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RESEARCH ARTICLE

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Development and validation of a simple online-SPE method coupled to high-resolution mass spectrometry for the analysis of stanozolol-N-glucuronides in urine samples

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Abstract

Stanozolol is still the most commonly used illicit anabolic-androgenic steroid (AAS) in professional sports. Therefore, accurate and fast analysis and long detection windows are of great interest in the field of antidoping analysis. In this work, a very simple, fast, and highly sensitive online solid-phase extraction method coupled with liquid chromatography–high-resolution tandem mass spectrometry (HPLC-HRMSMS) for the analysis of stanozolol-N-glucuronides was developed. This fully validated procedure is characterized by only a few manual steps (dilution and addition of internal standard) in the sample preparation. A limit of identification (LOI) of 75 pg/mL, high accuracy (87.1%–102.1%), precision (3.1%–7.8%), and sensitivity was achieved. Furthermore, good linearity (> 0.99) and robustness, as well as no carry-over effects, could be observed. In addition to excellent confirmation analysis performance, this method shows sufficient potential for the identification and characterization of unknown metabolites. Using this method, it was possible to unambiguously confirm the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine for the first time due to the access to reference material.

KEYWORDS

anabolic androgenic steroids, mass spectrometry, online solid-phase extraction, phase-II metabolite, stanozolol

1 | INTRODUCTION

Since the beginning of doping analysis, knowledge about the window of opportunity for the detection of illicit substances has been of great interest. The discovery of so-called long-term metabolites (LTM) of substances listed on the Prohibited List of the World Anti-Doping Agency (WADA),[1] is still one of the most important topics in current antidoping research.[2] In particular, the analysis of anabolic-androgenic steroids (AAS), which represent the most frequently detected class of illicitly used substances in professional sport,[3] has always been a major priority in the field of doping analysis.[2,4] Conventional steroid analysis is based on the enzymatic cleavage of steroid phase-II metabolite conjugates and the following analysis of remaining parent molecules and phase-I metabolites as their trimethylsilyl-derivatives with gas chromatography-tandem mass spectrometry (GC–MS/MS).[5,6] Although this very sensitive and highly efficient method is still the gold standard for routine steroid analysis in doping control, many previous studies have shown that the direct analysis of steroid phase-II

conjugates using liquid chromatography–tandem mass spectrometry (LC–MS/MS), is a highly promising approach for the detection of unknown steroid metabolites, respectively LTMs.[7–15] In most cases, phase-II metabolites are excreted in urine as glucuronide- and/or sulfate-conjugates. Both conjugates lead to an increase in water solubility, allowing urinary excretion.[16–19]

According to WADA statistics, stanozolol (17α-methyl-5α-androst-2-eno[3,2-*c*]pyrazol-17β-ol) is the most detected substance within the class of AAS.[3] It was first synthesized in 1959 and due to its special structure with a pyrazole ring fused to the androstane framework, it has a unique place in the family of AAS.[20] To date, many stanozolol metabolites have been described. Donike and Schänzer developed the detection of the metabolite 3′-OH-stanozolol as early as 1986 and successfully implemented the method for the Olympic Games in Seoul in 1988.[21] In the following years, many papers concerning stanozolol metabolites have been published and a high number of stanozolol metabolites have been described.[22–31] Both GC–MS/MS and LC–MS/MS methods were developed and improved and, as a result, the limits of identification (LOI) became lower and the detection windows for stanozolol larger. All these above-mentioned methods use an indirect analytical approach by detecting the remaining hydrolyzed parent molecules or phase-I metabolites. In 2012, Van Enoo et al. developed the first highly sensitive method for the direct detection of 3′OH-stanozolol-glucuronide by LC–MS/MS.[32] Further published methods either use a solid-phase-extraction (SPE) as sample preparation followed by LC–MS/MS analysis or a direct, so-called dilution-and-shoot approach, without any sample preparation, for the analysis of phase-II conjugates.[9,14,15,33,34] The objectives of SPE are the trapping and concentration of analytes and the removal of interfering substances from biological matrices in order to improve the detection in the following instrumental analysis. Therefore, SPE has become one of the most important preparation techniques for the analysis of small molecules in biological samples. However, sample preparation with SPE can be very time and resource consuming. The present work aimed to combine the advantages of SPE with a fast and simple dilute-and-shoot method. The result is the development of a simple, fully automatic online-SPE-LC-HRMS/MS method for the analysis of steroid-glucuronides, in particular for stanozolol-glucuronides. The method validation shows a highly sensitive and specific procedure with minimal sample preparation effort.

