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Artificial urine as sample matrix for calibrators and quality controls in determination of testosterone to epitestosterone ratio

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Introduction

Testosterone (T) and epitestosterone (EpiT) are endogenous steroids, and according to the list of prohibited substances and technical documents established by WADA [1,2] the urinary ratio T/EpiT > 4 of the glucuronide-conjugated steroid fraction indicates abnormal steroid profile and initiates further investigations in the laboratory. Due to endogenous origin, sample matrix influences the response factors of T and EpiT, linearity of the calibration curve and precision of the calculated ratio of T/EpiT [3,4]. For the accurate determination of T/EpiT self-made artificial urine was applied to obtain sample matrix that behaves in the sample preparation process and in the chromatographic/mass spectrometric analysis in a manner similar to an authentic urine sample.

Materials and method

Self-made artificial urine

Aqueous solution consisted of urea 14 g/l, ammonium phosphate 1.7 g/l, creatinine 700 mg/l, glycine 700 mg/l, alanine 700 mg/l, oxalic acid 700 mg/l, bovine albumine 350 mg/l, glucose 350 mg/l, sodium chloride 250 mg/l. The pH of the solution was 6.

Calibrators

For the calibration curves both T and EpiT were spiked in artificial urine as aglycones at concentrations of 1, 5, 25, 50 and 100 ng/ml.

Quality controls

Four different quality control (QC) samples were included into each analysis batch. T and EpiT were spiked as aglycones in artificial urine for “QC Low” and “QC High” samples at concentrations of 5 ng/ml and 80 ng/ml, respectively. For “QC Cut-off” sample the ratio T/EpiT=4 was spiked for each batch at specific level, which depended on the concentration of the sample to be confirmed. The fourth sample, “QC Real” was a long-term quality control,

i.e. an authentic urine sample containing T and EpiT glucuronides (T/EpiT=3.1, T=34.1 ng/ml, EpiT=11.0 ng/ml).

Sample preparation

Internal standards d_3-T and d_3-EpiT were spiked (30 ng/ml) in 2 ml urine aliquot prior to solid phase extraction (SPE) with Sep-Pak C18 (Waters). After the loading of the sample the SPE cartridge was washed with water and the analytes were eluted with methanol. Enzymatic hydrolysis was carried out by using β-glucuronidase (*Eschericia coli* K12), and incubation in 0.1 M phosphate buffer (pH 7) at 50 °C for 60 min. Steroid aglycones were extracted at pH 10 with *n*-pentane, which was evaporated to dryness prior to derivatization with the mixture containing MSTFA/NH_4I/dithioerythritol (1000:2:4, v/w/w) at 60 °C for 15 min.

GC/MS-analysis

The analysis was performed on an Agilent 6890/5973N GC/MS-instrument using Agilent HP-1 (16 m, 0.2 mm id., 0.11 µm film) column. Injection of 3 µl was done in split mode (15:1) at 280 °C, and helium was applied as carrier gas in constant flow mode (1 ml/min). Temperature ramp of the oven was from 180 °C to 230 °C at 3 °C/min and further to 310 °C at 30 °C/min and the specific ions m/z 432, 417, 327, 435 were monitored using dwell time of 15 msec.

Results and conclusions

An authentic urine sample and samples spiked in artificial urine at two absolute concentration levels were both used as internal quality controls. With respect to between-day precision of T and EpiT concentrations (QC Low and QC High) and T/EpiT ratio (QC Real) similar behavior of between-day precision was observed for both matrices (Tables 1 and 2). Analyte to internal standard peak-height ratios using linear regression were obtained from calibration curves (1–100 ng/ml), which were linear and calibration was also proven repeatable between days (n=17) (Table 3, Figures 1 and 2).

Table 1. Between-day precision of quality control samples prepared in artificial urine.

	QC Low		QC High		QC Cut-off
	T (ng/ml)	EpiT (ng/ml)	T (ng/ml)	EpiT (ng/ml)	T/EpiT
Mean	4.5	4.8	78.0	83.9	3.8
CV (%)	8.1	6.8	6.4	4.5	4.4
N	20	20	20	20	20

Table 2. Between-day precision of authentic urine control sample “QC Real”.

	T (ng/ml)	EpiT (ng/ml)	T/EpiT
Mean	33.9	10.7	3.1
CV (%)	7.4	11.5	5.6
N	20	20	

Table 3. Statistical evaluation of calibration of testosterone and epitestosterone.

	Testosterone			Epitestosterone		
	Slope	Intercept	Correlation	Slope	Intercept	Correlation
Mean	1.501	0.000	0.997	1.125	0.008	0.997
SD	0.253	0.011	0.002	0.160	0.023	0.002
CV (%)	16.9		0.2	14.2		0.2
N	17	17	17	17	17	17

Figure 1. Calibration curve for testosterone (T). Concentration range 1-100 ng/ml.

