containing 20, 80, 140, 200, 260, 320, 400, 500 and 600 ng/ml. The linear regression equations and the correlation coefficients are presented in Table 1. These results demonstrate that both internal standards have similar performances. Hence it was decided to continue using methyltestosterone.

**TABLE 1.** Calibration graphs for testosterone and epitestosterone using different internal standards.

Analyte	Internal standard	Equation <sup>a</sup>	$r^2$
Epitestosterone	Methyltestosterone	$y = 0.0061x - 0.1097$	0.994
Epitestosterone	[16,16,17- <sup>2</sup> H <sub>3</sub> ]-testosterone	$y = 0.0047x - 0.0884$	0.996
Testosterone	Methyltestosterone	$y = 0.0056x - 0.0419$	0.998
Testosterone	[16,16,17- <sup>2</sup> H <sub>3</sub> ]-testosterone	$y = 0.0044x - 0.0358$	0.997

<sup>a</sup>  $y$  = Peak area ratio;  $x$  = concentration (ng/ml)

### Precision, trueness and recovery

The precision and trueness were estimated employing five different samples spiked at levels of 40 and 250 ng/ml, respectively, and five different samples with unknown levels of testosterone and epitestosterone. The overall method recovery was evaluated at 40 and 250 ng/ml using five spiked samples. The concentrations were calculated from the corresponding calibration graph. Statistical results are given in Table 2. The recovery for both steroids was always higher than 90 %.

**TABLE 2.** Trueness, precision and repeatability for T and E in urine (n = 5)

Concentration added (ng/ml)	Testosterone		Epitestosterone	
	Found (mean $\pm$ S.D.) (ng/ml)	R.S.D. (%)	Found (mean $\pm$ S.D.) (ng/ml)	R.S.D. (%)
40	37.8 $\pm$ 1.3	3.4	45.3 $\pm$ 1.1	2.4
250	225 $\pm$ 3	1.3	239 $\pm$ 14	5.9
Unknown	78.6 $\pm$ 2.3	2.9	32.6 $\pm$ 0.9	2.8

**Limit of quantification.**

The limit of quantification was defined, following the rules of the American Chemical Society [6], as the analyte signal to background noise (S/N) ratio of 10. The limit was 3 ng/ml for both epitestosterone and testosterone.

**Interferences.**

Several urinary endogenous steroids, available in our laboratory, were tested to establish possible interferences. None of the following compounds interfered with epitestosterone, testosterone and methyltestosterone: androsterone, ethiocholanolone, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, dihydrotestosterone, 4-androsten-3,17-dione, 11 $\alpha$ -hydroxy-androsterone, 11 $\alpha$ -hydroxy-ethiocholanolone, 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol, 5 $\beta$ -pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol, tetrahydrocortisol, and allo-tetrahydrocortisol

**CONCLUSIONS**

The proposed method is selective for the simultaneous determination of epitestosterone and testosterone due to the sample work-up and chromatographic separation. The relative standard deviations lower than 6 %, demonstrate the precision and repeatability of the method. The trueness is fairly good as the differences between the mean value and the true value are generally less than 10 %. This method has been tested by analysing 10 suspicious samples

with different levels of testosterone and epitestosterone, and has been used for quantitation epitestosterone levels higher than 200 ng/ml in three cases.

## ACKNOWLEDGMENTS

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