

アプリケーションノート

## Improving Detection of Anabolic Steroids in Sports: Simultaneous Detection of Intact Phase I and Phase II Urinary Metabolites by UPLC-MS/MS

---

G. Balcells, A. Esquivel, O.J. Pozo, J. Segura, R. Ventura

Barcelona Antidoping Laboratory, Bioanalysis Research Group, IMIM, Hospital del Mar Medical Research Institute, Department of Experimental and Health Sciences, Universitat Pompeu Fabra



*For forensic toxicology use only.*

---

## Abstract

This application note presents a novel LC-MS/MS screening method for the simultaneous detection of intact phase I and phase II (glucuronides and sulfates) urinary metabolites of anabolic androgenic steroids (AAS).

A UPLC-MS/MS screening method for the simultaneous detection of steroid metabolites is a feasible alternative compared to the conventional procedures. Incorporation of new phase II metabolites as described is straightforward and can significantly enhance the screening and detection capabilities of steroids in sport.

### Benefits

- Simultaneous detection of metabolites irrespective of their nature by UPLC-MS/MS
- Simple and fast SPE sample preparation prior to chromatographic analysis
- An extended window of detection through the monitoring of long term excreted metabolites
- Elimination of hydrolysis and derivatization steps
- High recovery efficiency for all types of metabolites (unconjugated, glucuronides, sulfates)

---

## Introduction

Anabolic androgenic steroids (AAS) are prohibited in sports. They are the most frequently detected substances in doping controls reflecting their wide misuse among athletes.<sup>1</sup> Screening of AAS is currently performed using a combination of both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), after hydrolysis of the urine using  $\beta$ -glucuronidase enzymes.<sup>2</sup> Therefore, only unconjugated metabolites and hydrolyzable glucuronic acid conjugates are detectable under these conditions. Other phase II metabolites such as sulfates or glucuronoconjugates stable to this hydrolysis cannot be detected using the current screening methods.

LC-MS/MS technology allows for the intact detection of all types of conjugates, and this application note presents a novel LC-MS/MS screening method for the simultaneous detection of intact phase I and phase II (glucuronides and sulfates) urinary metabolites of AAS.<sup>3</sup>

The proposed analytical strategy has the advantage of simplified sample preparation as hydrolysis and derivatization steps, which are required in conventional screening methods, are not needed. In addition, the method improves the detection capabilities through the monitoring of long term excreted phase II

metabolites not detectable using the current screening strategy.<sup>4-7</sup> Moreover, new long term phase II metabolites described in the future could be incorporated irrespective of their urinary nature. Finally, automation of the procedure, with on-line solid phase extraction system, could be easily implemented.

---

## Experimental

### LC conditions

|                   |  |
|-------------------|--|
| System:           | ACQUITY UPLC   |
| Column:           | ACQUITY UPLC BEH C <sub>18</sub> 2.1 x 100 mm, 1.7 µm  |
| Column temp.:     | 45 °C  |
| Flow rate:        | 0.3 mL/min   |
| Mobile phase A:   | 1 mM ammonium formate in water, containing 0.01% formic acid   |
| Mobile phase B:   | 1 mM ammonium formate in acetonitrile:water (95:5, v/v), containing 0.01% formic acid                                  |
| Gradient:         | 0 min, 20% B; 2 min, 20% B; 15 min, 40% B; 16 min, 70% B; 17 min, 95% B; 18 min, 95% B; 18.5 min, 20% B; 20 min, 20% B |
| Injection volume: | 10 µL  |
| Analysis time:    | 20 min   |
| Strong wash:      | Acetonitrile   |
| Weak wash:        | water/acetonitrile (95:5, v/v)   |

### MS conditions

|                    |   |
|--------------------|---|
| System:            | Xevo TQ MS  |
| Ionization modes:  | ESI+ and ESICapillary                               |
| voltage:           | 3.5 kV (ESI+) and 2.5 kV (ESI-)                     |
| Source temp.:      | 120 °C  |
| Desolvation temp.: | 450 °C  |
| Desolvation gas:   | 1200 L/h  |
| Cone gas:          | 50 L/h  |
| Acquisition mode:  | Multiple reaction monitoring (MRM) shown in Table 1 |

## Sample description

Drug-free urine samples were collected from healthy volunteers and used for method development.

Excretion study samples were obtained following methyltestosterone and stanozolol administration. A single oral dose of methyltestosterone (10 mg) or stanozolol (6 mg) was administered to three and four healthy male volunteers, respectively. Samples were collected before administration and up to 31 days thereafter. Routine doping samples positive for stanozolol were also analyzed.

## Sample preparation

After the addition of 20 µL of the internal standard (IS) (a methanolic solution containing methyltestosterone, nandrolone-d3 sulfate and testosterone-d3 glucuronide at 1 µg/mL, and androsterone-d4 glucuronide at 5 µg/mL), 2 mL of urine samples were vortex-mixed and passed through a C<sub>18</sub> cartridge previously conditioned with 2 mL of methanol and 2 mL of water. The cartridge was then washed with 2 mL of water, and the analytes eluted with 2 mL of methanol. The samples were evaporated to dryness under nitrogen stream in a bath at 40 °C. The extract was redissolved into 200 µL of a solution of ACN:water (10:90, v/v).

