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Direct quantification of steroid glucuronides by liquid chromatography tandem mass spectrometry

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Abstract

A method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the direct quantification of glucuronides of testosterone (TG), epitestosterone (EPG), androsterone (AG) and etiocholanolone (ETG) has been developed. The method allowed for the direct determination of these analytes avoiding hydrolysis and derivatization, which are usual steps in commonly used methods based on gas chromatography-mass spectrometry (GC-MS). Two different approaches have been tested for sample preparation: direct injection after filtration and acidic liquid-liquid extraction (LLE) with ethyl acetate. The values obtained by both approaches were satisfactory for accuracy and precision with limits of detection lower than 1ng/ml for TG and EPG. The applicability of the method has been checked by the analysis of 100 urine samples. The results were compared with those obtained with the common GC-MS method. Results showed a good correlation between both methods.

Introduction

The abuse of T is normally controlled by the ratio between urinary concentrations of T and epitestosterone (E) (T/E ratio). Other ratios including concentrations of androsterone and etiocholanolone can also be useful to provide evidence of T, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA) and alfa reductase inhibitor abuse [1]. In order to establish these ratios accurately, quantitative methods for these analytes are needed.

Endogenous steroids are normally excreted in urine after conjugation in phase II metabolism reactions, of which the most common one is glucuronidation. The determination of these endogenous steroids is normally performed by GC-MS after enzymatic hydrolysis, liquid-

liquid extraction (LLE) and derivatization [2]. Besides being time consuming, this approach can sometimes generate problems associated with the quantitative recoveries in all steps and can potentially make that the result obtained for a sample varies between laboratories.

Although some methods describe the direct detection of steroid conjugates by GC-MS after derivatization [3], liquid chromatography-tandem mass spectrometry (LC-MS/MS) is reported as a more suitable alternative either in negative ionization mode due to the acidity of the glucuronide moiety [4] or in positive ionization owing to the proton affinity of the steroid keto function [5]. However, in all reported methods a preconcentration step is needed to reach the required sensitivity (low ng/ml). The use of a preconcentration step can affect the quantitative characteristics of an LC-MS/MS method because of endogenous interferences [6] which can increase the ion suppression.

The aim of this study is to develop a quantitative method for the direct determination of steroid glucuronides based on LC-MS/MS allowing for a reduction in sample volume, total analysis time and sample manipulation. Two sample pre-treatment approaches are tested in order to meet this aim: filtration and LLE. Finally, the applicability of the developed methods is tested by comparing the results with those obtained by the GC-MS method routinely applied in our laboratory.

Materials and Methods

Chemicals and reagents

Glucuronides of testosterone- (TG), epitestosterone (EPG), androsterone (AG), etiocholanolone (ETG), 19-norandrosterone (NAG), 19-noretiocholanolone (NETG), [²H₃]testosterone (TG-d₃), [²H₃]epitestosterone (EPG-d₃), [²H₄]androsterone (AG-d₄) and [²H₄]19-norandrosterone (NAG-d₄) were purchased from NMI (Pymble, Australia).

The β-glucuronidase preparation (type *Escherichia coli* K12) was purchased from Roche (Mannheim, Germany). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was bought from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Ethyl acetate, HPLC grade methanol and HPLC grade water were purchased from Acros (Geel, Belgium) and Fischer Scientific (Loughborough, UK), respectively.

Standard stock solutions were prepared in methanol. A mixture of adequate amounts of labelled stock solutions (1 µg/ml for TG-d₃ and EPG-d₃, 10 µg/ml for AG-d₄ and NAG-d₄) was used as internal standard.

Instrumentation

20 µL of sample were injected into a HPLC Finnigan Surveyor MS pump plus (Thermo, San Jose, CA, USA) interfaced to a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo). The LC separation was performed on a Varian Omnispher C₁₈ column (100 x 2 mm I.D., 3 µm) (Varian, Sint-Katelijne-Waver, Belgium), at a flow rate of 250 µL/min using a ChromSep guard column (10 x 2 mm I.D., 5 µm) (Varian).

Water (A) and methanol (B) both containing 1 mM ammonium acetate and 0.1% formic acid were selected as mobile phase solvents. A gradient program was used, the percentage of B was changed linearly as follows: 0 min., 40%; 0.5 min., 40%; 2 min., 55%; 7 min., 65%; 7.2 min., 65%; 7.5 min., 40%; 9 min, 40%.

