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Determination of sulphate- and glucuronide-conjugated anabolic steroids by direct injection of urine to UPLC-MS/MS

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Abstract

Gas chromatography-mass spectrometry (GC-MS) is normally used for the determination of endogenous anabolic steroids in human urine; however, the number of LC-MS methods for the determination of anabolic steroids has increased in the past years [1-5]. We have developed a new UPLC-MS/MS method for direct injection of human urine to determine 12 conjugated steroids simultaneously.

Materials and method

Chemicals and reagents

Glucuronides (G) and sulphates (S) of testosterone (TG, TS), epitestosterone (EPG, EPS), dihydrotestosterone (DHTG, DHTS), dehydroepiandrosterone (DHEAG, DHEAS), androsterone (AG, AS), etiocolanolone (ETG, ETS), [$^2H_3$]testosterone (TG-d_3, TS- d_3), [$^2H_3$]epitestosterone (EPG-d_3, EPS- d_3), [$^2H_3$]dihydrotestosterone (DHTG-d_3, DHTS-d_3) [$^2H_4$]androsterone (AG-d_4, AS-d_4), [$^2H_5$]etiocolanolone (DHTS-d_5), testosterone (T), epitestosterone (ET), [$^2H_3$]testosterone (T-d_3) and [$^2H_3$]epitestosterone (ET-d_3) were purchased from NMI (Pymble, Australia). Methanol was bought from Lab-Scan (Poch Sa, Swinskiego, Poland) and ammonium acetate and ammonia from Merck KGaA (Darmstadt, Germany). Steroid free urine blank was collected from a prepubertal girl. Standard stock solutions containing steroid glucuronides and sulphates were prepared in methanol. Calibrators (n=8) were prepared by serial diluting of stock solution to adequate concentrations of intermediate solutions followed by addition of steroid free urine blank (1:20 urine). Internal standard solution was prepared in water by diluting the deuterium labelled conjugates listed above to adequate concentrations.

Sample preparation

direct injection (D.I.)

To 400 µl sample 20 µl internal standard was added by Xiril X-100 pipetting robot.

solid phase extraction (SPE)

To 1 ml sample 20 µl internal standard was added by a Xiril X-100 pipetting robot to SPE Oasis HLB 96-well plate. The SPE was washed and centrifuged subsequently, and the analytes were eluted with acetone. After evaporation, the residue was reconstituted with aqueous solution of 20 % MeOH.

Chromatographic Separation

Waters Acquity UPLC system (Waters Assoc, Milford, MA) was used to perform the separation on Waters Acquity UPLC BEH RP18 column 100 mm x 2.1 mm with 1.7- µm particles. The mobile phases were 5 mM NH_4Ac adjusted by ammonia to pH 9.6 (A) and MeOH (B). Separation was performed by the following gradient; initial mobile phase composition was 20 % B with a linear gradient to 50 % B at 13 min followed by another increased gradient to 95 % B at 15.0 min held there to 18.0 min. The organic modifier was decreased to initial condition (20 %) at 18.1 min and the final equilibration time was 20.0 min

Mass spectrometric method

The analytes were ionized by ion electrospray and detected in multiple reaction monitoring (MRM) mode with simultaneous polarity switching by a Waters Quattro Premier triplequadropole instrument (Waters Assoc., Manchester, UK). Two diagnostic ions for each substance (one for internal standards) were used and the selected precursor/product ion transitions are listed in Table 1.