---

## Results and Discussion

## Optimization of the mass spectrometric detection conditions

All steroid metabolites showed to be readily ionizable.<sup>2</sup> In positive mode, unconjugated and glucuronide metabolites with a 3-keto function ionized as  $[M+H]^+$ . Glucuronides lacking this feature showed the adduct  $[M+NH_4]^+$  as the major ion. In negative mode, all glucuronides formed the ion  $[M-H]^-$  resulting from deprotonation of the acidic group. The sulfate conjugates also yielded the  $[M-H]^-$  ion; in all cases, this species gave the most intense signals. Collision-induced dissociation (CID) gave common ions or losses depending on the analytes chemical features (Table 1 and Figure 1).

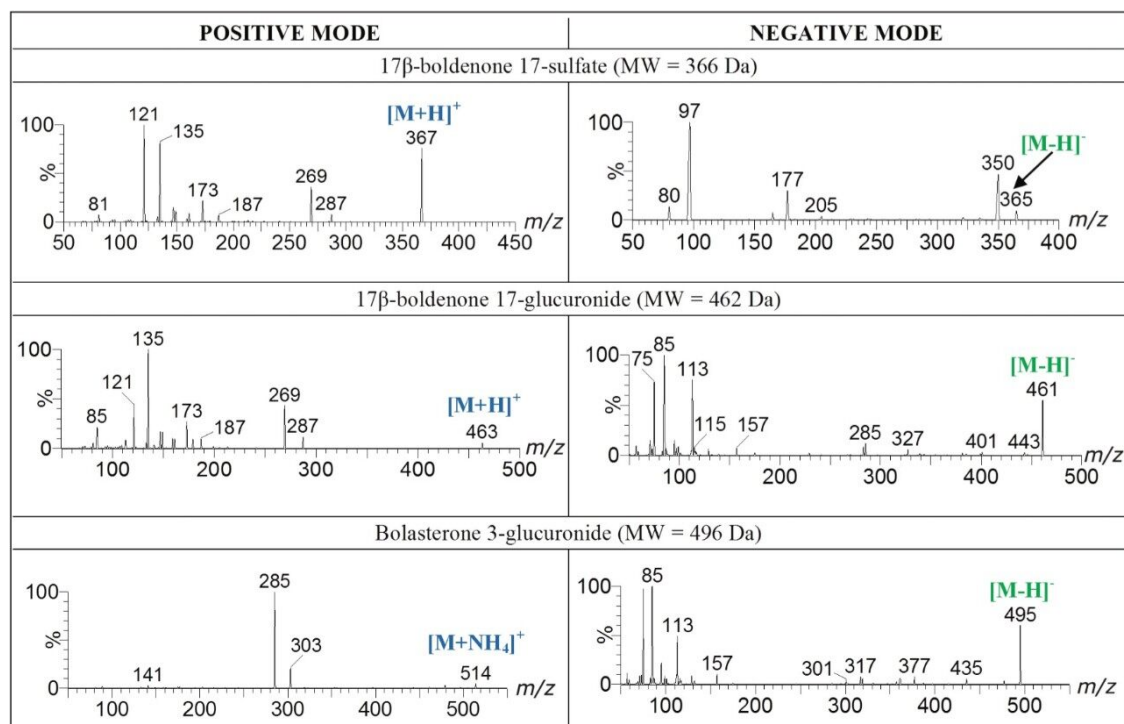


Figure 1. Product ion mass spectra of representative sulfate and glucuronide metabolites in positive and negative electrospray ionization modes.

