

# Detection of Stanozolol Glucuronides in Human Sports Drug Testing by Means of High-Resolution, Accurate-Mass Mass Spectrometry

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## Key Words

Sports doping, antidoping testing, Q Exactive Focus, long-term metabolite, anabolic agents, 16-oxo-stanozolol, stanozolol glucuronide, epistanozolol

## Goal

To demonstrate the utility of direct dilute-and-shoot analysis of glucuronic acid conjugates of stanozolol by means of liquid chromatography and high-resolution, accurate-mass mass spectrometry in sports antidoping testing. To characterize and validate, by means of commercially available 3'-OH-stanozolol glucuronide, "dilute and inject" and confirmation methods, which will allow for the unambiguous identification of stanozolol misuse in routine doping-control samples.

## Introduction

The analysis of the anabolic steroid stanozolol (Figure 1a) has proved to be challenging for gas chromatography mass spectrometry (GC-MS) methods due to stanozolol's peculiar physicochemical properties. The uncovering of stanozolol abuse by means of its major urinary metabolite 3'-OH-stanozolol (Figure 1b) as accomplished by Schänzer and Donike<sup>1</sup> initiated investigations into the metabolic fate of this anabolic agent. The molecular features of stanozolol and its metabolites demand sophisticated derivatization and separation steps for GC/MS-based methodologies. Methods based on liquid chromatography with electrospray-ionization tandem mass spectrometry (LC-MS/MS), on the other hand, provide benefits such as lower limits of detection (LODs) and detection windows with expanded metabolite identification. 3'-OH-stanozolol glucuronide (Figure 1c) is the latest metabolite analyzed at 25–50 pg/mL in human urine. In the present study, the use of high-resolution, accurate-mass mass spectrometry for the detection of 3'-OH-stanozolol glucuronide is outlined. Complementary information on N-conjugated glucuronide metabolites of stanozolol and 17-epistanozolol and the use of these in routine doping controls is provided.

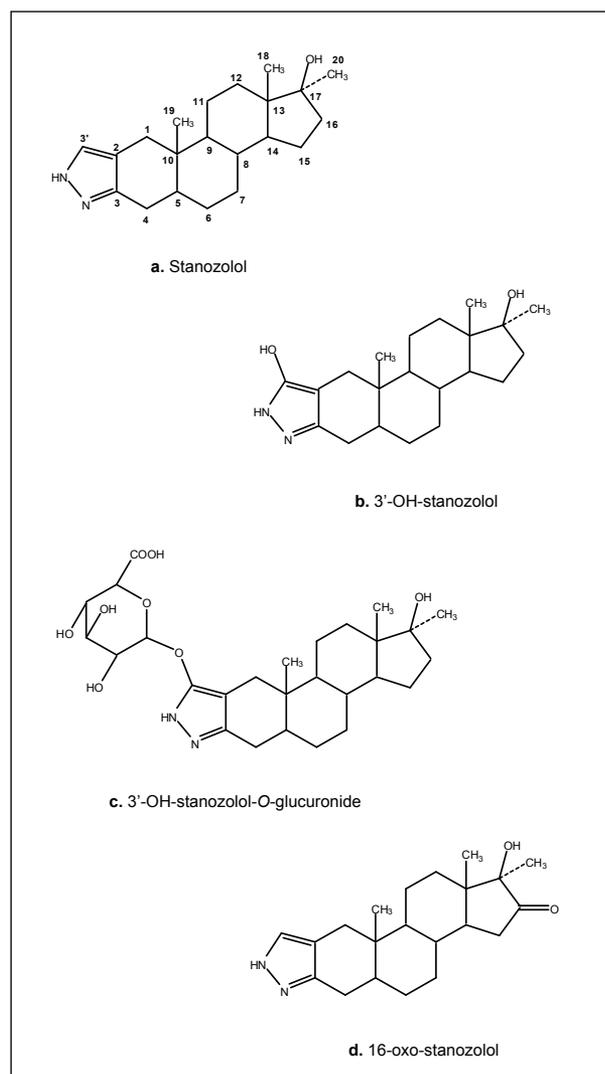


Figure 1. Chemical formulae of stanozolol (a.  $C_{21}H_{32}ON_2$ , mol wt = 328.2515), 3'-OH-stanozolol (b.  $C_{21}H_{32}O_2N_2$ , mol wt = 344.2464), 3'-OH-stanozolol-O-glucuronide (c.  $C_{27}H_{40}O_8N_2$ , mol wt = 520.2785), and 16-oxo-stanozolol (d.  $C_{21}H_{30}O_2N_2$ , mol wt = 342.2307)

## Experimental

### Administration Samples

Two healthy male volunteers (56 and 61 years of age) received a single oral dose of 5 mg of stanozolol (Winstrol®). Urine samples were collected prior to (blank) and up to 28 days post administration of the drug. The urine specimens were stored at -20 °C until preparation and analysis. The study was approved by the local ethical committee and written consent was obtained from both participants.

