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Stanozolol-N-glucuronide metabolites in human urine samples as suitable targets in terms of routine anti-doping analysis

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Abstract

The exogenous anabolic-androgenic steroid (AAS) stanozolol stays one of the most detected substances in professional sports. Its detection is a fundamental part of doping analysis, and the analysis of this steroid has been intensively investigated for a long time. This contribution to the detection of stanozolol doping describes for the first time the unambiguous proof for the existence of 17-epistanozolol-1'-N-glucuronide and 17-epistanozolol-2'-N-glucuronide in stanozolol-positive human urine samples due to the access to high-quality reference standards. Examination of excretion study samples shows large detection windows for the phase-II metabolites stanozolol-1'-N-glucuronide and 17-epistanozolol-1'-N-glucuronide up to 12 days and respectively up to almost 28 days. In addition, we present appropriate validation parameters for the analysis of these metabolites using a fully automatic method online solid-phase extraction (SPE) method already published before. Limits of identification (LOIs) as low as 100 pg/ml and other validation parameters like accuracy, precision, sensitivity, robustness, and linearity are given.

KEYWORDS

anabolic androgenic steroids, glucuronide, high-resolution mass spectrometry, phase-II metabolite, stanozolol

1 | INTRODUCTION

The family of anabolic-androgenic steroids (AAS) belongs to one of the most common illicitly used substance class in the world of professional sports. Within this large group of different drugs, the synthetic steroid stanozolol (17 α -methyl-5 α -androst-2-eno[3,2-c]pyrazol-17 β -ol) attributes to the highest number of positive cases according to World Anti-Doping Agencies (WADA) statistics.^{1,2} This exogenous steroid is well known analytically and various strategies for its detection are described in the literature. Because this steroid was synthesized in the late 1950s, there was plenty of time to develop many

different approaches to analyze stanozolol and its metabolites.³ In 1986, the team around Donike and Schänzer developed the first method for the analysis of the metabolite 3'-OH-stanozolol applying gas chromatography–mass spectrometry (GC–MS).⁴ In the following 35 years, many other techniques, primarily based on mass spectrometric techniques coupled to on either gas (GC–MS) or liquid chromatography (LC–MS), for analyzing a large number of different stanozolol metabolites, were published.^{5–20}

In general, the traditional approach for the simultaneous analysis of several different steroids is to perform enzymatic hydrolysis to cleave highly polar phase-II conjugates, like glucuronic acids and

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sulfates, followed by liquid–liquid extraction and the analysis of remaining phase-I metabolites and parent molecules with GC- or LC-MS.^{21,22} For the measurement with GC-MS, the analytes are additionally derivatized with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) to reduce their polarity. This kind of approach is the gold standard nowadays and is commonly performed by anti-doping laboratories worldwide for the routine initial testing procedure (ITP), often including the detection of stanozolol parent or phase-I metabolites.

However, with the emergence of more powerful LC-MS devices, a new, modern way of steroid analysis was developed. With this approach, time- and resource-consuming steps of enzymatic hydrolysis, extraction and derivatization are omitted. Phase-II conjugates of steroids are analyzed directly without further extraction or concentration steps.^{15–20,23–29} In 2015, the team around G. Balcells already proposed the analysis of a high number of relevant phase-II metabolites for anti-doping screening purposes.¹⁶ Nowadays, high-resolution (HR) LC-MS devices are frequently used in order to increase sensitivity and selectivity of the measurement. In 2013, Van Eenoo et al. showed the promising potential of this approach for the detection of stanozolol abuse for the first time.¹⁷ The team developed an approach for the direct analysis of 3'-OH-stanozolol glucuronide in human urine.

This idea was adopted by developing a simple but powerful method for the detection of phase-II metabolites of steroids, as previously published. This approach was optimized by placing a fully automated online solid-phase extraction (SPE) procedure upstream of the analytical measurement with LC-HRMS.¹⁸ Next to the aspect of

saving time and resources by direct analysis of phase-II conjugates, no enzymatic hydrolysis step using, for example, β -glucuronidase from *Escherichia coli* is required. Consequently, issues like incomplete or inhibited hydrolysis to yield phase-I metabolites, as necessary for GC-MS methods, are no longer relevant. Literature and own experience demonstrates that, for example, stanozolol-N-glucuronides are hardly hydrolyzed with enzymes commonly used in anti-doping laboratories.¹⁹ As a consequence, these metabolites are usually not detected in routine ITP at all.