Initially developed for the confirmation analysis of stanozolol-glucuronides in routine doping control, our method shows very good selectivity and mass accuracy, allowing us to use it for the identification and characterization of new, unknown metabolites. In 2013, Schänzer et al. demonstrated the utility of direct detection of stanozolol glucuronides by high-resolution mass spectrometry (HRMS) coupled to LC in routine doping control and they additionally found and described two new metabolites, stanozolol-N-glucuronide and 17-epistanozolol-N-glucuronide.[33] These metabolites are resistant to enzymatic hydrolysis with beta-glucuronidase and have a high potential for long-term detection. Stanozolol and corresponding metabolites have two feasible N-atoms (1′N/2′N) for the conjugation of glucuronic acid. However, the exact position of the glucuronic acid on the pyrazole ring was not clarified. In 2015, Thevis et al. suggested the existence of both 1′N- and 2′N-stanozolol-glucuronides, based on experiments with collision cross-section computation, but they could not unequivocally confirm the position of the N-glucuronides.[34] In this work, with the presented method and synthesized reference standards[35] we aimed to confirm unambiguously the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine samples after administration of stanozolol.

2 | EXPERIMENTAL

2.1 | Chemicals, reagents, and solutions

Methanol (MeOH) and water used for HPLC analysis (HPLC grade) were purchased from Biosolve Chimie (Dieuze, France). Formic acid (FA) for HPLC was bought from Merck (Darmstadt, Germany). Water (MQ) for sample dilution was obtained by a Milli-Q water purification system (Millipore, Reference A+, Burlington, Massachusetts, USA). Methanol for standard solutions was supplied by Chem-Lab (Zedelgem, Belgium). The internal standard (IS) 16,16,17α-d3-testosterone-glucuronide was purchased from the National Measurement Institute Australia (Sydney, Australia). Both 1′N- and 2′N-stanozolol-glucuronide standards were synthesized and characterized by nuclear magnetic resonance spectroscopy in a previously published study.[35] The chemical structures of all substances involved are illustrated in Figure 1. A concentration of 1 ng stanozolol-glucuronide corresponds to approximately 0.65 ng free stanozolol.

Both the IS solution and standard stock solutions were prepared by dissolving 1 μg of standard substance in 1 mL MeOH (1 μg/mL). A standard working solution was made by diluting stock solutions with MeOH. All solutions were stored at −20°C. For reference samples, the methanolic working solution was directly added to blank urine.

2.2 | Urine samples

The positive urine samples shown in this work were collected by accredited sample collection authorities in compliance with WADA's collection guidelines.[36] The anonymized samples were received, analyzed, and subsequently provided by the WADA accredited antidoping laboratory Seibersdorf Labor GmbH. Other already characterized stanozolol metabolites had previously been confirmed in these samples. Before the analysis, the athletes gave permission to use the urine samples for research purposes. This is in accordance with the International Standard for Laboratories (ISL).[37] Additionally, WADA proficiency test samples were used, which are excretion samples and sent to antidoping laboratories as part of the educational external quality assessment scheme (EQAS). Blank urine samples were collected from healthy female and male volunteers working at Seibersdorf Labor GmbH. All urine samples were stored frozen at −20°C until analysis.

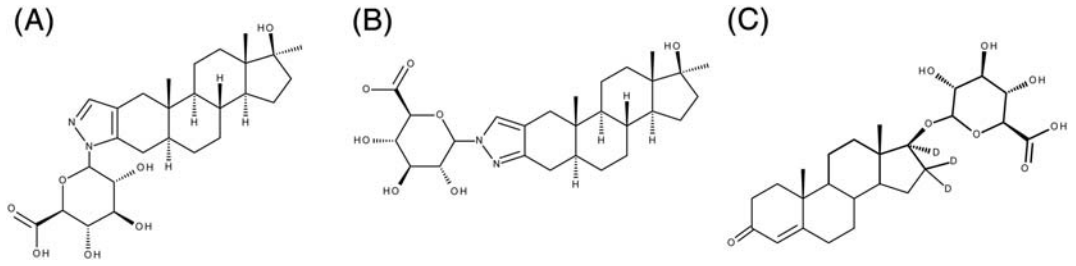

FIGURE 1 Chemical structures of (A) 1′N-stanozolol-glucuronide (1 N-STANG), (B) 2′N-stanozolol-glucuronide (2 N-STANG), and (C) internal standard: 16,16,17α-d3-testosterone-glucuronide (D3-TESG)

2.3 | Sample preparation

For the analysis, 0.5 mL of urine was diluted with 0.5 mL MQ and 30 µL IS (final concentration: 30 ng/mL) solution was added. Afterwards, the samples were vortexed for 10 seconds.

