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Calibration issue for the quantitative determination of endogenous steroids: the comparison of 6 matrices

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1. Introduction

Recently [1] the endogenous steroids have become one of the most misused doping agents in sports. The effect of their administration is quite similar to the exogenous steroids, but determination, on the contrary, is not easy [2]. The calibration of the gas chromatography – mass-spectrometry (GC/MS) system for quantitative detection of the endogenous steroids in urine is connected with different problems [2-7]. They are as follows: 1) Huge amount of testosterone metabolites present in urine, thus the calibration on the urine as a matrix needs preliminary purification. 2) The calibration curves are not linear for some types of matrices [4]. 3) The response of known amount of steroids differs from matrix to matrix, and the response in urine is the highest.

In our research we have compared 6 different matrices: blank male urine, "free urine", SPE-stripped urine, child urine, water and methanolic solution of standards. As the basic level of the calibration curve coefficient we decided to take the blank male urine fortified with different amounts of steroids (standard addition method).

2. Experimental

GC–MS spectra were recorded on Agilent 6890 Series GC System coupled to Agilent 5973N Mass Selective Detector with electron impact ionization (70 eV).The column used was Rxi – 1 ms, 12 m length, 0.20 mm inner diameter, 0.33 μm film thickness) Oven temperature was programmed as follows: 188˚C to 232˚C by 2˚C/min; 232˚C to 300˚C by 12˚C/min; 5.33min final hold. Injection volume was 2 μl in split mode (1:10). Helium was used as carrier gas at 0.6 ml/min. All calculations were made by ChemStation software package.

2.1. Chemicals, solvents and materials

Steroids were purchased from National Measurement Institute (Australia), Steraloids (USA), Dr. Ehrenstorfer GmbH (Germany), and from LGC Standards (Germany).

Stock solutions of reference substances were prepared in methanol. Cartridges for solid phase extraction (BondElut C18, 10 ml, 500 mg) were purchased from Varian Inc (USA).

2.2. Preparation of urine matrices

For precise experiment one pool of urine was used as the following matrices: i.e. blank urine, "free urine", SPE urine". Child urine was from a child under 7 years old. Calibration on blank urine was considered as the most correct and all other responses were compared with it. Only two of these matrices needed additional preparation. *Free urine:* One ml phosphate buffer (0.8M pH 6.4) was added to 5 ml blank urine, than extracted with 5 ml of diethyl ether at vortex extractor for 5 min. The organic layer was discharged, and urine was collected for the further experiments. *SPE urine:* Each cartridge was conditioned with methanol and deionized water. Then 25 ml of urine was passed through each cartridge and collected.

2.3. Preparation of calibrator solutions

For reliable and precise calibration all compounds were divided in four solutions according to their common concentration in urine [6]. Each level of calibration was obtained by addition of 30 µl of calibrator to the matrix.

2.4. Sample preparation for the calibration

Calibrator solution with the ISTD (Methyltestosterone, 500 ng/ml) was added to each testtube and evaporated under vacuum before sample preparation. Then 3 ml of matrix was added and vortexed gently, after that 1 ml of phosphate buffer (0.8 M, pH6.4), 1 ml of carbonate buffer (pH 10.4) and 5 ml of diethyl ether were added and the samples were vortexed for 5 min. Organic layer was separated and evaporated at 50˚C for 40 min. Finally, 50-µl MSTFA/NH4I/DTT (1000:3:2) reaction mixture was added to the vial and heated at 70˚C for 30 min before GC–MS analysis.

3. Results and discussion

3.1. Calibration curve coefficients

First of all we have defined the main coefficients (slope of calibration curve, as calculated using linear regression, forced through the origin). Each calibration level was prepared in triplicate. Calibration coefficients are presented in Table 1.

Table 1. Calibration coefficients of the investigated steroids. 1 – blank male urine, 2 – "free urine", 3 – SPE urine, 4 – child urine, 5 – water, 6 – methanolic solutions.

Steroid	1	2	3	4	5	6	Concentrations range, ng/ml
Testosterone (T)	**2.39**	2.48	2.38	2.42	2.13	2.06	10 – 500
Epitestosterone (E)	**2.23**	2.33	2.23	2.27	2.03	1.86	10 – 500
5α-Androstane-3α,17β-diol	**0.44**	0.44	0.43	0.42	0.39	0.38	10 – 500
5β-Androstane-3α,17β-diol	**0.44**	0.45	0.44	0.43	0.39	0.36	10 – 500
Dehydroepiandrosterone (DHEA)	**0.98**	1.03	0.99	1.00	1.03	0.78	10 – 500
Dihydrotestosterone (DHT)	**0.55**	0.55	0.50	0.54	0.46	0.50	10 – 500
Androsterone (A)	**0.84**	0.83	0.90	0.94	0.92	0.83	200 - 8000
Etiocholanolone (Etio)	**0.83**	0.88	0.91	0.88	0.87	0.83	200 - 8000
Pregnanediol	**14.5**	15.2	15.8	16.4	14.7	15.2	200 - 8000
Pregnanetriol	**2.50**	4.00	4.08	5.64	5.23	5.76	200 - 8000
Cholesterol	**0.88**	0.95	0.98	0.99	0.94	0.91	200 - 8000
Androstendione	**2.44**	2.54	2.50	2.36	2.61	2.43	10 – 500
11-oxo-Etiocholanolone	**0.47**	0.50	0.56	0.59	0.63	0.50	10 – 500
Epiandrosterone	**2.15**	2.31	2.23	2.14	2.18	2.04	10 – 500
Estradiol	**2.04**	2.21	2.12	2.16	1.85	1.73	10 – 500
Estriol	**0.41**	0.44	0.43	0.40	0.36	0.39	10 – 500
Estrone	**1.59**	1.70	1.66	1.54	1.76	1.31	10 – 500
3α,5-cyclo-5α-androstane-6β-ol-17-one	**0.85**	0.89	0.83	0.72	0.49	0.49	10 – 500

As follows from Table 1**,** the second and third matrices appear as the most correct for the majority of compounds. Those coefficients are important for the quantitative determination of the steroids, and if the coefficients of a matrix is close to the 1st matrix coefficients then this matrix is more precise and useable.

3.2. The ratio of the calibration curve coefficients

Method of steroid profile determination means not only the direct concentration measurement, but the determination of the main ratios like T/E, 5a/5b – Adiols**,** 11βOHA/11βOHE, A/Etio. For that part of research we have evaluated only three matrices excluding water and methanol solutions because of their deviating coefficients. In Table 2 all main ratios are presented. It is clear that all 3 matrices are precise enough for the steroid profile determination.

Table 2. The ratios of the main calibration curve coefficients. 1 – blank male urine, 2 – "free urine", 3 – SPE urine, 4 – child urine.

Ratio	1	2	3	4	Nominal ratios, as prepared
T/E	**1.07**	1.06	1.07	1.07	1.00
5α/5β – Adiols	**0.98**	0.98	0.98	0.97	1.00
11βOHA/11βOHE	**1.48**	1.47	1.48	1.50	1.45
A/Etio	**1.02**	1.02	0.98	1.08	1.00

4. Conclusions

Six matrices for the quantitative determination of endogenous steroids were compared. The best results were observed for the first three matrices namely free urine, SPE-stripped urine and child urine. The minimal z-score was for the "free urine", and two others were similar but showered overall higher z-scores. This experiment showed the good agreement of the first three matrices for the endogenous steroid calibration. The use of "free urine" matrix proved to be the most suitable because of the simplicity of urine pretreatment and the best z-score for the slopes for the most of endogenous steroids.

5. References

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