| Precurso  | Metabolite *  | RT (min) | Mode (ESI) | Precursor ion      |                                      | CV (V) | CE (eV) | Product ion (m/z) |
|---|---|----------|------------|--------------------|--------------------------------------|--------|---------|-------------------|
|   |   |          |            | (m/z)              | Adduct                               |        |         |                   |
| Bolasterone   | bolasterone 3-G                                       | 10.55    | NEG        | 495                | [M-H] <sup>-</sup>                   | 50     | 45      | 85                |
|   |   |          | POS        | 514                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 15     | 40      | 175               |
| Boldenone/boldione  | 17β-boldenone 17-G                                    | 6.16     | POS        | 463                | [M+H] <sup>+</sup>                   | 20     | 25      | 121               |
|   |   |          | POS        | 463                | [M+H] <sup>+</sup>                   | 20     | 20      | 135               |
|   | 5β-androst-1-ene-17β-ol-3-one 17-G                    | 9.65     | NEG        | 463                | [M-H] <sup>-</sup>                   | 30     | 40      | 85                |
|   |   |          | POS        | 465                | [M+H] <sup>+</sup>                   | 25     | 25      | 187               |
|   | 17β-boldenone 17-S                                    | 6.61     | NEG        | 365                | [M-H] <sup>-</sup>                   | 55     | 30      | 350               |
|   |   |          | NEG        | 365                | [M-H] <sup>-</sup>                   | 55     | 40      | 177               |
| epiboldenone 17-S   | 7.32  | NEG      | 365        | [M-H] <sup>-</sup> | 55                                   | 30     | 350     |                   |
| Calusterone   | 5β-androstan-7β,17α-dimethyl-3α,17β-diol 3-G          | 11.80    | NEG        | 495                | [M-H] <sup>-</sup>                   | 50     | 45      | 75                |
|   |   |          | NEG        | 495                | [M-H] <sup>-</sup>                   | 50     | 45      | 85                |
| Dehydrochloromethyltestosterone                                   | 6β-hydroxy-4-chloro-metandienone                      | 9.00     | POS        | 351                | [M+H] <sup>+</sup>                   | 15     | 20      | 147               |
|   |   |          | POS        | 351                | [M+H] <sup>+</sup>                   | 15     | 50      | 91                |
| Drostanolone  | 2α-methyl-5α-androstan-3α-ol-17-one 3-G               | 14.79    | NEG        | 479                | [M-H] <sup>-</sup>                   | 55     | 35      | 85                |
|   |   |          | NEG        | 479                | [M-H] <sup>-</sup>                   | 50     | 35      | 75                |
| Fluoxymesterone   | 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one | 17.02    | POS        | 319                | [M+H] <sup>+</sup>                   | 35     | 25      | 225               |
|   |   |          | POS        | 319                | [M+H] <sup>+</sup>                   | 35     | 25      | 281               |
| Mesterolone   | 1α-methyl-5α-androstan-3α-ol-17-one 3-G               | 12.95    | NEG        | 479                | [M-H] <sup>-</sup>                   | 55     | 35      | 85                |
|   |   |          | NEG        | 479                | [M-H] <sup>-</sup>                   | 55     | 35      | 75                |
| Metandienone  | 1α-methyl-5α-androstan-3α-,17β-diol 3-G               | 10.28    | NEG        | 481                | [M-H] <sup>-</sup>                   | 50     | 35      | 75                |
|   |   |          | NEG        | 481                | [M-H] <sup>-</sup>                   | 50     | 35      | 85                |
|   | 6β-hydroxy-metandienone                               | 5.58     | POS        | 281                | [M+H-2H <sub>2</sub> O] <sup>+</sup> | 30     | 30      | 171               |
|   |   |          | POS        | 299                | [M+H-H <sub>2</sub> O] <sup>+</sup>  | 25     | 25      | 121               |
|   | Epimetandienone                                       | 16.42    | POS        | 301                | [M+H] <sup>+</sup>                   | 20     | 15      | 149               |
|   |   |          | POS        | 301                | [M+H] <sup>+</sup>                   | 20     | 10      | 283               |
| 18-nor-17β-hydroxymethyl-17α-methylandro-1,4,13-triene-3-one 18-S | 7.52  | NEG      | 377        | [M-H] <sup>-</sup> | 60                                   | 40     | 80      |                   |
|   |   | NEG      | 377        | [M-H] <sup>-</sup> | 60                                   | 30     | 362     |                   |
| Metenolone  | 1-methylen-5α-androstan-3α-ol-17-one 3-G              | 11.47    | NEG        | 477                | [M-H] <sup>-</sup>                   | 50     | 35      | 75                |
|   |   |          | POS        | 496                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 15     | 15      | 285               |
| Methyltestosterone  | 17α-hydroxy-17β-methylandro-4,6-dien-3-one            | 16.57    | POS        | 301                | [M+H] <sup>+</sup>                   | 25     | 20      | 225               |
|   |   |          | POS        | 301                | [M+H] <sup>+</sup>                   | 25     | 30      | 210               |
|   | methyl-5β-androstan-3α,17β-diol 3-G                   | 9.91     | POS        | 500                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 15     | 40      | 161               |
|   |   |          | POS        | 500                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 15     | 20      | 271               |
|   | 17α-methyl-5α-androstan-3α,17β-diol 3/17-G            | 10.01    | NEG        | 481                | [M-H] <sup>-</sup>                   | 50     | 35      | 75                |