Nitrogen was used as sheath gas, ion sweep gas and auxiliary gas, which were set at flows of 50, 2 and 20 units, respectively. Spray voltage of 4000 V was used. The capillary temperature was set at 350 °C and the source collision induced dissociation (CID) at 2 units. The collision gas was argon (Air Liquide, Desteldonk, Belgium) with a collision gas pressure of 0.2 Pa.

GC/MS analysis was carried out on an Agilent 5973 mass spectrometer (Palo Alto, CA, USA) directly coupled to an Agilent 6870 gas chromatograph equipped with a J&W-Ultra 1 column (J&W, Folsom, CA, USA), length of 17 m, internal diameter of 0.2 mm and a film thickness of 0.11 µm. The GC system was operated in constant flow mode at a flow rate of 0.7 ml/min (initial linear velocity 43 cm/s). The oven temperature was as follows: 120 °C (0 min) at 60°C/min → 183 °C (1.05 min) at 3 °C/min → 232 °C (0 min) at 40 °C/min → 310 °C (3 min). Half a microliter was injected in the splitless mode. The mass spectrometer was operated in the selected ion monitoring (SIM) mode with the ions shown elsewhere [2].

Sample preparation

For filtration, 200 µl urine were introduced onto an Ultrafree-MC filter (Millipore, Bedford, MA, USA). After the addition of 50 µl internal standard the sample was centrifuged during 10 min at 15 000 g with a Universal 32R centrifuge (Hettich, Tuttlingen, Germany). Twenty µl of the filtrate were injected directly into the system.

In the LLE, 1 ml urine was acidified with 200 µL 1 M HCl and 50 µl internal standard were added. A LLE was performed with 4 ml ethyl acetate. After centrifugation, the organic layer was separated and evaporated under oxygen free nitrogen at 40 °C. The remaining residue was dissolved into 100 µl of mobile phase.

For GC-MS, 2 ml urine were hydrolysed (56 °C during 2.5 hours) after addition of phosphate buffer (pH=7) and 50 µl of glucuronidase solution. After cooling to room temperature, the pH of the urine was adjusted to 9.2 by addition of sodium hydrogen carbonate and potassium carbonate. A liquid-liquid extraction was performed with 5 ml diethyl ether. After centrifugation, the organic layer was separated and evaporated under nitrogen at 40°C. The dry residues were derivatised using 100 µl MSTFA/NH₄I/ethanethiol (320/1/2; v/w/v) at 80°C for 60 min and 0.5 µL were injected into the system.

Extraction recovery

Steroid-free urine was obtained by passing 200 ml urine through a column packed with 50 g of XAD-2 (Serva, NY, USA) previously conditioned with methanol and water. The absence of steroids was checked by GC-MS analysis.

For extraction recovery experiments, steroid-free urine was extracted following the LLE method described above and the transferred organic layer spiked at 50 ng/ml. Additionally, steroid free urine was spiked at 50 ng/ml and extracted. The extraction recovery was calculated by comparison of the area obtained for the samples spiked before and after extraction. These experiments were carried out at different pH values between 1 and 5.2.

Due to the endogenous presence of some of the selected analytes, the extraction recovery was calculated also in real urine samples by means of labelled internal standards. For this purpose, the previous procedure was followed using ten different urines and spiking with the labeled internal standards.

Ion suppression

The study of the ion suppression was also performed in steroid free urine and real urine samples extracted by filtration and LLE. The blank extracts were spiked after the extraction with the analytes (5 ng/ml for NAG and NETG, 50 ng/ml for TG and EPG and 1000 ng/ml for AG and ETG) for steroid-free urine and with the deuterated analogues for real urine samples. The ion suppression due to the matrix components was calculated by comparing the responses between these spiked extracts and a standard at the same concentration prepared in mobile phase.

Application to real samples

In order to evaluate the applicability of the method for routine purposes, 100 samples previously analysed by the GC-MS method were analysed using the LC-MS/MS methods.