We have observed that the excretion profile for stanozolol-N-glucuronides is consistent in most positive samples, depending on the drug's application time. Figure 1 shows a typical extracted ion chromatogram (XIC, $m/z = 505.3 \rightarrow 329.3$) for stanozolol-mono-glucuronides of a positive urine sample and the known corresponding metabolite structures, which are based on the metabolically unchanged molecule of stanozolol.

The structures behind Peaks A–C were already suggested by Schänzer et al. in 2013 and Thevis et al. in 2015.^{19,20} Peak A represents stanozolol-17'-O-glucuronide, and Peaks B and C represent two N-glucuronides of stanozolol. These two metabolites were identified and characterized in our previous work.¹⁸ These two phase-II metabolites were identified as stanozolol-1'-N- (B) and stanozolol-2'-N-glucuronide (C). Aim of the present study was to use this method for characterization of the two remaining metabolites D and E. Schänzer and Thevis already suggested the appearance of a 17-epistanozolol-glucuronide in above-mentioned studies. However, in both cases, an unambiguous identification was not successful due to the lack of

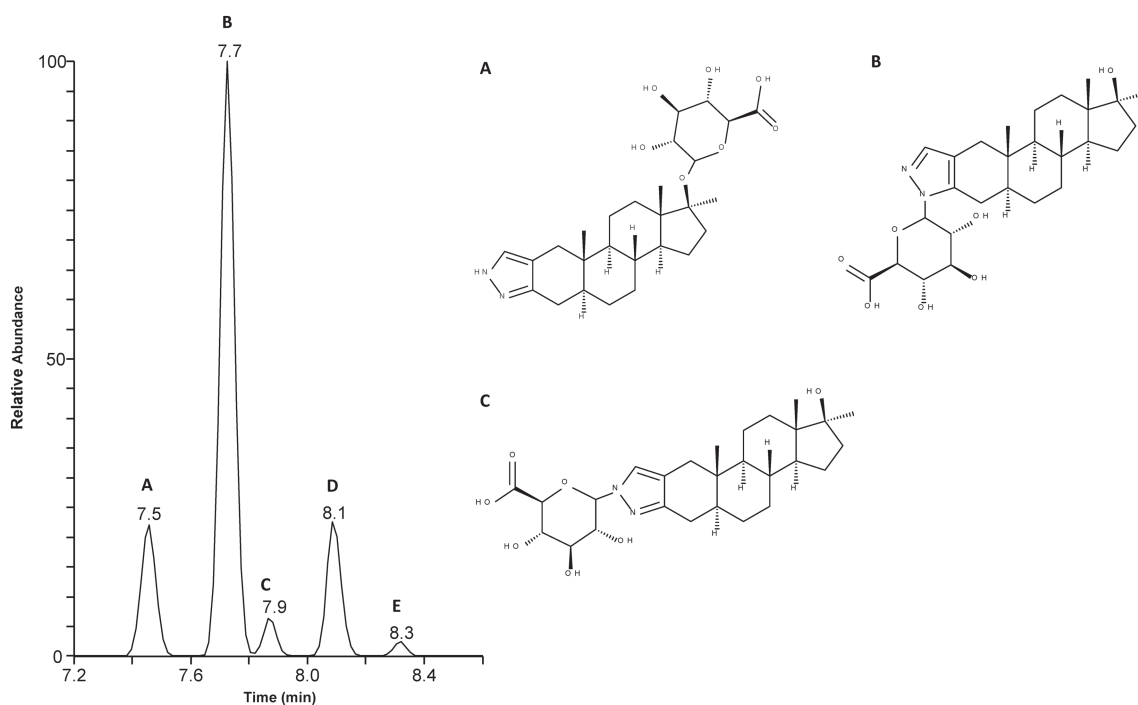


FIGURE 1 Extracted ion chromatogram (XIC) ($m/z = 505.3 \rightarrow 329.3$) of stanozolol-mono-glucuronides and corresponding chemical structures: (a) stanozolol-17'-O-glucuronide, (b) stanozolol-1'-N-glucuronide, (c) stanozolol-2'-N-glucuronide, (d) 17-epistanozolol-1'-N-glucuronide, and (e) 17-epistanozolol-2'-N-glucuronide (see text below)

high-quality reference material. Furthermore, differentiation of 17-epistanozolol-1'-N- and 17-epistanozolol-2'-N-glucuronide metabolites was not performed. The structures of these two new metabolites and the parent molecule are shown in Figure 2.