|   |   |          | NEG        | 481                | [M-H] <sup>-</sup>                   | 50     | 35      | 85                |
|   | 17α-methyl-5β-androstan-3α,17β-diol 3-S               | 9.47     | NEG        | 385                | [M-H] <sup>-</sup>                   | 60     | 40      | 97                |
|   |   |          | NEG        | 385                | [M-H] <sup>-</sup>                   | 60     | 40      | 97                |
| 17β-methyl-5α-androstan-3α,17α-diol 3-S                           | 15.16   | NEG      | 385        | [M-H] <sup>-</sup> | 60                                   | 40     | 97      |                   |
|   |   | NEG      | 385        | [M-H] <sup>-</sup> | 60                                   | 40     | 97      |                   |
| Nandrolone  | 19-noretiocholanolone 3-G                             | 10.20    | POS        | 470                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 20     | 25      | 241               |
|   |   |          | POS        | 470                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 20     | 20      | 259               |
|   | 19-norandrosterone 3-G                                | 10.68    | NEG        | 451                | [M-H] <sup>-</sup>                   | 50     | 35      | 85                |
|   |   |          | NEG        | 451                | [M-H] <sup>-</sup>                   | 50     | 35      | 75                |
|   | 17β-nandrolone 17-G                                   | 5.91     | POS        | 451                | [M+H] <sup>+</sup>                   | 30     | 30      | 85                |
|   |   |          | NEG        | 449                | [M-H] <sup>-</sup>                   | 45     | 30      | 113               |
|   | 17α-nandrolone 17-S                                   | 6.88     | NEG        | 353                | [M-H] <sup>-</sup>                   | 55     | 35      | 97                |
|   |   |          | NEG        | 353                | [M-H] <sup>-</sup>                   | 55     | 35      | 97                |
|   | 17β-nandrolone 17-S                                   | 6.04     | NEG        | 353                | [M-H] <sup>-</sup>                   | 55     | 35      | 97                |
|   |   |          | NEG        | 355                | [M-H] <sup>-</sup>                   | 55     | 35      | 97                |
| 19-noretiocholanolone 3-S   | 9.70  | NEG      | 355        | [M-H] <sup>-</sup> | 55                                   | 35     | 97      |                   |
|   |   | NEG      | 355        | [M-H] <sup>-</sup> | 55                                   | 35     | 97      |                   |
| Oxandrolone   | Oxandrolone   | 12.68    | POS        | 307                | [M+H] <sup>+</sup>                   | 20     | 30      | 93                |
|   |   |          | POS        | 307                | [M+H] <sup>+</sup>                   | 25     | 10      | 289               |
| Epioxandrolone  | Epioxandrolone  | 16.22    | POS        | 307                | [M+H] <sup>+</sup>                   | 20     | 30      | 93                |
|   |   |          | POS        | 307                | [M+H] <sup>+</sup>                   | 20     | 10      | 289               |
| Stanozolol  | 3'-hydroxystanozolol 3'-G                             | 9.22     | POS        | 345                | [M+H-gluc] <sup>+</sup>              | 60     | 45      | 97                |
|   |   |          | NEG        | 519                | [M-H] <sup>-</sup>                   | 45     | 30      | 343               |
|   | 4β-hydroxy-stanozolol 4-G                             | 7.08     | POS        | 521                | [M+H] <sup>+</sup>                   | 25     | 25      | 309               |
|   |   |          | NEG        | 519                | [M-H] <sup>-</sup>                   | 45     | 25      | 193               |
|   | 16β-hydroxy-stanozolol 16-G                           | 4.64     | POS        | 521                | [M+H] <sup>+</sup>                   | 25     | 65      | 81                |
|   |   |          | POS        | 521                | [M+H] <sup>+</sup>                   | 25     | 40      | 345               |
|   | stanozolol-O-G  | 7.74     | POS        | 505                | [M+H] <sup>+</sup>                   | 25     | 45      | 329               |
|   |   |          | POS        | 505                | [M+H] <sup>+</sup>                   | 25     | 65      | 81                |
|   | stanozolol-N-G  | 7.55     | POS        | 505                | [M+H] <sup>+</sup>                   | 25     | 45      | 329               |
|   |   |          | POS        | 505                | [M+H] <sup>+</sup>                   | 25     | 65      | 81                |
| 17-epistanozolol-N-G  | 10.90   | POS      | 505        | [M+H] <sup>+</sup> | 25                                   | 45     | 329     |                   |
|   |   | POS      | 505        | [M+H] <sup>+</sup> | 25                                   | 65     | 81      |                   |
| d3-NAN-S (IS)   | nandrolone-d3 17-S                                    | 6.11     | NEG        | 356                | [M-H] <sup>-</sup>                   | 55     | 40      | 98                |
| d3-T-G (IS)   | testosterone-d3 17-G                                  | 7.30     | POS        | 468                | [M+H] <sup>+</sup>                   | 35     | 25      | 97                |
| d4-And-G (IS)   | androsterone-d4 3-G                                   | 12.08    | NEG        | 466                | [M-H] <sup>-</sup>                   | 45     | 30      | 85                |
|   |   |          | POS        | 488                | [M+NH <sub>4</sub> ] <sup>+</sup>    | 15     | 40      | 95                |
| MET (IS)  | Methyltestosterone                                    | 14.81    | POS        | 303                | [M+H] <sup>+</sup>                   | 30     | 25      | 109               |