2.4 | Online solid-phase extraction (online SPE)

The online-SPE is based on a standard UHPLC dual-pump system, in which an additional extraction column is attached before the analytical column via a valve system. As an extraction column, an Accucore Phenyl-Hexyl, 10 × 3 mm column with 2.6 µm particle size and 80 Å pore size was used (Fischer Scientific, Loughborough, UK). As illustrated in Figure 2, the extraction column was installed upstream to a two-position, six-port UHPLC valve (MXT715–000, Rheodyne LLC, Bensheim, Germany). The column was connected to the valve by installing a Universal Uniguard Holder 2.1/3.0 mm ID (Thermo Scientific, Bellefonte, USA). For step 1 (see also Figure 2), the sample loading and washing, the valve is set to position 1–2, which directs the liquid stream through the extraction column and into a waste container. This procedure traps analytes on the

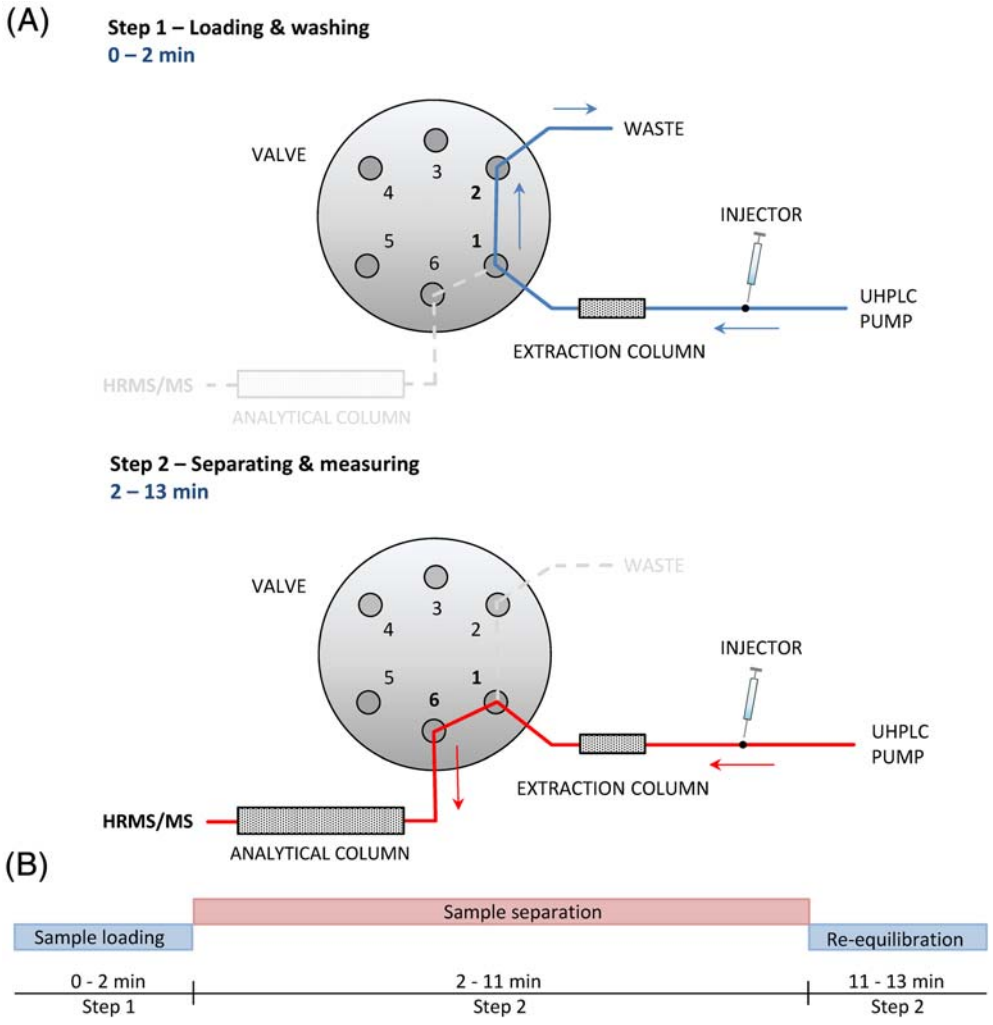

FIGURE 2 Online solid-phase extraction procedure:
(A) Schematic illustration of the valve switch system; above: Step 1, Loading of analytes on extraction column and washing; below: Step 2, Eluting analytes from extraction column, separating on analytical column and HRMS measurement
(B) Temporal progression of the experimental procedure [Colour figure can be viewed at wileyonlinelibrary.com]

pre-column and flushes matrix compounds, such as proteins or salts, into the waste. After 2 minutes the valve switches from position 1–2 to 1–6, thereby leading the stream via the analytical column to the mass spectrometer. Simultaneously with valve switching (step 2), the solvent gradient is started and the elution of analytes from the extraction column begins. After finishing the separation, the system is flushed and re-equilibrated. Connections between single components were established with Viper Capillary finger-tight fittings (Thermo Fisher, Austin, Texas, USA).

2.5 | Liquid chromatography – high-resolution mass spectrometry (LC-HRMS)

Measurements were performed on a Vanquish Horizon UHPLC$^+$ System coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher, Austin, Texas, USA). As an analytical column, a Kinetex EVO C-18, 100 × 2.1 mm column with 2.6 μm particle- and 100 Å pore size was used (Phenomenex, Aschaffenburg, Germany). Chromatography was carried out with mobile phases containing water with 0.2% v/v FA (solvent A) and methanol with 0.1% v/v FA (solvent B). The separation was performed with a constant flow of 0.4 mL/min and constant temperature at 25°C. After loading and washing the pre-column with 10% solvent B for 2 minutes, the solvent gradient continues as follows: start with 10% solvent B up to 100% over 7 minutes, hold 100% B for 2 minutes and again 10% B for 2 minutes to flush and re-equilibrate the system. The sample injection volume was 25 μL.