Results and Discussion

MS/MS optimization

All selected glucuronides exhibited ionization in both positive and negative modes. According to Kuuranne et al [7], TG and ETG were predominantly detected as $[M+H]^+$ in positive ionization mode due to the high proton affinity of these steroids. On the other hand, AG, ETG, NAG and NETG were mainly detected as $[M+NH_4]^+$ and $[M+Na]^+$ as a result of their lower proton affinity. In negative ionization mode all analytes exhibited an $[M-H]^-$ ion together with small amounts (lower than 30%) of $[M+HCOO]^-$.

Table 1. Precursor and product ions obtained in positive ionization mode

| Analyte | Precursor ion | | Product ion | | |
|--------------|---------------|------------------|------------------|--------------------|----------------------|
| | m/z | specie | m/z | Collision E (eV) | |
| TG and EPG | 465 | $[M+H]^+$ | 289 | 20 | $[M+H-Gluc]^+$ |
| | | | 271 | 25 | $[M+H-H_2O-Gluc]^+$ |
| | | | 253 | 25 | $[M+H-2H_2O-Gluc]^+$ |
| | | | 109 | 30 | C_7H_9O (A ring) |
| | | | 97 ^a | 30 | C_6H_9O (A ring) |
| AG and ETG | 484 | $[M+NH_4]^+$ | 291 | 10 | $[M+H-Gluc]^+$ |
| | | | 273 | 20 | $[M+H-H_2O-Gluc]^+$ |
| | | | 255 | 20 | $[M+H-2H_2O-Gluc]^+$ |
| | | | 177 | 20 | $[Gluc+H]^+$ |
| | | | 159 | 25 | $[Gluc+H-H_2O]^+$ |
| | | 141 ^a | 30 | $[Gluc+H-2H_2O]^+$ | |
| NAG and NETG | 470 | $[M+NH_4]^+$ | 277 | 15 | $[M+H-Gluc]^+$ |
| | | | 259 ^a | 20 | $[M+H-H_2O-Gluc]^+$ |
| | | | 241 | 20 | $[M+H-2H_2O-Gluc]^+$ |
| | | | 177 | 20 | $[Gluc+H]^+$ |
| | | | 159 | 25 | $[Gluc+H-H_2O]^+$ |
| | | | 141 | 25 | $[Gluc+H-2H_2O]^+$ |

Results in positive ionization mode were better than in negative ionization mode. The main product ions observed for each selected analyte in positive ionization mode are shown in Table 1. The ions observed are mainly due to losses of the glucuronide moiety and losses of water. Concretely, for all compounds the ions $[M+H-Gluc]^+$, $[M+H-H_2O-Gluc]^+$ and $[M+H-2H_2O-Gluc]^+$ were the most abundant ones (Figure 1). For TG and ETG two additional ions were found at m/z 97 and m/z 109 which are generated from the A ring of the steroid structure [8]. Although these ions exhibited low abundance at 20 eV, they become predominant when the collision energy was increased to 30 eV (Figure 2). In negative ionization mode, only product ions at m/z 113, 85 and 75 were found to have intensities higher than 10%. These ions can arise from fragmentation of the glucuronide moiety.