At the Institute of Applied Synthetic Chemistry, Technical University of Vienna, Austria, these two metabolites were synthesized in an amount suitable to confirm their structures with nuclear magnetic resonance (NMR) spectrometry. The detailed description of the synthesis procedure and NMR analysis is still in progress and will soon be published elsewhere. Unambiguous identification and characterization of 17-epistanozolol-1'-N- (II) and 17-epistanozolol-2'-N-glucuronide (III) is given by using mass spectrometric techniques to compare these reference standards with stanozolol positive human urine samples from excretion experiments or actual athletes. Additionally to the characterization of these two new metabolites, the potential of all four N-associated metabolites for a routine anti-doping analysis of stanozolol is demonstrated. A comprehensive validation and the application of the validated method to an excretion study for stanozolol demonstrates the fitness for purpose of this analytical method as well as the window of detection for stanozolol abuse.

2 | EXPERIMENT

2.1 | Chemicals, reagents, and solutions

Water (high-performance liquid chromatography, HPLC grade) and Methanol (MeOH, HPLC grade) used for HPLC analysis were bought from Biosolve Chimie (Dieuze, France). Formic acid (FA) used for HPLC was purchased from Merck (Darmstadt, Germany). Water (MQ) used for sample dilution was provided by a Milli-Q water purification system (Millipore, Reference A+, Burlington, Massachusetts, USA). Methanol used to prepare standard solutions was supplied by Chem-Lab (Zedelgem, Belgium). The 16,16,17 α -d3-testosterone-glucuronide used as the internal standard (IS) was bought from the National Measurement Institute Australia (Sydney, Australia). All stanozolol metabolite standards were synthesized by the team of Peter Gärtner at the Technical University of Vienna and

characterized by NMR spectroscopy. Chemical structures are shown in Figure 2.

Stock solutions with a concentration of 1 μ g/ml for IS and standard substances were prepared by dissolving 1 μ g of standard substance in 1-ml MeOH. Standard working solutions were prepared by diluting stock solutions with MeOH. Until use, solutions were stored at -20°C . Reference samples were prepared by adding working solutions directly to blank urine.

2.2 | Urine samples

According to WADA's collection guidelines, all positive urine samples used in this project were collected by accredited sample collection authorities.³⁰ The samples have previously been analyzed by the accredited anti-doping laboratory Seibersdorf Labor GmbH. All samples are unanimously confirmed positive for stanozolol. The samples were subsequently anonymized and approved for research. Previously, the athletes gave permission to use the urine samples for research purposes, according to the International Standard for Laboratories (ISL).³¹ Samples used for the excretion study were provided by the accredited anti-doping laboratory Cologne, Institute of Biochemistry—German Sport University Cologne, Germany. For these samples, a male healthy volunteer received a single oral dose of 5 mg of stanozolol (Winstrol[®]). Urine samples were then collected up to 28 days after administration of the substance. A written agreement was received from the participant and the project was accepted by the local ethical committee.¹⁹ The anonymized blank urine samples were provided from healthy female and male volunteers. Until analysis, all urine samples were stored at -20°C .

2.3 | Sample preparation

For sample preparation, 250 μ l of urine was diluted with 250 μ l of MQ, 15 μ l of IS (30 ng/ml) solution was added, followed by vortexing samples for 10 s.

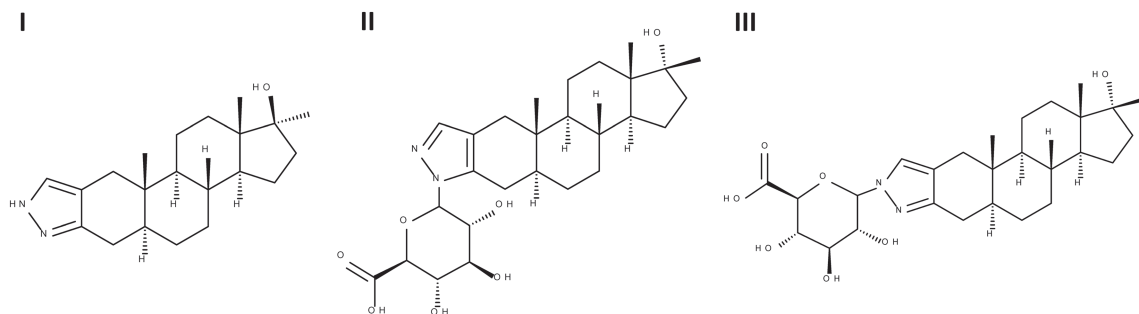


FIGURE 2 Chemical structures of I: Stanozolol, II: 17-epistanozolol-1'-N-glucuronide, and III: 17-epistanozolol-2'-N-glucuronide