High-resolution mass spectrometry (HRMS) was carried out in positive electrospray ionization (ESI+) in the modes full scan and parallel reaction monitoring (PRM) with the following common settings: The spray voltage was 3.8 kV and the capillary temperature was set to 320°C. Nitrogen was used as the sheath gas (pressure 25 units) as well as auxiliary gas (pressure 8 units) and the auxiliary gas heater temperature was set to 310°C. The s-lens RF level was set to 55 and the sweep gas flow rate was 0. The mass resolution was set to 70 000 at m/z 200 and automatic gain control (AGC) to 2×10^5 ions. The maximum IT was set to 100 ms. Internal calibration with the lock-mass m/z 391.2843 (di-isooctyl phthalate) was used. Full scanning was performed in the range of m/z 300–600. PRM measurements were carried out in separate runs. Isolation windows were set to 1 m/z. Collision energies (CE) were optimized to obtain the most abundant signal intensities. Spectrometric parameters were optimized by injection of the methanolic compound solutions. The chosen diagnostic ions and corresponding CEs are summarized in Table 1. Data were processed and monoisotopic masses were calculated with Thermo Xcalibur Qual Browser 4.1.45. All systems were controlled with Xcalibur 4.0 (Thermo Fischer).

2.6 | Method validation

The method introduced in this work was validated for qualitative and semi-quantitative purposes according to the ISL using the parameters presented below. Samples described in the following sections were measured with the above described PRM-method. To create extraction ion chromatograms (XIC), product ion 1 and 2 shown in Table 1 with an ion extraction range of 2 ppm were used. For all quantitative parameters the peak area of product ion 1 was taken. Values were corrected with the internal standard and calculated with a calibration curve, which was established for each measurement sequence. Data processing was carried out with Thermo Xcalibur Quan Browser 4.1.45 and calculations were performed with Microsoft Excel 2010.

A concentration of 1 ng/mL, used for most of the parameters, represents 50% of the minimum required performance level for free stanozolol, defined by WADA.[38] For specificity, robustness, and limit of identification (LOI), the comparison of retention times and ratios of relative abundances of two ion transitions must fulfil WADA identification criteria.[39]

2.7 | Specificity

Five different male and five different female urine samples from healthy volunteers were spiked with 1 ng/mL standard. Additionally, five male and five female blank urine samples were analyzed (n = 10). The absence of interferences for both diagnostic ions was verified. Retention times and relative abundances of two ion transitions (peak area) were compared.