Table 1. Compounds and internal standards included in the screening method: retention time (RT), ionization mode (POS, positive; NEG, negative), precursor ion, cone voltages (CV), collision energies (CE) and product ions.

\* S: sulfate; G: glucuronide

An MRM method was set up including one or more ion transitions for each steroid metabolite (Table 1). To select the ion transitions to monitor each analyte, ten different urine samples spiked with the analytes at different concentrations were analyzed, and the selection was based on the signal to noise ratio (>3:1) and the signal intensity of each metabolite. In the case of sulfate conjugates, the ion transitions that yielded the highest signal were those to the product negative ion  $m/z$  97 and, for most sulfate metabolites, only these transitions were monitored (Table 1). However, for other sulfates with molecular masses closer to steroidal endogenous compounds such as  $17\beta$ -boldenone 17-sulfate, the ion transition  $[M-H]^-$  to  $m/z$  97 showed low specificity and negative ion transitions resulting from characteristic fragmentations ( $m/z$  365>350,  $m/z$  365>177) had higher selectivity (Figure 2).<sup>4</sup>

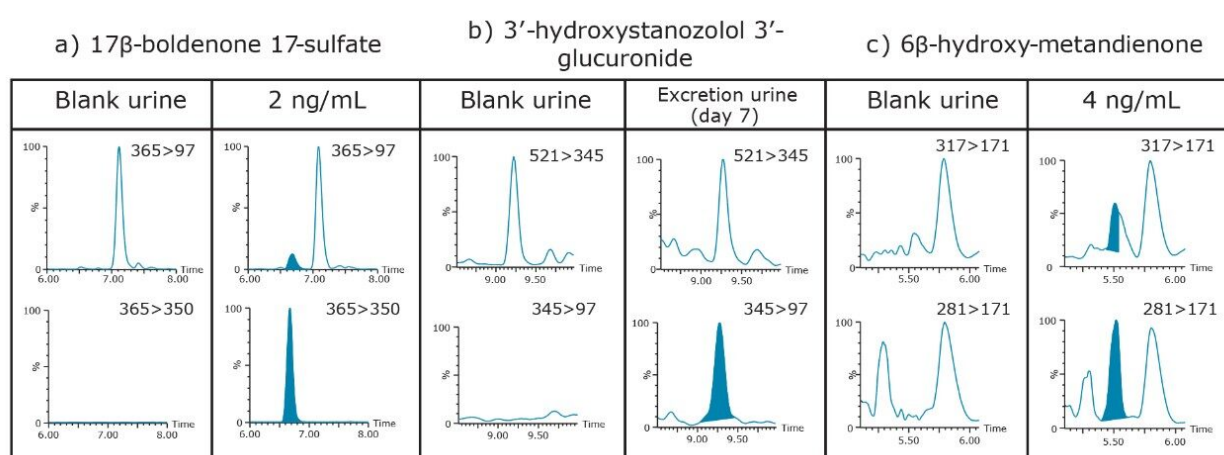


Figure 2. Differences in the detection of (a)  $17\beta$ -boldenone 17-sulfate, (b)  $3'$ -hydroxystanozolol  $3'$ -glucuronide, and (c)  $6\beta$ -hydroxy-metandienone in urine using the ion transitions involving common ions/losses (top) and using specific transitions or in-source fragments (bottom).

For the majority of glucuronides, the ion transitions were related to the glucuronide moiety and the best signal intensity was generally obtained in negative ionization mode (product ions  $m/z$  75, 85, 113) (Figure 1). Some ion transitions demonstrated significant background interferences, therefore a strategy based on the CID of in-source fragments was used to have more selective transitions. As shown in Figure 2, the MRM transition  $[M+H]^+$  ( $m/z$  521) to  $m/z$  345 for  $3'$ -hydroxystanozolol  $3'$ -glucuronide had a big interfering peak that was not present when monitoring the ion transition  $[M+H-gluc]^+$  ( $m/z$  345) to  $m/z$  97.

The same strategy was used for some unconjugated compounds, such as  $6\beta$ -hydroxy-metandienone, for which the use of the in-source fragment  $[M+H-2H_2O]^+$  ( $m/z$  281) as precursor ion improved its detectability compared to the use of  $[M+H]^+$  ( $m/z$  317) (Figure 2).

## Validation of the method

The method was validated for qualitative purposes. Results are listed in Table 2. The limit of detection (LOD) was defined as the lowest concentration at which an analyte was detected in ten different urine samples with a signal to noise ratio of at least 3:1. The LODs were in the range 0.25–4.00 ng/mL for 18 of the 23 analytes. Extraction recovery was evaluated by spiking six urines with the analytes before and after sample preparation, and was calculated by comparing the areas obtained in the samples spiked before sample preparation with the mean of the areas obtained in samples spiked after sample preparation. Extraction recoveries were above 77% for all 23 validated analytes.

Intraday precision was assessed by analysis, on the same day, of six replicates of a urine sample spiked at low and upper concentrations levels. Results were expressed as % relative standard deviation (RSD) of the measured area ratios between the analyte and the IS. Intraday precision was better than 21% for all analytes.