2.4 | Online SPE coupled to liquid chromatography HR mass spectrometry (online-SPE-LC-HRMS)

An online-SPE-LC-HRMS approach was chosen as analytical method. The method is described in detail in a previous publication.¹⁸ Analytes extraction is carried out fully automatically upstream the injection into the Vanquish Horizon UHPLC+ system (Thermo Fisher, Austin, Texas, USA). An Accucore Phenyl-Hexyl, 10 × 3-mm column with 2.6- μ m particle and 80-Å pore size (Fischer Scientific, Loughborough, UK) was used as extraction column. As analytical column, a Kinetex EVO C-18, 100 × 2.1-mm column with 2.6- μ m particle- and 100-Å pore size (Phenomenex, Aschaffenburg, Germany) was applied. For chromatography, mobile phases containing water with 0.2% v/v FA (Solvent A) and methanol with 0.1% v/v FA (Solvent B), constant flow of 0.4 ml/min, constant temperature at 25°C, and an injection volume of 25 μ l were used. Following gradient was carried out: 10% Solvent B for 2 min to load and wash the pre-column, 10% Solvent B up to 100% over 7 minutes, hold 100% B for 2 min and again 10% B for 2 min to flush and re-equilibrate the system.

HR mass spectrometric measurements were carried out on a Q-Exactive Orbitrap system (Thermo Fisher, Austin, Texas, USA) in positive electrospray ionization mode (ESI+) using the following settings: spray voltage was set to 3.8 kV, and capillary temperature was 320°C. Nitrogen was used as sheath gas (pressure 25 units) and as auxiliary gas (pressure 8 units, temperature 310°C). Sweep gas flow rate was set to 0 and s-lens radio frequency (RF) level was 55. A mass resolution of 70,000 at m/z 200 and automatic gain control (AGC) to 2×10^5 ions were carried out.

Parallel reaction monitoring (PRM) was chosen as measuring method. To extract ion chromatograms (XIC), transitions shown in Table 1 with an ion extraction range of 5 ppm were used. Isolation windows were set to ± 1 m/z . Collision energies (CEs) were optimized by injection of methanolic working solutions of reference substances. Diagnostic ions and corresponding CEs are also shown in Table 1. The software Thermo Xcalibur Qual Browser 4.1.45 was used for data procession and calculation of monoisotopic masses. All systems were supervised with Xcalibur 4.0 (Thermo Fischer).

2.5 | Method validation

Method validation parameters for qualitative and semi-quantitative purposes were used according to the ISL. The following parameters were acquired: specificity, precision, robustness, linearity, accuracy, matrix effects, carryover and limit of identification (LOI). Detailed

descriptions of all parameters are given below. Method validation was carried out by using the above described PRM method. Peak areas gained from product ion 1 were used for all semi-quantitative parameters. Concentrations were corrected with the IS and calculated with an internal calibration curve measured in each sequence. Data processing used the software Thermo Xcalibur Quan Browser 4.1.45 and parameters were calculated with Microsoft Excel 2010. The minimum required performance level (MRPL) for free stanozolol is 2 ng/ml, as defined in the WADA Technical Document TD2019MRPL.³² Therefore, 50% of MRPL, 1 ng/ml, were used for most validation parameters. According to the WADA identification criteria, comparison of retention times and ratios of relative abundances of two ion transitions were used to evaluate the specificity, robustness and LOI.³³ For comparisons, matrix-free (MQ) samples were spiked with reference substances at the respective concentrations.

2.6 | Specificity

Five different female and five different male blank urine samples from healthy volunteers were analyzed ($n = 10$). Furthermore, a second set of these 10 samples were spiked with 1-ng/ml standard working solution. Relative abundances (peak area) of two ion transitions and retention times were compared in order to verify the absence of interferences for both diagnostic ions.

2.6.1 | Precision

Three sets of 10 replicates of blank urine samples were spiked with standard working solution at three different concentrations, low 1 ng/ml, medium 10 ng/ml, and high 50 ng/ml ($n = 3 \times 10$) and were analyzed. Coefficient of variation (CV) of areas (normalized with IS) for intra- and inter-day precision for three concentration levels was calculated by measuring samples on three consecutive days.