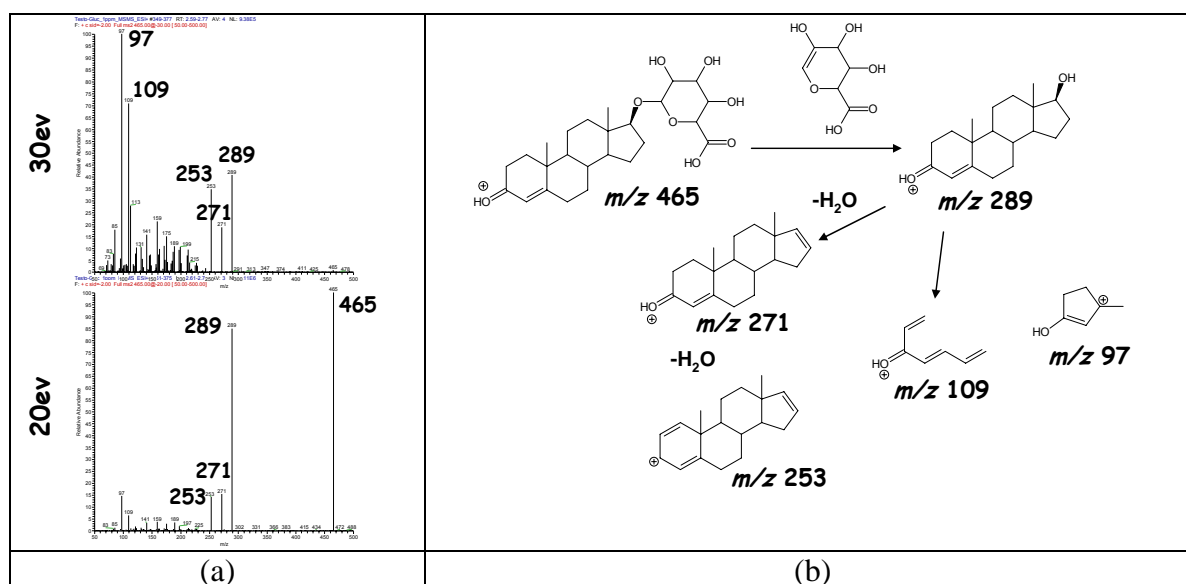


Figure 1. (a) Product ion spectra obtained in positive ionization mode for TG at two collision energies. (b) Proposed fragmentation pathway for TG

Extraction recovery

The effect of the pH on the extraction was studied for the LLE approach. Due to the acidic properties of the glucuronides, a high pH dependency for the extraction was found. All analytes showed recoveries higher than 70% at pH < 2 in steroid free urine. A pH 1 was selected due to the higher recoveries obtained mainly for TG and NETG. At higher pH values the calculated recoveries were lower than 50%.

The extraction recovery for 10 real urine samples was checked with deuterated internal standards. Recoveries were satisfactory (higher than 80%) for all urine samples (Table 2). Additionally, the standard deviation was found to be lower than 20% showing small variability between the recoveries obtained for different samples.

Table 2. Recoveries and ion suppressions obtained for deuterated analogues in 10 urines

| Analyte | LLE rec. (%) | RSD (%) | LLE ion suppression (%) | RSD (%) | Filtration ion suppression (%) | RSD (%) |
|---------|-----------------|------------|----------------------------|------------|-----------------------------------|------------|
| TG-d3 | 80 | 6 | 61 | 48 | 19 | 18 |
| EPG-d3 | 87 | 9 | 30 | 29 | 4 | 5 |
| AG-d4 | 89 | 14 | 39 | 35 | 3 | 11 |
| NAG-d4 | 93 | 14 | 0 | 26 | 3 | 8 |

Selection of the optimal transition

Most of the product ions included in the single reaction monitoring (SRM) method for the determination of steroid glucuronides are related to the glucuronide moiety. Although these ions have been described as structure specific [7], their specificity can be questioned because

any endogenous glucuronide, sharing the same mass as the analyte, would produce the same ions and therefore would interfere in the analysis. This problem was observed in the determination of TG and EPG using the transitions $465 \rightarrow 289$ and $465 \rightarrow 271$ corresponding to $[M+H-Gluc]^+$ and $[M+H-H_2O-Gluc]^+$, respectively. Although their detection at the required level was satisfactory in steroid-free urine (Figure 2a), numerous endogenous interferences were noticed in real samples hampering the quantification of TG at concentrations lower than 10 ng/ml (Figure 2b). These endogenous interferences were also found in previous studies using these transitions [5].

The use of more specific transitions is an efficient way to circumvent endogenous interferences. In this case, the specific transition $465 \rightarrow 97$ allowed for the detection of TG and EPG at the required level (Figure 2) improving the method sensitivity.