Table 1. Ion transitions used to detect the analytes

Substance	Precursor	Product ions	Collision offset voltage (V)	Internal standard
TG	$[M+H]^+$ (465)	271/289	19/21	D3
TS	$[M+H]^+$ (369)	109/97	28/28	D3
T	$[M+H]^+$ (290)	97/109	22/24	D3
EPG	$[M+H]^+$ (465)	271/289	15/11	D3
EPS	$[M+H]^+$ (369)	109/97	30/58	D3
EP	$[M+H]^+$ (290)	97/109	22/24	D3
DHTG	$[M+NH_4]^+$ (485)	141/273	26/12	D3
DHTS	$[M-H]^-$ (369)	97/80	38/76	D3
DHEAG	$[M+NH_4]^+$ (482)	253/271	27/15	-
DHEAG	$[M-H]^-$ (367)	97/80	31/50	-
AG	$[M+NH_4]^+$ (484)	255/273	26/18	D4
AS	$[M-H]^-$ (369)	97/80	38/76	D4
ETG	$[M+NH_4]^+$ (484)	255/273	26/18	-
ETS	$[M-H]^-$ (369)	97/80	38/76	D5

Results and discussion

Baseline resolution of the conjugates with same ion transitions (see table 1) was achieved, as show in Figure 1. EPS was detected in positive mode to avoid the endogenous DHEAS as an interfering peak, shown as a non-integrated peak for the QC-sample (Q) in the DHEAS chromatogram in figure 1. Limit of detection for the D.I. method (signal to noise =3) was determined for all substances with a range from 0.2 to 2 ng/ml, for TS and AS, respectively. The sensitivity of the SPE method was found to be 5 times higher. The intra-day precisions was determined for all analytes (QC sample, n=8) and varied from 2.6 % for ETS (D.I. of 80 ng/ml) to 13 % for DHEAG (D.I. of 8 ng/ml). Cross validation of the D.I. and SPE method, see figure 2, was performed by samples from a testosterone study (n=30) and showed an excellent correlation (1.00 and 0.98 for TG and EPG, respectively). Various athlete samples (n=9) were spiked with 13 ng/ml EPG and 43 ng/ml TG and analysed by D.I. method. The observed recoveries were 103 % and 104 %, respectively. Samples analyzed by GC-MS during year 2008 with a T/E ratio >4 (n=26) were reanalyzed with the UPLC-MS/MS method, (D.I.). A chromatogram of one of the samples is shown in figure 1 where all conjugates were found, at concentrations from 12 ng/ml (EPS) to 4000 ng/ml (AG). All samples were determined to a T/E ratio of >4 with the UPLC-MS/MS method. Figure 3 demonstrates a plot of the cross validation of the two methods and the correlation was acceptable (0.96 and 0.91 for TG and EPG, respectively). Results from the testosterone study will be presented later.