2.6.2 | Robustness

Blank urine samples with various specific gravities (0.005, 0.010, 0.020, 0.025, and 0.030) and different pH values (3, 4.5, 6, 7.5, and 9) were spiked with 1-ng/ml standard working solution and were analyzed. Additionally, increasing injection volumes (15, 20, 25, 30, and 35 μ l) were tested ($n = 15$). Comparison of retention times and relative abundances of two ion transitions was carried out.

TABLE 1 Mass transitions used for parallel reaction monitoring (PRM) for 17-epistanozolol-1'-N- and 17-epistanozolol-2'-N-glucuronide (e1N-SG and e2N-SG) and IS d3-testosterone-glucuronide (D3-TG)

Substance	Formula	Precursor ion (m/z)	Species	Product ion 1 (m/z)/(eV)	Product ion 2 (m/z)/(eV)
e1N-SG	C27H40N2O7	505.2908	[M + H] ⁺	329.2587/60	81.0447/70
e2N-SG	C27H40N2O7	505.2908	[M + H] ⁺	329.2587/60	81.0447/70
D3-TG	C25H32D3O8	468.2671	[M + H] ⁺	109.0645/35	97.0651/35

2.6.3 | Linearity

Calibration curves were generated by measuring four replicates of urine samples spiked with standard working solution at six different concentrations (1, 10, 25, 50, 75, and 100 ng/ml, $n = 4 \times 6$). The Software Thermo Quan Browser was used to calculate linearity (R^2).

2.6.4 | Accuracy

Three sets of 10 replicates of blank urine samples were spiked with standard working solution at three different concentrations, low 1 ng/ml, medium 10 ng/ml, and high 50 ng/ml ($n = 3 \times 10$) and were measured. Accuracy (determined concentration/nominal concentration*100%) was calculated.

2.6.5 | Matrix effects

Six different blank urine samples and one matrix-free sample (MQ) were spiked with 1-ng/ml standard working solution and measured. Average matrix effects (ion suppression or enhancement) were calculated by comparing signal area (normalized with IS) of urine samples to the matrix-free sample.

2.6.6 | Carryover

Blank urine sample was spiked with 400-ng/ml standard working solution and measured directly prior to a blank urine sample. The intensity of signal area (normalized with IS) in the blank sample was calculated (%).

2.6.7 | Limit of identification

Three sets of three different blank urine samples were spiked with standard working solution at three concentrations (0.05, 0.075, and 0.1 ng/ml, $n = 3 \times 3$), close to an estimated LOI and were analyzed. According to WADA specifications, LOI was defined as the lowest concentration level at which the analytical signal meets the regulations for relative abundance and retention times. The acronym LOI, used by WADA, is coequal with the more known term limit of detection (LOD).

3 | RESULTS AND DISCUSSION

3.1 | Method validation

The method validation parameters of the 17-epistanzolol-N-glucuronides are quite similar to the values observed for stanazolol-N-glucuronides in our previous work.¹⁸ In Table 2, the

TABLE 2 Summary of validation results for 17-epistanzolol-1'-N- and 17-epistanzolol-2'-N-glucuronide (e1N-SG and e2N-SG)

n	c (ng/ml)	Specificity		Intra-day pr. CV		Inter-day pr. CV		Accuracy		Linearity r^2	Robustness	Carryover	ME (RSD)	LOI
		10/10	10/10	10	10	10	10	10	10					
1	1	10/10	10/10	4.8%	3.5%	6.7%	7.4%	6.3%	99.6%	91.3%	99.6%	0%	177% (11%)	100 pg/ml
	10	10/10	10/10	3.9%	3.1%	4.3%	3.2%	2.7%	90.6%	94.2%	102.1%	0%	184% (11%)	100 pg/ml
	100	10/10	10/10	4.8%	3.5%	6.7%	7.4%	6.3%	99.6%	91.3%	99.6%	0%	177% (11%)	100 pg/ml
	100	10/10	10/10	3.9%	3.1%	4.3%	3.2%	2.7%	90.6%	94.2%	102.1%	0%	184% (11%)	100 pg/ml

Abbreviations: c, concentration; CV, coefficient of variation; LOI, limit of identification; ME, matrix effects; n, number of samples; pr., precision; RSD, relative standard deviation.

determined validation parameter values for 17-epistanozolol-1'-N- and 17-epistanozolol-2'-N-glucuronide are summarized.