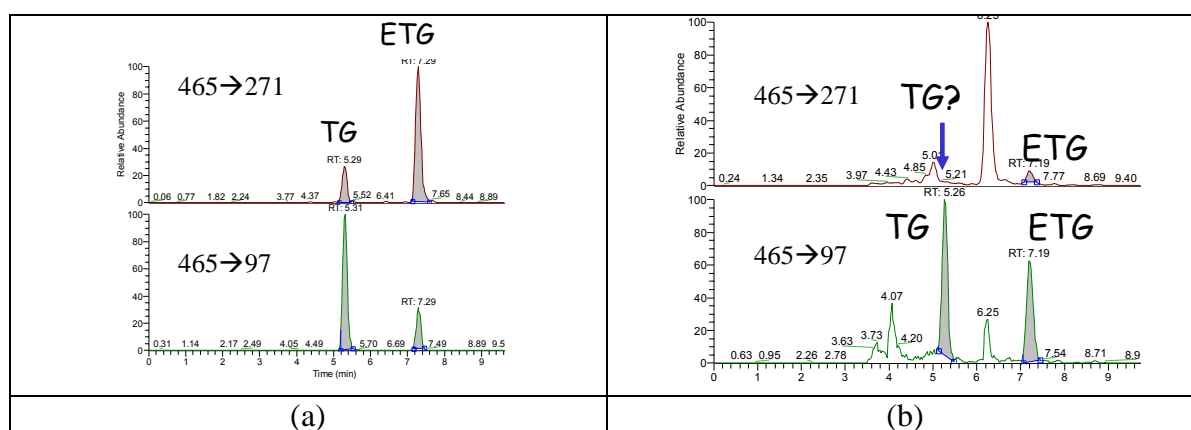


Figure 2. LC-MS/MS chromatograms for TG and ETG by acquisition of two transitions (a) standard in steroid free urine. (b) Real urine containing 3ng/ml TG and 7 ng/ml ETG

Suppression of the ionization

The experiments performed with steroid free urine showed the absence of important ion suppression in both LLE and filtration experiments. Hence, suppressions lower than 10% were obtained for all compounds under these conditions. However, the steroid free urine did not contain endogenous steroids which can be the most relevant contribution for ion suppression. Therefore, similarly to extraction recovery experiments, the ion suppressions for the deuterated internal standards were calculated for both approaches in 10 different real urine samples (Table 2).

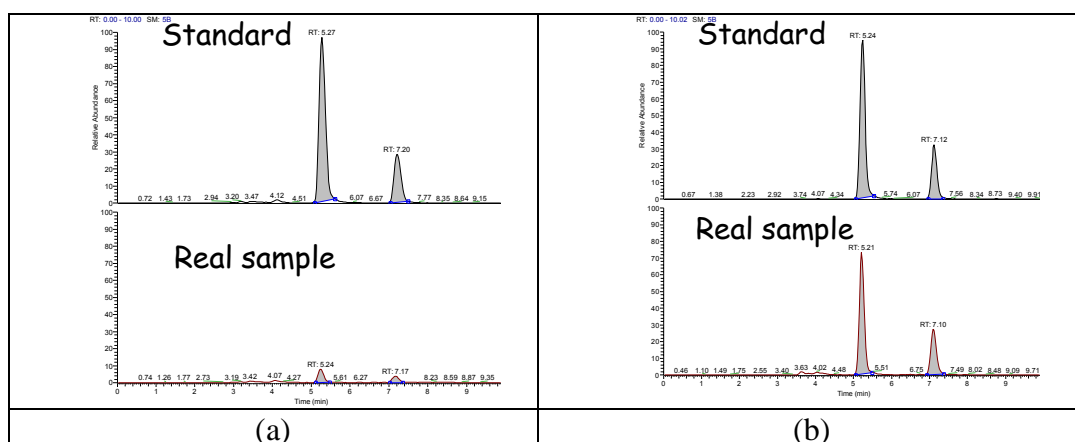


Figure 3. Ion suppression obtained by (a) LLE. (b) after filtration

The results obtained for the filtration approach showed minimal suppression or enhancement (lower than 10%) for all analytes except for TG which exhibited a suppression of 20%. The standard deviation was in all cases lower than 20% indicating a slow variation between matrices. However, in the LLE approach, the observed ion suppression was substantially higher than for filtration reaching average values of 61% in the case of TG (Figure 3). This fact can be attributed to the concentration of interferences. The matrix variability was also more pronounced with LLE where standard deviations higher than 25% were observed. TG showed the highest variability with a deviation of 48% and suppressions from 35% to 85%. These results make the usefulness of the LLE approach as preconcentration step questionable because in some samples the ion suppression exceeds 80% which would almost compensate for the 10 fold concentration factor obtained during this approach.

The matrix variability and ion suppression can be easily corrected by the use of labelled internal standards, when available. This was the case for TG, EPG, NAG and AG. However, the unavailability of deuterated analogues for ETG and NETG made that AG-d4 and NAG-d4 were used for the correction of ETG and NETG respectively.