2.7.1 | Precision

Ten replicates of 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 × 10). Samples were measured on 3 consecutive days and the coefficient of variation (CV) for intra- and inter-day precision was calculated.

TABLE 1 Mass transitions applied for parallel reaction monitoring

Substance	Formula	Precursor ion	Species	Product ion 1	Product ion 2
		[m/z]		[m/z] / [eV]	[m/z] / [eV]
1 N-STANG	C27H40N2O7	505.2908	[M + H]+	329.2587/60	81.0447/70
2 N-STANG	C27H40N2O7	505.2908	[M + H]+	329.2587/60	81.0447/70
D3-TESG	C25H32D3O8	468.2671	[M + H]+	109.0645/35	97.0651/35

2.7.2 | Robustness

Urine samples spiked with 1 ng/mL standard working solution at various pH values (3, 6,9) and specific gravities (0.005, 0.010, 0.015, 0.025, 0.032) were measured and different injection volumes (15 μL, 25 μL, 35 μL) were tested. Retention times and relative abundances of two ion transitions were compared.

2.7.3 | Linearity

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 × 6), were measured and a calibration curve was generated. Linearity (r^2) was calculated with the software Thermo Quan Browser.

2.7.4 | Accuracy

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

2.7.5 | Matrix effects

Six urine samples obtained from six different volunteers and one matrix-free sample (MQ), spiked with 1 ng/mL standard working solution, were measured and average ion suppression or enhancement effects were calculated by comparison of the signal area (normalized with IS) of urine samples with the matrix-free samples.

2.7.6 | Carryover

A urine sample spiked with 200 ng/mL standard working solution was measured directly prior to a blank urine specimen. The presence of signals in the blank sample was calculated (%).

2.7.7 | Limit of identification (LOI)

Three urine samples collected from three different volunteers, spiked with standard working solution at three concentrations (0.025, 0.05, 0.075 ng/mL, n = 3 × 3), close to an estimated LOI, were measured. The LOI was defined as the lowest concentration level at which a compound could be clearly identified. Therefore, retention times and relative abundances of two ion transitions were compared. The term LOI, used by WADA, is to be equated to the limit of detection (LOD).

TABLE 2 Summary of validation results for 1 N-STANG and 2 N-STANG

	Specificity	Intra-day pr. CV			Inter-day pr. CV			Accuracy			Linearity r^2	Robustness	Carryover	ME (RSD)	LOI
n	10	10	10	10	10	10	10	10	10	10	6 x 4	3 + 5 + 3	1	6	n = 3
c [ng/mL]	1	1	10	50	1	10	50	1	10	50	1–100	1	200	1	-
1 N-STANG	10/10	3.1%	5.5%	4.8%	3.4%	5.6%	4.5%	87.0%	91.6%	99.7%	0.999	11/11	0%	160% (12%)	75 pg/mL
2 N-STANG	10/10	4.9%	3.7%	3.3%	7.8%	6.8%	5.0%	90.2%	94.2%	102.1%	0.998	11/11	0%	151% (15%)	75 pg/mL

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

2.7.8 | Identification of stanozolol-N-glucuronides

In antidoping analysis, an unequivocal identification of newly discovered metabolites is extremely important. A proper way to achieve this is a combination of chromatography, high-resolution mass spectrometry, and nuclear magnetic resonance spectroscopy. In this work, reference standards of 1′N- and 2′N-stanozolol-glucuronide were compared with human urine samples that were confirmed to be positive for stanozolol.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