Passing the WADA identification criteria in 10 of 10 samples for both metabolites reflects this method's high specificity. No interfering signals could be observed. Furthermore, suitable intra- (CV 1.9%–4.8%) and inter-day (CV 2.7%–7.4%) precision values and satisfying accuracy parameters (90.6%–102.1%) were achieved. R^2 values (0.999 and 0.997) confirm a linear signal response development with increasing substance concentration for both metabolites. Suitable robustness in 15 of 15 samples was accomplished for the 17-epistanozolol-2'-N metabolite. However, for the 17-epistanozolol-1'-N metabolite, only 14 of 15 samples passed the identification criteria. The sample with an injection volume of 35 μ l could not pass the criteria. In this sample, product ion 2 (m/z 81) showed a disproportionately increased abundance compared to product ion 1 (m/z 329), leading to a bigger area ratio than a reference sample without matrix and with smaller injection volume. No carryover effect at all was observed after injection of a high concentration sample. Probably due to the lack of comprehensive sample preparation, high matrix effects (177% and 184%) were observed, which, however, do not seem to have a negative influence on precision and accuracy of the method. Nevertheless, for pure

quantitative measurements a matching deuterated IS is recommended. Fulfilling WADAs identification criteria, we could detect both 17-epistanozolol-1'-N-glucuronide and 17-epistanozolol-2'-N-glucuronide at the lowest concentration of 100 pg/ml. By applying alternative criteria for the calculation of the LOI, for example, a signal/noise ratio of >3 , the LOIs would be even lower (50 pg/ml). These suitable validation parameters promise a reliable use of this method for the confirmation of stanozolol doping in routine anti-doping analysis.

3.2 | Identification of 17-epistanozolol-N-glucuronides

In order to identify the two metabolites in question, 17-epistanozolol-1'-N- and 17-epistanozolol-2'-N-glucuronide, HRMS/MS measurements were performed with the above-described PRM method on stanozolol positive urine samples, blank urine samples, and urine samples spiked with reference standards. Extracted ion chromatograms (XIC) with the transition $m/z = 505.2908 \rightarrow 329.2578$ are shown in Figure 3-I.