Method validation

The developed method was validated obtaining satisfactory results (correlation coefficients higher than 0.99, accuracy, between 90-103% for all compounds in all levels assayed with RSD always lower than 20% and LODs between 0.05 and 0.25 ng/ml) as can be seen elsewhere [9].

Application to routine samples

The suitability of the developed method for routine analysis was checked by analyzing 100

urine samples which did not test positive for NAG and NETG by the GC-MS method. An example of a real sample is shown in Figure 4.

The endogenous steroids TG, EPG, AG and ETG were detected in all samples using both approaches with concentrations in the range from less than 1 ng/ml-90 ng/ml for TG, 1 ng/ml -125 ng/ml for EPG, 400 ng/ml -more than 4000 ng/ml for AG and 500 ng/ml to more than 4000 ng/ml for ETG. The chromatograms obtained after applying the filtration approach for a sample are shown in Figure 5. Concentrations obtained using the filtration approach were compared with those obtained by GC-MS method. Results were found to be comparable with correlation coefficients higher than 0.9 for all compounds. The slopes of all curves were between 1.13 and 1.36 (Figure 5).

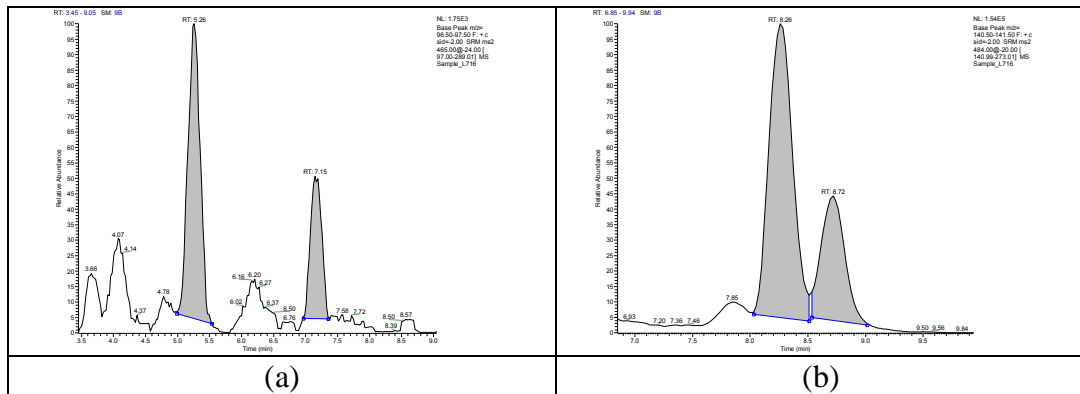


Figure 4. LC-MS/MS chromatograms for a urine sample containing (a) X ng/ml TG and Y ng/ml ETG and (b) X ng/ml AG and Y ng/ml EG