### Sample Preparation

Ninety microliters of urine were enriched with 10 µL of an acetonitrile solution containing the internal standard methyltestosterone (1 µg/mL). The samples were vortexed for 10 s and subjected to LC-MS/MS analysis.

Confirmatory analyses were conducted by applying 1 mL of urine to a solid-phase extraction (SPE) cartridge preconditioned with 2 mL of water and 2 mL of methanol. After the sample had passed through, the resin was washed with 2 mL of water and the analytes eluted with 2 mL of methanol. The organic phase was evaporated to dryness and reconstituted in 100 µL of solvents A (0.1% formic acid) and B (acetonitrile) (1:1, *v/v*) for LC-MS/MS analysis.

### LC-MS/MS

The analyses were conducted using a Thermo Scientific™ Accela™ 1250 liquid chromatograph interfaced via a heated electrospray ionization (HESI-II) source to a Thermo Scientific™ Q Exactive™ Focus mass spectrometer. The LC was equipped with a Nucleodur® C18 Pyramid analytical column, 50 x 2 mm, particle size 1.8 µm, (Macherey-Nagel, Düren, Germany) and a corresponding precolumn (4 x 2 mm, particle size 3 µm). The mobile phases 0.1% formic acid (A) and acetonitrile (B) were used to perform a gradient elution at 200 µL/min from 99% of A to 100% of B in 7 min, followed by re-equilibration for 3 min.

The mass spectrometer settings were as follows:

Ionization mode	Positive
Spray voltage	4000 V
Source temperature	300 °C
Full Scan	
Resolution setting	35,000 (FWHM) at <i>m/z</i> 200
Mass range	<i>m/z</i> 100–1000
Targeted Higher Energy Collisional Dissociation (HCD)	
Preselected ions	<i>m/z</i> 505.25 (for stanozolol- and 17-epistanozolol glucuronide) <i>m/z</i> 521.25 (for 3'-OH-stanozolol glucuronide)
Resolution setting	35,000 (FWHM) at <i>m/z</i> 200
Mass ranges	<i>m/z</i> 50–535 and 50–550
Automatic gain control	2 x 10 <sup>5</sup>
Maximum IT fill time	200 ms
Isolation window	1.2 Da
Applied collision energy	55 and 72 eV
Collision gas	Nitrogen

### Method Characterization

Due to the lack of certified reference material for the newly identified conjugates, the specificity (20 blank urine samples from 10 male and 10 female volunteers), limit of detection (LOD), and ion suppression/enhancement were determined with 3'-OH-stanozolol glucuronide only. In the case of the confirmatory assay, the recovery, linearity, and intra- and interday precision (at 25, 100, and 200 pg/mL), together with the identification capability, were also determined with 3'-OH-stanozolol glucuronide.

## Results and Discussion

### Stanozolol Metabolites

Administration study urine samples were collected after oral application of 5 mg of stanozolol and subjected to LC-MS/MS with high-resolution, accurate-mass capability in both MS and MS/MS modes. In agreement with earlier initial testing protocols, urine samples were injected into the LC-MS/MS system without further sample preparation, except for the addition of the internal standard methyltestosterone (at 100 ng/mL). Targeted product ion scan experiments [parallel reaction monitoring (PRM)] were performed on precursor ions of various different metabolites, including particularly the glucuronide(s) of stanozolol and its 17-epimer (precursor ion [M+H]<sup>+</sup> at *m/z* 505.29) and hydroxylated phase-I-metabolites (precursor ion [M+H]<sup>+</sup> at *m/z* 521.29). These yielded a series of signals, which were assigned to stanozolol metabolites by means of accurate masses of the intact protonated molecules and the respective aglycons obtained via collisional activation. A typical post-administration sample (5 days) and a blank urine specimen are shown in Figure 2.