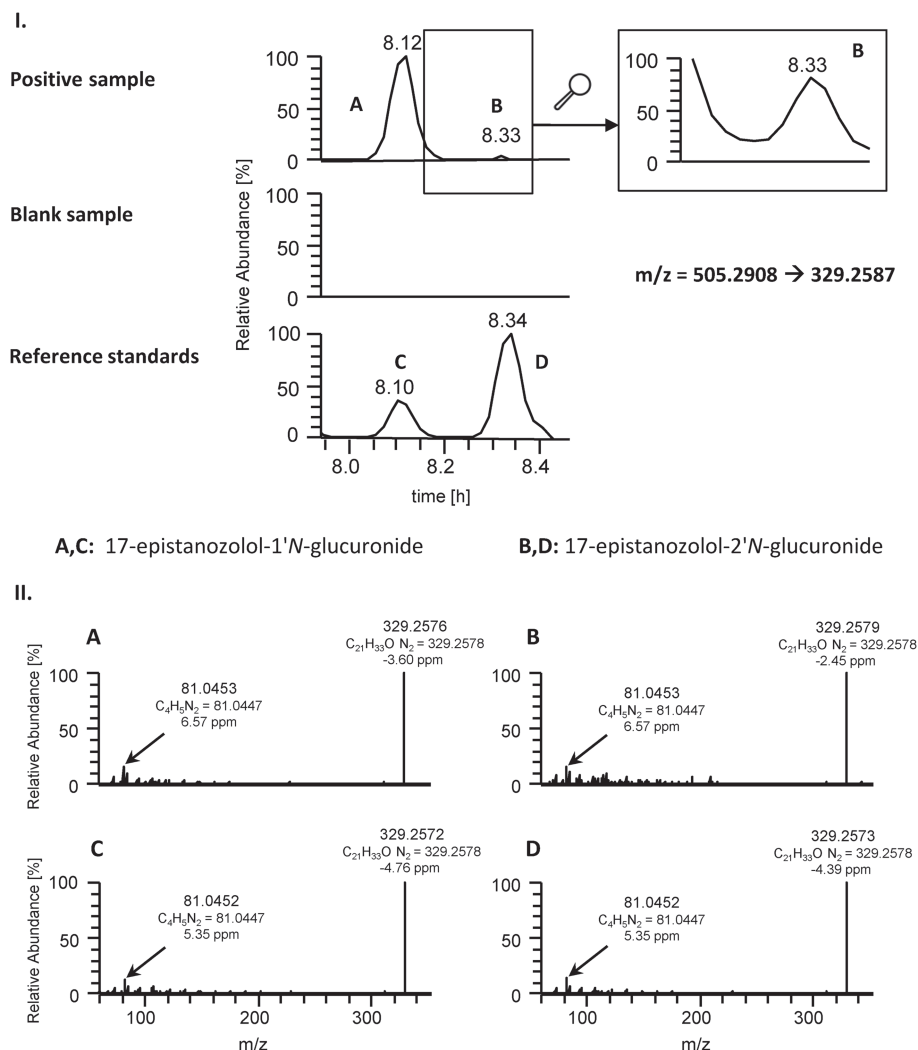


FIGURE 3 Results of parallel reaction monitoring (PRM) measurements; I: XIC of positive urine, blank urine and reference standards; m/z 505.2908 \rightarrow 329.2587 (60 eV), ESI+, 5-ppm mass tolerance II: Corresponding PRM spectra of 17-epistanozolol-1'-N- (a, c) and 17-epistanozolol-2'-N-glucuronide (b, d)

The analysis of a number of positive urine samples showed that 17-epistanozolol-2'-N-glucuronide (B) is excreted only in significantly lower concentrations, mostly below the detection limit of this method, compared to 17-epistanozolol-1'-N-glucuronide (A). In the positive sample shown as an example in this paper, it was possible to provide evidence for the appearance of 17-epistanozolol-2'-N-glucuronide (B). In order to visualize the corresponding peak, the chromatogram was expanded at the relevant position. In the blank sample, no signals have been observed. The urine sample spiked with reference standards (2.5 ng/ml) shows excellent signals for both 17-epistanozolol-1'-N-glucuronide (C) and 17-epistanozolol-2'-N-glucuronide (D). In Figure 3-II, the corresponding PRM mass spectra are shown. All four signals exhibit a highly similar mass spectrometric pattern. Both metabolites form the two stanozolol-glucuronide specific product ions at m/z 329 and 81. The product ion at m/z 329 is formed by the cleavage of the glucuronic acid and represents the resulting stanozolol aglycone molecule. The product ion at m/z 81 is suggested to consist of a robust heterocyclic pyridazine hexagonal ring structure. It is formed by fusing the pyrazole ring with an additional C atom from the sterane backbone during the fragmentation process.¹³ In all four cases, the deviation of the theoretical mass from the experimental mass was less than 5 ppm for the ion at m/z 329. For the ion at m/z 81, the mass deviation is below 7 ppm, explainable by the higher amount of interfering signals in the area of smaller masses.

Comparing retention times and at least two MS/MS transitions of the targeted analyte in a positive sample and a reference sample is required to fulfill WADA identification criteria. The relative abundance of diagnostic ions can be determined from peak areas or heights. In this work, peak areas were used. Table 3 shows the comparative calculations of retention times and abundances, as well as the criteria to be met.

With 0.2% difference for 17-epistanozolol-1'-N-glucuronide and 0.1% for 17-epistanozolol-2'-N-glucuronide, for both metabolites, the relative differences of retention times were significantly below the maximum tolerance of 1%. Furthermore, the relative area abundances' differences were 0.6% and 0.8%, which is also far below the tolerated 5% aberrance. These data provide the unequivocal proof of the existence of 17-epistanozolol-1'-N-glucuronide and 17-epistanozolol-2'-N-glucuronide in human urine after ingestion of the exogenous steroid stanozolol.

3.3 | Excretion study

W. Schänzer et al. demonstrated the utility of stanozolol-glucuronides to improve the detection of stanozolol abuse by analyzing excretion study samples in their work in 2013 for the first time.¹⁹ In the following years, further research teams confirmed the usefulness of these metabolites for long-term detection of stanozolol administration in their studies with a higher number of volunteers using oral and intramuscular administration

TABLE 3 Comparison of retention times and relative abundances of two MS/MS transitions for a positive stanozolol sample and reference standards for 17-epistanozolol-1'-N- and 17-epistanozolol-2'-N-glucuronide (e1N-SG and e2N-SG)

Substance	Retention time IS (min)		Retention time samples (min)		Rel. Difference max. tolerated: 1%	Substance	Transition (m/z)	Relative abundance		Difference	Maximum tolerance window
	sample	reference	sample	reference				sample	reference		
e1N-SG	7.45	7.45	8.12	8.10	0.2%	e1N-SG	→ 329.2587 (60 eV) → 81.0447 (70 eV)	100%	100%	0.6%	10.9%–20.9% (±5)
e2N-SG	7.45	7.45	8.33	8.34	0.1%	e2N-SG	→ 329.2587 (60 eV) → 81.0447 (70 eV)	15.9%	15.3%	0.8%	9.8%–19.8% (±5)
								14.8%	15.7%		