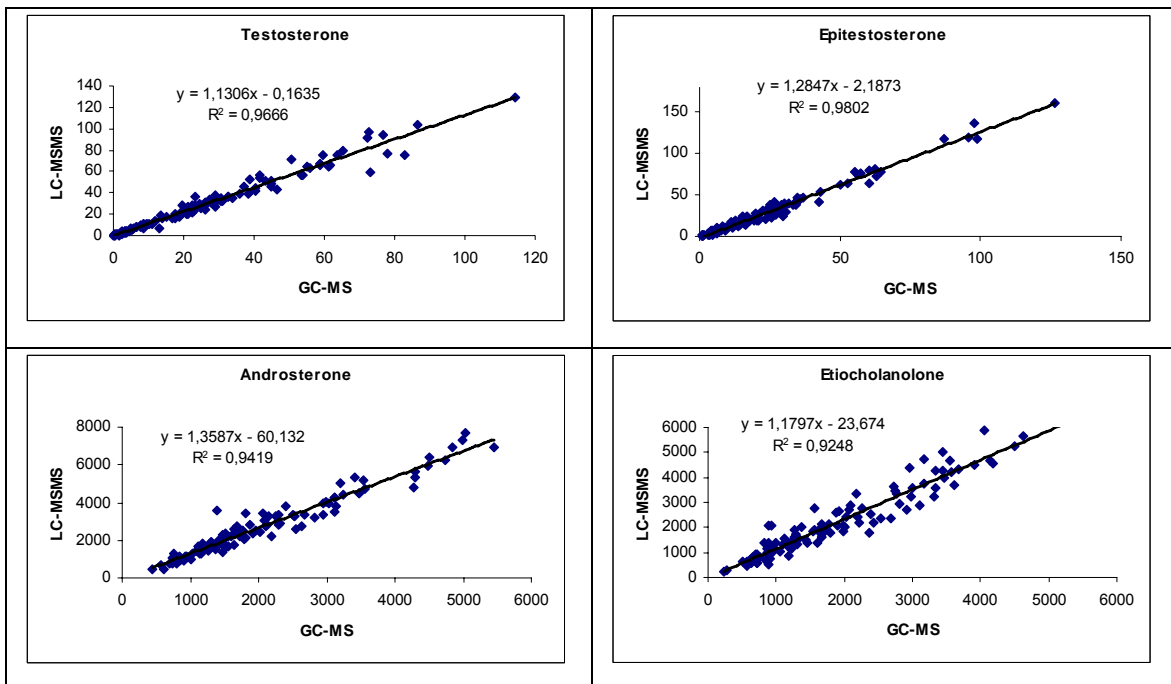


Figure 5. Correlation between the results obtained by LC-MS/MS and GC-MS

Conclusions

Two quantitative methods based on LC-MS/MS for the determination of some anabolic steroid glucuronides were developed. The selection of highly specific transitions allowed for an increased sensitivity of the method after optimizing the MS/MS conditions. This was found to be critical for TG and EPG where a large number of endogenous interferences were found. More accurate results were found with the filtration approach due to the higher ion suppression observed after LLE. This method is ideal for routine doping analysis due to the high sensitivity, low amount of sample handling, short total analysis time (15 minutes/sample) and volume of urine (200µl) used.

Acknowledgements

Postdoctoral grants by the Spanish Ministerio de Educacion y Ciencia (O.J.P.) and the Flemish Ministry of Culture, Youth, Sports and Brussels (P.V.E., W.V.T., K.D.) are gratefully acknowledged. WADA is thanked for partially funding this work.

References

- [1] World Anti-Doping Agency. Technical Document-TD2004EAAS (2004) http://www.wada-ama.org/rtecontent/document/end_steroids_aug_04.pdf (access date 16.05.2008)
- [2] Ayotte C, Goudreault D, Charlebois A. (1996) Testing for natural and synthetic anabolic agents in human urine. *J Chromatogr. B* 68, 3-25
- [3] Choi M.H, Kim K.R, Chung B.C. (2000) Simultaneous determination of urinary androgen glucuronides by high temperature gas chromatography-mass spectrometry with selected ion monitoring. *Steroids* 65, 54-59
- [4] Buiarelli F, Coccioli F, Neri B, Terracciano A. (2004) Development of a liquid chromatography tandem mass spectrometry method for the identification of natural androgen steroids and their conjugates in urine samples. *Anal. Chim. Acta* 526, 113-120
- [5] Bean K.A, Henion J.D. (1997) Direct quantification of anabolic steroid conjugates in human urine by combined high-performance liquid chromatography and tandem mass spectrometry. *J. Chromatogr. B*, 690, 65-75
- [6] Pozo O. J., Van Eenoo P., Deventer K., Delbeke F. T. (2007) Development and validation of a qualitative screening method for the detection of exogenous anabolic steroids in urine by liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem* 389 1209-1224.
- [7] Kuuranne T., Vahermo M., Leinonen A., Kostianen R. (2000) Electrospray and atmospheric pressure chemical ionization tandem mass spectrometric behaviour of eight anabolic steroid glucuronides. *J. Am. Soc. Mass Spectrom.* 11, 722-730.
- [8] Thevis M., Schänzer W. (2007) Mass spectrometry in sports drug testing: Structure characterization and analytical assays. *Mass Spectrom. Rev.* 26, 79-107.
- [9] Pozo O.J, Van Eenoo P, Van Thuyne W, Deventer K, Delbeke F.T. (2008) Direct quantification of steroid glucuronides in human urine by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* 1183, 108-118.