Matrix effect was evaluated using post-column infusion and ranged from 92 to 147%.

| Compound*  | LOD (ng/mL) | Extraction recovery (%). mean $\pm$ SD | Intraday precision |      |       |      | %Matrix effect (RSD %) |
|--|-------------|--|--------------------|------|-------|------|------------------------|
|  |             |  | ng/mL              | RSD% | ng/mL | RSD% |                        |
| 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one                                  | 0.25        | 77.8 $\pm$ 12.70                       | 0.25               | 9.1  | 2.5   | 2.3  | 92 (2.35)              |
| epimetandienone  | 0.5         | 85.2 $\pm$ 10.7                        | 0.5                | 11.1 | 5     | 1.6  | 116 (4.31)             |
| oxandrolone  | 1           | 123.9 $\pm$ 1.9                        | 1                  | 20.2 | 5     | 13.7 | 116 (4.95)             |
| epioxandrolone   | 1           | 109.9 $\pm$ 6.3                        | 1                  | 15.7 | 5     | 8.2  | 109 (1.48)             |
| 6 $\beta$ -hydroxy-4-chloro-metandienone   | 4           | 92.2 $\pm$ 4.6                         | 4                  | 10.6 | 20    | 6.1  | 100 (8.77)             |
| 6 $\beta$ -hydroxy-metandienone  | 4           | 95.7 $\pm$ 4.9                         | 4                  | 16.2 | 20    | 7.3  | 112 (8.02)             |
| 17 $\beta$ -boldenone 17-S   | 0.25        | 93.2 $\pm$ 7.7                         | 0.25               | 4.5  | 2.5   | 9.6  | 118 (4.35)             |
| 17 $\beta$ -nandrolone 17-S  | 0.25        | 96.2 $\pm$ 1.4                         | 0.25               | 12.9 | 2.5   | 7.2  | 125 (7.81)             |
| 17 $\alpha$ -nandrolone 17-S   | 0.5         | 87.1 $\pm$ 2.1                         | 0.5                | 8.8  | 5     | 4.0  | 138 (3.07)             |
| 19-norandrosterone 3-S   | 0.5         | 93.8 $\pm$ 1.5                         | 0.5                | 10.8 | 5     | 3.8  | 141 (6.69)             |
| 19-noretiocholanolone 3-S  | 0.5         | 92.30 $\pm$ 9.6                        | 0.5                | 8.3  | 5     | 12.7 | 114 (6.81)             |
| 19-noretiocholanolone 3-G  | 0.5         | 77.1 $\pm$ 13.5                        | 0.5                | 19.8 | 5     | 18.9 | 141 (8.50)             |
| 17 $\beta$ -boldenone 17-G   | 0.5         | 93.6 $\pm$ 5.2                         | 0.5                | 21.4 | 5     | 7.7  | 123 (10.09)            |
| 3'-hydroxystanozolol 3'-G  | 0.5         | 87.6 $\pm$ 7.1                         | 0.5                | 9.9  | 5     | 5.7  | 120 (2.13)             |
| 2 $\alpha$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ -ol-17-one 3-G                     | 2           | 86.8 $\pm$ 4.0                         | 2                  | 7.8  | 10    | 1.6  | 104 (2.55)             |
| 1 $\alpha$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ -ol-17 $\beta$ -diol 3-G           | 4           | 98.3 $\pm$ 3.8                         | 4                  | 5.2  | 20    | 12.1 | 119 (5.30)             |
| 19-norandrosterone 3-G   | 4           | 95.0 $\pm$ 6.7                         | 4                  | 10.7 | 20    | 9.1  | 147 (9.19)             |
| 1-methylen-5 $\alpha$ -androstan-3 $\alpha$ -ol-17-one 3-G                             | 4           | 82.8 $\pm$ 3.5                         | 4                  | 18.0 | 20    | 2.1  | 144 (5.36)             |
| 1 $\alpha$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ -ol-17-one 3-G                     | 8           | 88.7 $\pm$ 4.4                         | 8                  | 13.1 | 20    | 3.8  | 132 (2.19)             |
| 17 $\beta$ -nandrolone 17-G  | 10          | 96.0 $\pm$ 4.4                         | 10                 | 17.8 | 50    | 5.8  | 115 (5.03)             |
| 5 $\beta$ -androst-1-ene-17 $\beta$ -ol-3-one 17-G                                     | 16          | 94.9 $\pm$ 4.7                         | 16                 | 10.6 | 40    | 8.5  | 112 (4.31)             |
| 5 $\beta$ -androstan-7 $\beta$ ,17 $\alpha$ -dimethyl-3 $\alpha$ ,17 $\beta$ -diol 3-G | 20          | 93.5 $\pm$ 2.0                         | 20                 | 18.8 | 50    | 6.0  | 119 (1.78)             |
| bolasterone 3-G  | >20         | 92.9 $\pm$ 1.4                         | 20                 | 12.6 | 50    | 1.9  | 130 (2.61)             |

Table 2. LOD, extraction recovery, intraday precision, and matrix effect results of the validation for qualitative purposes.

\*S: sulfate; G: glucuronide

## Analysis of excretion study samples and positive samples



The method was applied to samples obtained after administration of different anabolic steroids. As examples, results obtained for methyltestosterone and stanzolol are presented.