Abbreviation: IS = internal standard.

of stanozolol.^{15,16} However, due to the lack of proper reference substances, in all cases, metabolite elimination data were presented based on relative signal intensities rather than metabolite concentrations. The re-analysis of the same excretion samples used in the work of W. Schänzer provided similar if not equal results including substance concentrations as shown in Figure 4. However, this study focuses only on the analysis of stanozolol-N-glucuronides. The chart shows the concentrations of the four different N-glucuronide metabolites in human urine over time in hours. In order to ensure better comparability of concentrations, values were adjusted for the urine specific gravity according to WADA recommendations and are presented on a logarithmic scale.³⁴

These data clearly confirm the large excretion window of 17-epistanozolol-1'-N-glucuronide, which is up to almost 4 weeks. Compared to all other known stanozolol metabolites, this metabolite has the largest timeframe for detection.²⁹ Stanozolol-1'-N-glucuronide was detectable up to 12 days. The two 2'-N-glucuronides show shorter detection windows up to only 2 days. A major difference in the concentrations of the metabolites can also be observed. At the maximum, stanozolol-1'-N-glucuronide is excreted in about 25 times higher concentration compared to 17-epistanozolol-1'-N-glucuronide. As already mentioned above, 17-epistanozolol-2'-N-glucuronide is only excreted in comparably low concentrations, which is clearly demonstrated in these samples. Almost all data points for this metabolite are below the LOI of the method. Consequently, the concentrations of 17-epistanozolol-2'-N-glucuronide below the LOI of 0.1 ng/ml, presented in Figure 4, do not meet the WADA criteria and shall be interpreted as indicative. This metabolite is regarded as of minor importance for the long-term detection of stanozolol doping, but may nevertheless provide information about the time of application of stanozolol, if successfully detected.

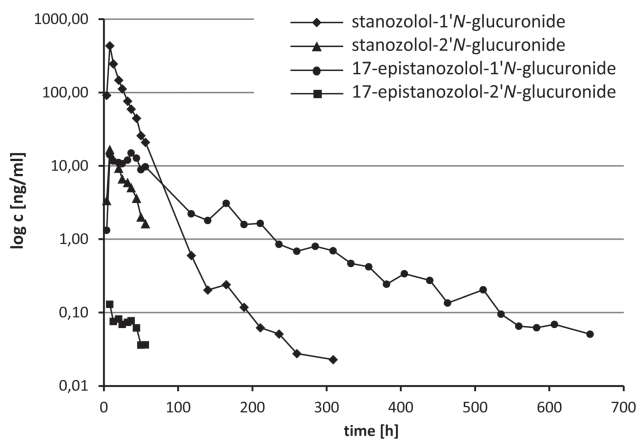


FIGURE 4 Elimination curve of four stanozolol-N-glucuronides monitored in the excretion study samples after oral application of 5 mg of stanozolol; y axis: Concentration in nanograms per milliliter on logarithmic scale, x axis: Time in hours

4 | CONCLUSION

With the previously developed fully automated SPE-LC-HRMS method, a simple and fast procedure yielding excellent validation parameters for the analysis of 17-epistanozolol-N-glucuronides has been established. Using this method, the presence of 17-epistanozolol-1'-N-glucuronide and 17-epistanozolol-2'-N-glucuronide in human urine after intake of stanozolol was unequivocally confirmed. Furthermore, due to access to high-quality reference samples, an elimination curve based on the absolute metabolite concentrations of all four stanozolol-N-glucuronides in human urine excretion samples was shown for the first time. The long detection window of up to almost 28 days, the ease of analysis, and the access to synthesized reference standards qualify these metabolites as suitable targets for routine stanozolol analysis.

The fact that these N-glucuronides, some of which exhibit very large detection windows, are resistant to β -glucuronidase means that the long detection time frames of stanozolol are not fully utilized today, because normal ITP relies on the use of β -glucuronidase.

Furthermore, the direct analysis of glucuronide metabolites delivers promising results for many other substances, too. Therefore, consideration should be given to complementing the usual ITP with an approach involving the direct analysis of glucuronide metabolites of doping substances without the use of glucuronidase.

Direct analysis of steroid phase-II metabolites is deemed to bring many advantages to the field of anti-doping analysis. Therefore, the characterization of new unknown metabolites and the subsequent production of reference substances should stay in focus of current research.

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