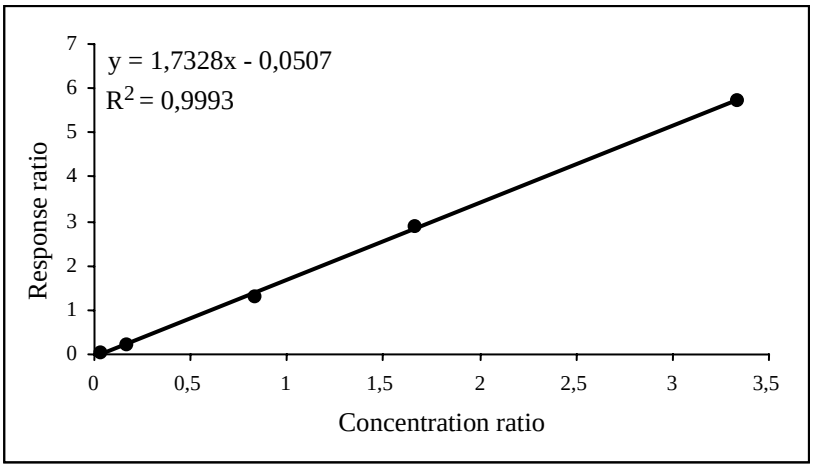
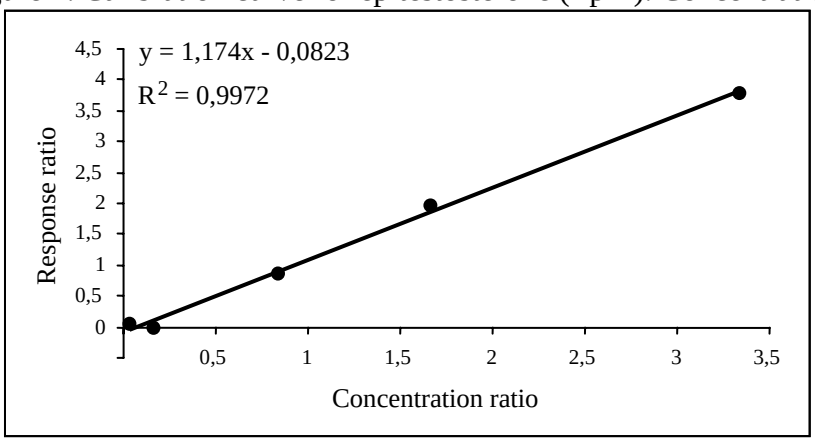
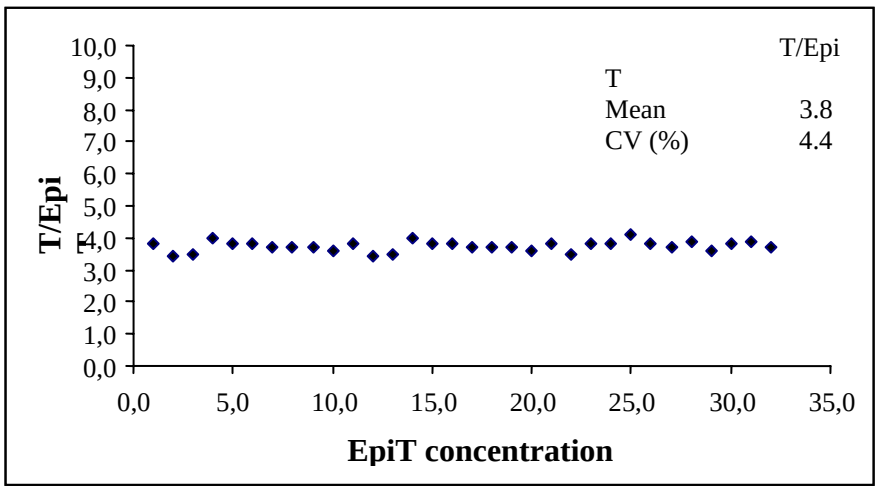


Figure 2. Calibration curve for epitestosterone (EpiT). Concentration range 1-100 ng/ml.



Based on the results from long-term calibration and quality assurance data from the routine T/EpiT measurements (QC Cut-off), the ratio T/EpiT was found also stabile over a wide range of absolute concentration of T and EpiT (Figure 3).

Figure 3. Stability of T/EpiT ratio of the QC Cut-off sample.



As a conclusion, this approach to apply calibrators and quality control samples spiked in artificial urine matrix has proven extremely well applicable for the high quality T/EpiT measurements.

References

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