Samples obtained after administration of methyltestosterone were analysed using the MRM method to determine the detection times of the six described metabolites. The developed methodology allowed for the detection of 17 $\beta$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ ,17 $\alpha$ -diol 3-sulfate (METm3-S) up to 23 days (Figure 3), whereas the current screening methods (analysis by GC-MS/MS after hydrolysis and derivatization step) detect 17 $\alpha$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol and 17 $\alpha$ -methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol (METm1 and METm2) metabolites up to 4 and 6 days respectively. Therefore the detection capabilities for methyltestosterone have been significantly improved. Unaltered glucuronide METm1-G and METm2-3-G metabolites were detected in urine up to 1 and 5 days, respectively. Detection of the unconjugated metabolite 17 $\alpha$ -hydroxy-17 $\beta$ -methylandro-4,6-dien-3-one (METm5) was around one week. Other recently reported sulfate metabolites (METm2-S, 17 $\alpha$ -methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol 3-sulfate, and METm4-S, 17 $\beta$ -methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\alpha$ -diol 3-sulfate) were detected up to 8 and 13 days, respectively (Figure 3).<sup>5</sup>

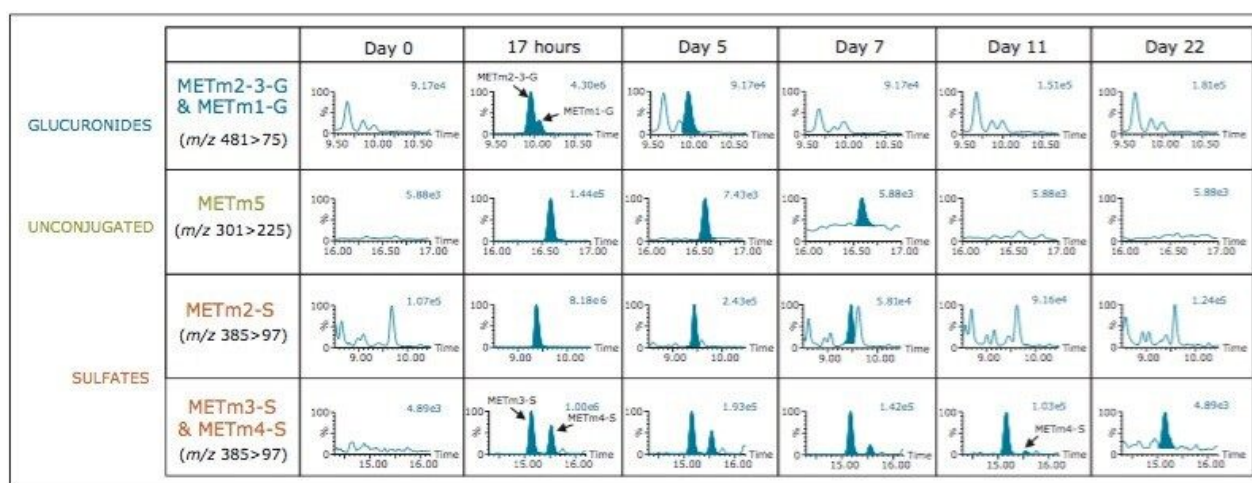


Figure 3. Results of methyltestosterone excretion study: chromatograms of the characteristic ion transitions of the metabolites 17 $\alpha$ -methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol 3-G (METm2-3-G), 17 $\alpha$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol 3/17-G (METm1-G), 17 $\alpha$ -hydroxy-17 $\beta$ -methylandro-4,6-dien-3-one (METm5), 17 $\alpha$ -methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol 3-S (METm2-S), 17 $\beta$ -methyl-5 $\alpha$ -androstan-3 $\alpha$ ,17 $\alpha$ -diol 3-S (METm3-S) and 17 $\beta$ -methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\alpha$ -diol 3-S (METm4-S), obtained after analysis of a pre-administration sample and samples collected at 1, 5, 7, 11 and 22 days after methyltestosterone administration.

Regarding stanzolol, as shown in Figure 4, six different unaltered glucuronides: 3'-hydroxystanzolol 3'-glucuronide (3STAN-G), 4 $\beta$ -hydroxy-stanzolol 4-glucuronide (4STAN-G), 16 $\beta$  hydroxy stanzolol 16-glucuronide (16STAN-G), stanzolol-N-glucuronide (STAN-N-G), stanzolol-O-glucuronide (STAN-O-G) and 17-epistanzolol-N-glucuronide (eSTAN-N-G); were simultaneously monitored. One of them, eSTAN-N-G,

has been recently described as a long-term metabolite and it is not detectable in the current screening conditions because it is stable to enzymatic hydrolysis.<sup>7</sup> Our method allows the detection of this metabolite up to 21 days after administration and, therefore, it improves the detection capabilities for stanozolol misuse.