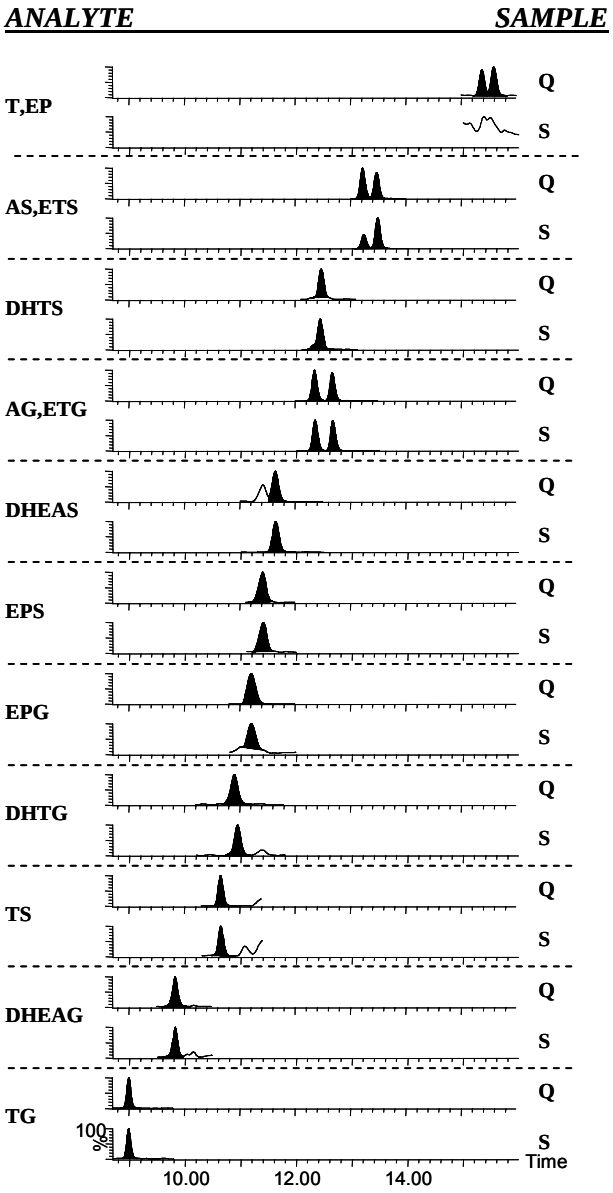

Figure 1. Two overlaid chromatograms, a QC-sample (Q) and an athlete sample (S) with T/E ratio >4.

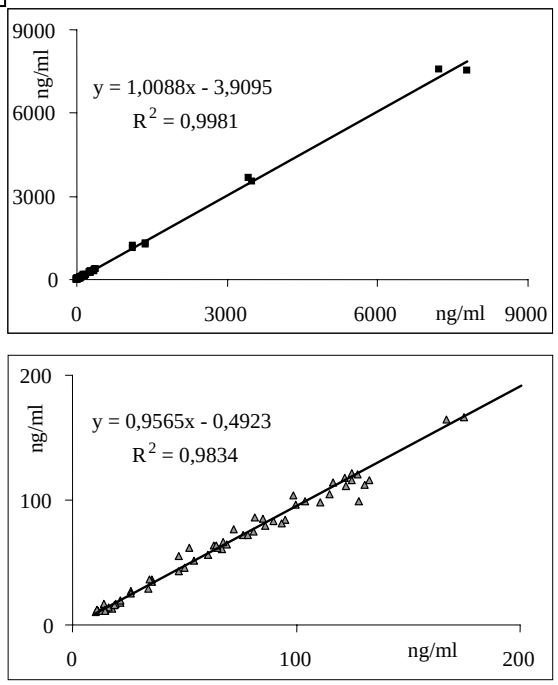

Figure 2. Cross validation of direct injection (x-axis) and SPE method (y-axis). The upper and lower graph corresponds to TG and EPG, respectively.

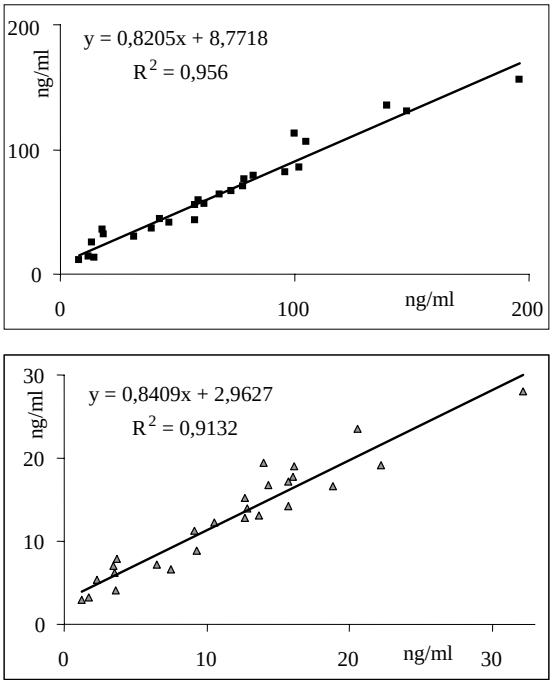

Figure 3. Cross validation of the LC-MS/MS (x-axis) and the GC-MS method (y-axis). The upper and lower graph corresponds to TG and EPG, respectively.

Conclusions

Direct injection or solid phase extraction in combination with UPLC-MS/MS can be used for quantification of some anabolic steroid conjugates and a compliment to the traditional GC-MS method for T/E ratio.

References

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