The obtained method validation parameters are summarized in Table 2. Good specificity and robustness were demonstrated in all 10 of 10 and 11 of 11 samples for both 1′N- and 2′N-stanozolol-glucuronide, respectively. In all samples, no interfering signals were observed and WADA identification criteria for ion ratios and retention time were fulfilled. The method shows excellent precision and accuracy values. The CV for intra-day precision ranged from 3.1% to 5.5% for both metabolites and the CV for inter-day precision was between 3.4% and 7.8%. Accuracy varied between 87.0% and 102.1%. An improvement in the accuracy with increasing concentration of the substance could be observed. A linear signal response of both metabolites with increasing concentration in the sample matrix was shown. No carryover could be observed after injection of high substance concentrations. The high matrix effects of above 150% may be explained by the lack of elaborate sample preparation, but it does not seem to have any influence on the precision and accuracy of the method. Rather a signal enhancement was observed in all samples. Therefore for quantitative issues, a matrix-matched calibration is needed. We could detect both metabolites with a concentration of 25 pg/mL and S/N > 3 in all samples. However, at this concentration level, the ion ratios did not fulfill WADA's identification criteria. Even though in analytical chemistry S/N > 3 is often consulted for the LOI (LOD) definition, we decided to consider the stricter LOI criteria defined by WADA. This applies to a concentration of 75 pg/mL glucuronide, which corresponds to approximately 50 pg/mL of free stanozolol. Most likely, an additional conventional SPE sample separation prior to analytical measurement could significantly improve the LOI if it is required.

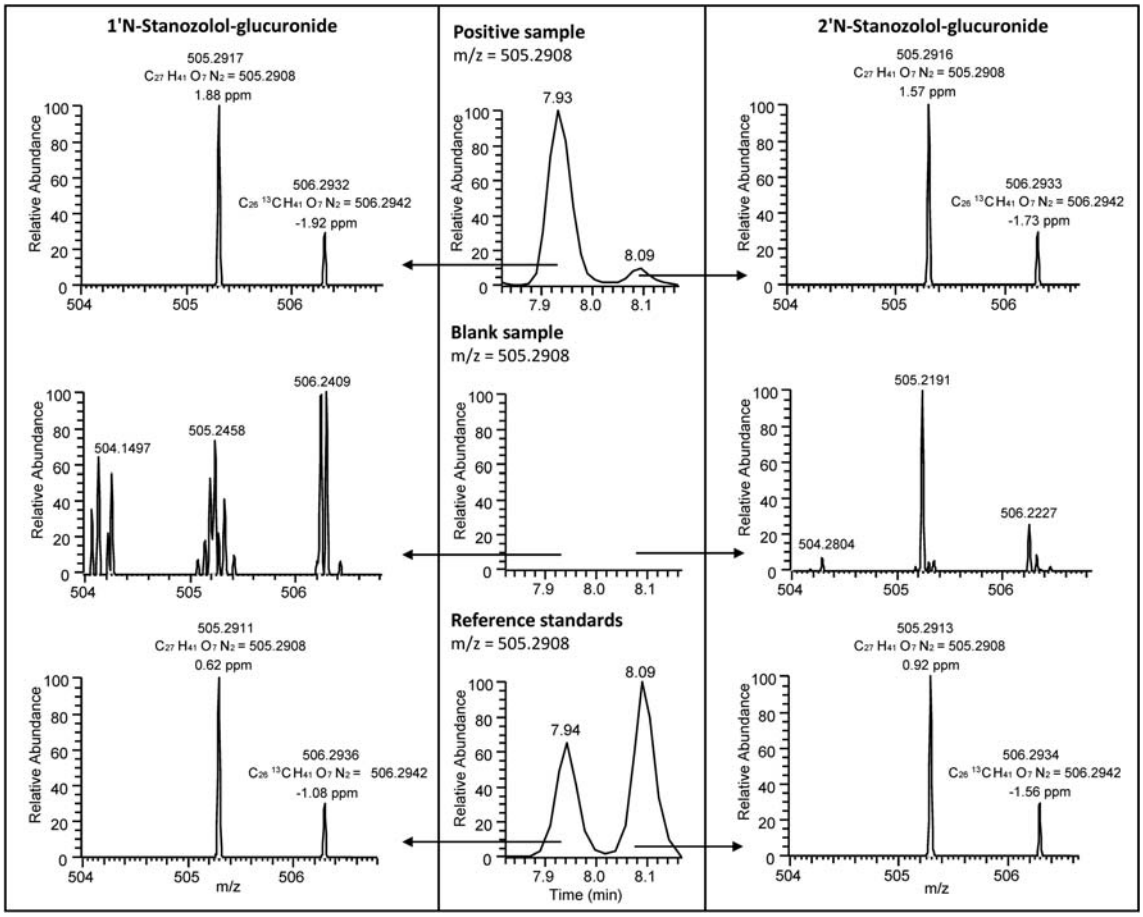

FIGURE 3 Results of full-MS scan; XIC (middle, m/z 505.2908, ESI+, 2 ppm mass tolerance) and corresponding HRMS spectra (left and right) of 1 N-STANG and 2 N-STANG in positive urine, blank urine, and reference standards

3.2 | Identification of synthesized products

The metabolites 1′N- and 2′N-stanozolol-glucuronide were identified by using mass spectrometric techniques. For definitive proof of the existence of these two metabolites, positive doping samples for stanozolol were compared with reference standards and blank urine samples. The above-described method was used for the acquisition of both full-MS scans and MS/MS measurements.