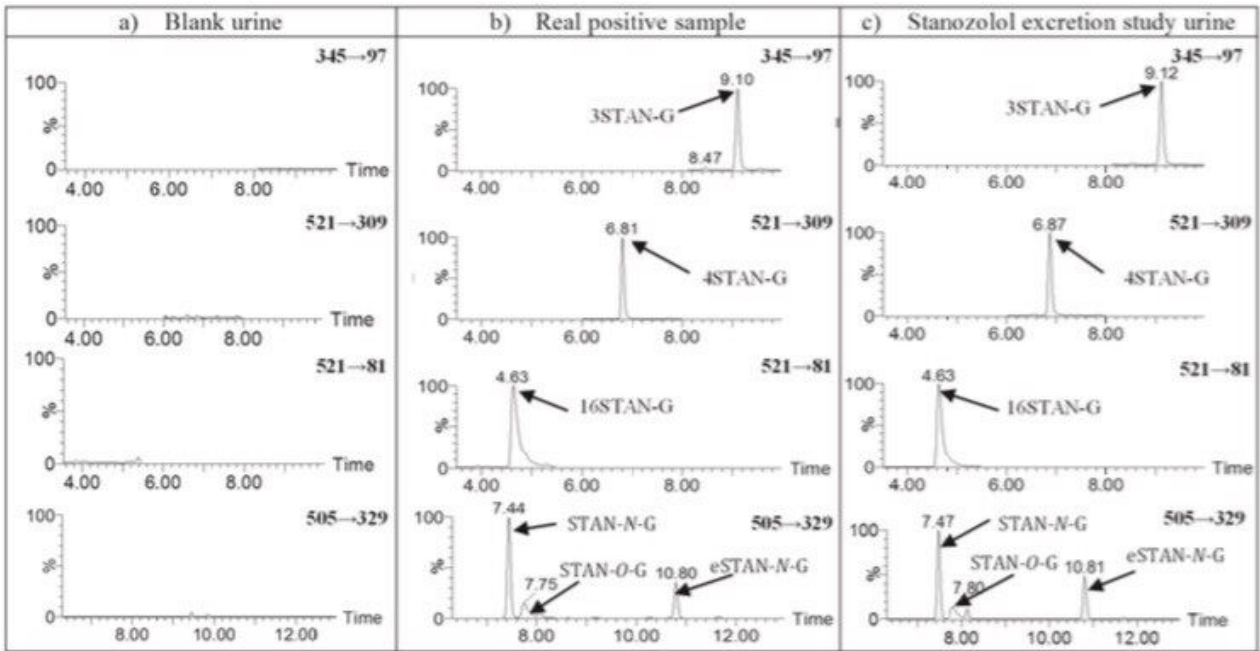


Figure 4. Results of the analysis of a blank urine (a) authentic positive sample (b) and stanozolol excretion study urine (c): chromatograms of the characteristic ion transitions of stanozolol metabolites: 3'-hydroxystanozolol 3' glucuronide (3STAN-G), 4 $\beta$ -hydroxy-stanozolol 4-glucuronide (4STAN-G), 16 $\beta$ -hydroxy-stanozolol 16-glucuronide (16STAN-G), stanozolol-N-glucuronide (STAN-N-G), stanozolol-O-glucuronide (STAN-O-G) and 17-epistanozolol-N glucuronide (eSTAN-N-G).

## Conclusion

A UPLC-MS/MS screening method for the simultaneous detection of steroid metabolites is a feasible alternative compared to the conventional procedures. Incorporation of new phase II metabolites as described, is straightforward and can significantly enhance the screening and detection capabilities of steroids in sport.

---

## References

1. World Antidoping Agency (WADA). 2013 Adverse Analytical Findings and Atypical Findings Reported by Accredited Laboratories. Available from: <https://www.wada-ama.org/en/resources/laboratories/2013-anti-doping-testing-figureslaborato-report#.VBmQFhZnrzB>. (accessed April, 2015).
2. C. Gomez, A. Fabregat, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism. *TrAC-Trend Anal. Chem.* 53 (2013) 106.
3. G. Balcells, O.J. Pozo, A. Esquivel, A. Kotronoulas, J. Joglar, J. Segura, R. Ventura. Screening for anabolic steroids in sports: analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *J Chromatogr A.* 1389 (2015) 65.
4. C. Gomez, O.J. Pozo, H. Geyer, J. Marcos, M. Thevis, W. Schanzer, J. Segura, R. Ventura. New potential markers for the detection of boldenone misuse. *J. Steroid Biochem. Mol. Biol.* 132 (2012) 239.
5. C. Gomez, O.J. Pozo, J. Marcos, J. Segura, R. Ventura. Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids.* 78 (2013) 44.
6. C. Gomez, O.J. Pozo, L. Garrostas, J. Segura, R. Ventura. A new sulphate metabolite as a long-term marker of metandienone misuse. *Steroids.* 78 (2013) 1245.
7. W. Schanzer, S. Guddat, A. Thomas, G. Opfermann, H. Geyer, M. Thevis. Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/ high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal.* 5 (2013) 810.

---

## Featured Products

[ACQUITY UPLC System <https://www.waters.com/514207>](https://www.waters.com/514207)

[MassLynx Mass Spectrometry Software <https://www.waters.com/513164>](https://www.waters.com/513164)

720005565, January 2016

©2019 Waters Corporation. All Rights Reserved.