3.3 | HPLC-full-HRMS

As a first step for the identification of synthesized products, HPLC-full-HRMS scans of positive samples for stanozolol, reference standards, and blank samples were measured. When the MS-range was set to m/z 505.2908 (mass tolerance 2 ppm) which corresponds to the theoretical mass of [stanozolol-mono-glucuronide + H] $^{+}$, the results showed a perfect match for both retention times and full-HRMS spectra for both metabolites. The deviations of the retention times were close to zero and the differences between theoretical mass and experimental mass were below 2 ppm in all cases. No signals at all could be observed in blank urine samples. The chromatographic and mass-spectrometric results are shown in Figure 3.

3.4 | HPLC-HRMS/MS

As a second step for the identification of unknown metabolites, PRM was performed on a positive stanozolol sample, reference standards, and blank urine samples. The precursor ion was filtered at m/z 505.2908. Fragmentation with 60 eV collision energy and a following scan were carried out. The chromatograms, again with an ion extraction range of 2 ppm, and resulting fragment spectra are shown in Figure 4. Again, the deviations of the retention times were close to zero and the differences between theoretical mass and experimental mass were below 4 ppm for the 329 fragments and below 6 ppm for the 81 fragments. More interferences in the smaller mass range, as visible in Figure 4, led to a higher deviation of the mass accuracy. All four signals show a highly similar fragmentation pattern, with the most abundant peak at m/z 329 and the second most abundant peak at m/z 81. The 329 ion represents the parent stanozolol molecule, generated after the loss of the glucuronic acid. The 81 ion is a characteristic fragment for stanozolol, which has previously been accurately described in the literature.[30] It represents a stable six-membered heterocyclic ring structure formed by the pyrazole ring and an additional carbon from the steroidal framework. Again, no signals could be observed in blank urine samples.

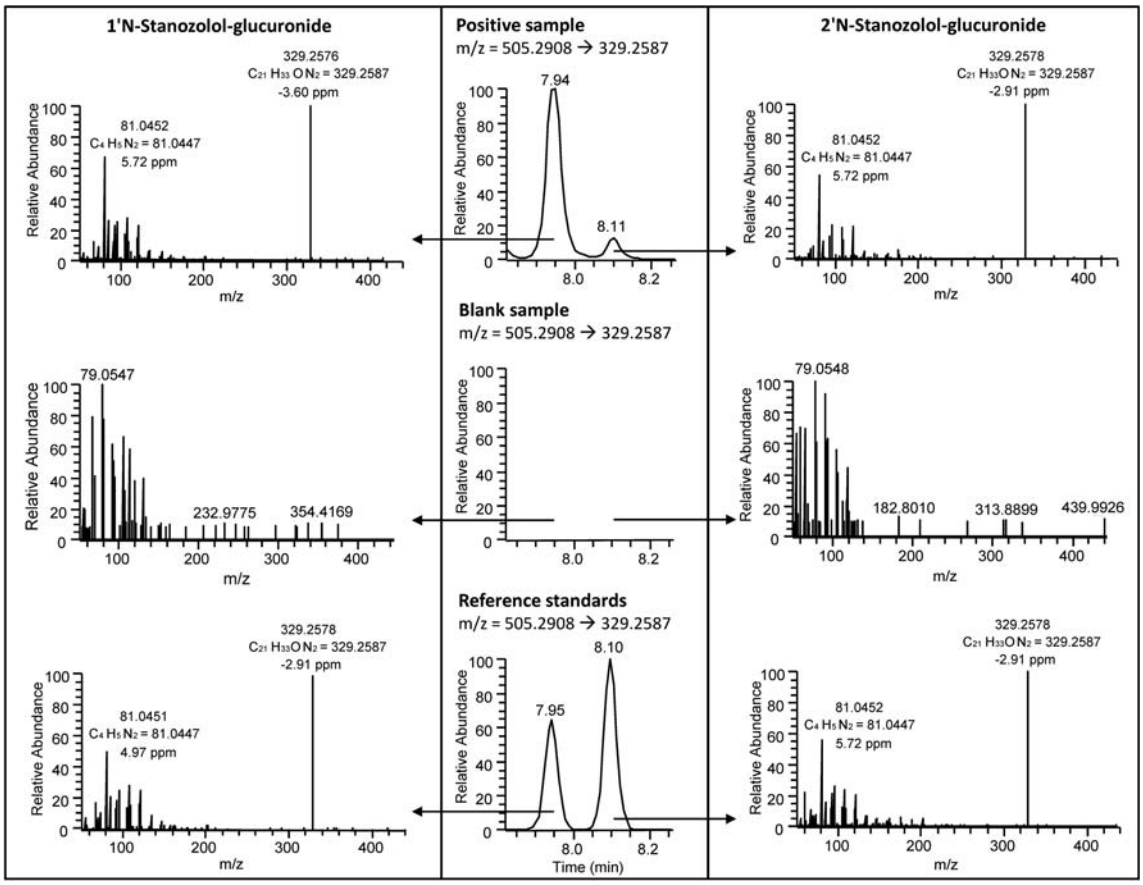

FIGURE 4 Results of PRM measurements; XIC (middle; m/z 505.2908 - > 329.2587 (60 eV), ESI+, 2 ppm mass tolerance) and corresponding PRM spectra (left and right) of 1 N-STANG and 2 N-STANG in positive urine, blank urine, and reference standards

TABLE 3 (A) Retention time comparison and (B) Relative abundances of two MS/MS transitions for a positive stanozolol sample and reference standards; IS, internal standard

A					
Substance	Retention time IS [min]		Retention time samples [min]		Rel. Difference Max. Tolerated: 1%
	Sample	Reference	Sample	Reference	
1 N-STANG	7.77	7.77	7.94	7.95	0.1%
2 N-STANG	7.77	7.77	8.11	8.10	0.1%

B					
Substance	Transition [m/z] 505.2908	Relative abundance		Difference	Maximum tolerance Window
		Sample	Reference		
1 N-STANG	→ 329.2587 (60 eV)	100%	100%	0.67%	8.5% - 18.5% (±5)
	→ 81.0447 (70 eV)	13.5%	12.9%		
2 N-STANG	→ 329.2587 (60 eV)	100%	100%	0.39%	8.3% - 18.3% (±5)
	→ 81.0447 (70 eV)	13.3%	12.9%		

According to the WADA identification criteria, the ratios of at least two MS/MS-transitions of the targeted analyte in a positive sample and a reference sample have to be compared. Additionally, the retention times have to match in both sample types. In Table 3 the compared retention times, the chosen transitions with corresponding relative abundances, and maximum tolerated values are shown. The abundance of the transitions was determined from the peak area. The relative abundance was calculated by dividing the area of the less intense signal by the area of the more intense signal (100%).

For both metabolites, the retention times in the positive sample match with the retention times in the reference sample. The relative difference in both cases is far below the tolerated level of 1%. Similar good matches were achieved for the relative abundances. In both cases, the relative difference is below 1%, which is much lower than the accepted ±5%. These data clearly confirm the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine.

4 | CONCLUSION

In the present study, we introduce a novel, highly functional analytical method for the analysis of stanozolol-glucuronides for doping control analysis. Sample preparation is reduced to diluting the sample with water and adding an internal standard solution. We established a very simple approach for installing an automatic online solid-phase extraction coupled with UHLPC-HRMS/MS. The method is characterized by satisfactory validation parameters. The LOI of 75 pg/mL, excellent specificity, precision, and accuracy as well as good linearity and robustness make our method interesting for very fast and sensitive confirmation procedures, but also for the identification and characterization of unknown metabolites. We could unambiguously identify the presence of 1′N- and 2′N-stanozolol-glucuronide in human urine with the present method. We suggest that these two metabolites can be used as additional information for initial testing and confirmation procedures for the analysis of stanozolol in human urine. In this paper, we focus on the analysis of N-stanozolol-glucuronides, but our method also showed highly promising results for 3-OH-stanozolol-glucuronides (data not shown).

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