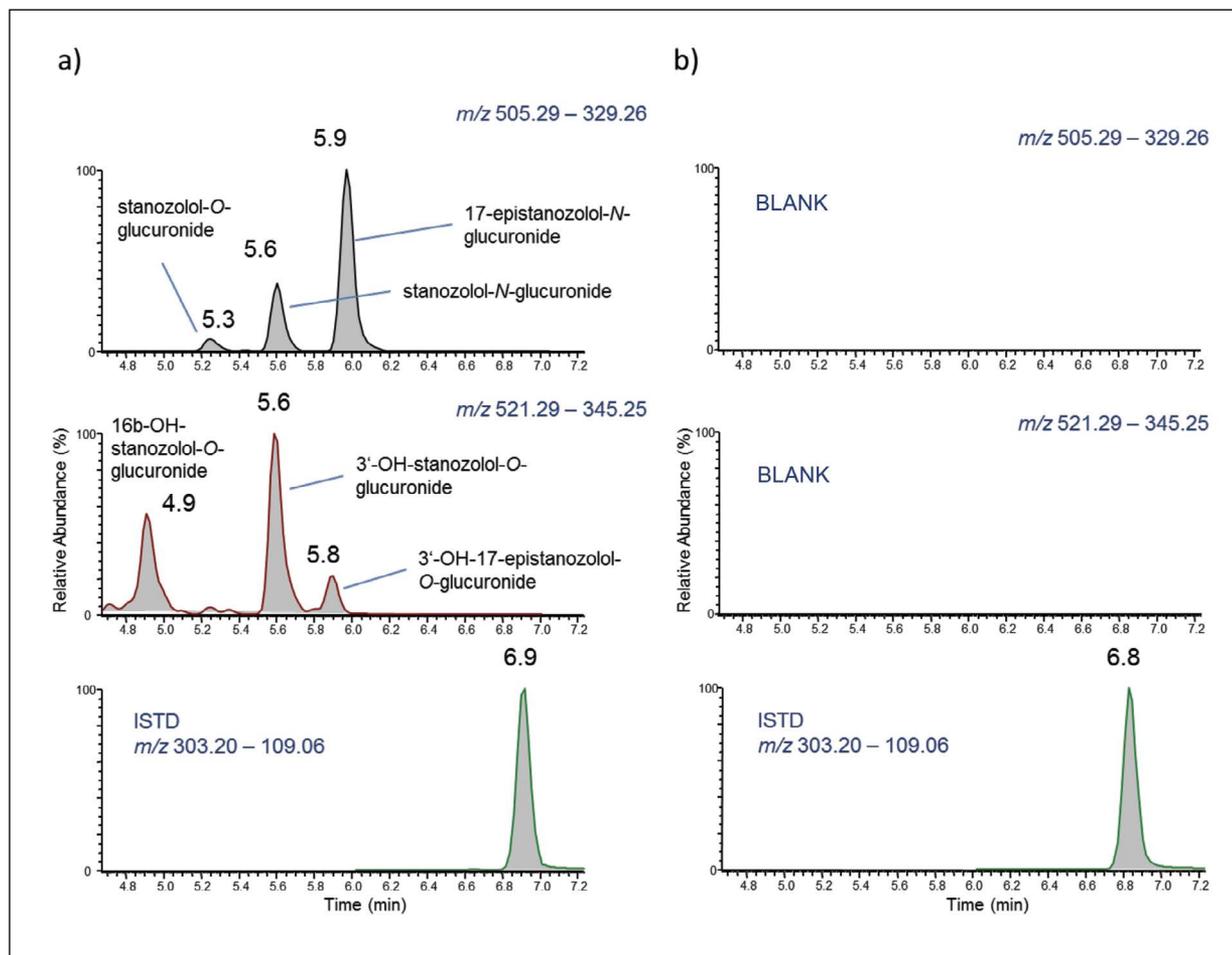


Figure 2. Extracted ion chromatograms of a) post-administration sample (5 days after administration of 5 mg of stanozolol) and b) blank urine sample. Considering accurate masses of precursor and product ions, hydrolysis experiments, and comparison to reference standards, structures were shown as indicated next to each peak.

The MS/MS experiments on  $m/z$  505.29 resulted in three distinct signals as depicted in Figure 2a (top). Stanozolol comprises a hydroxyl group at C-17 and was thus expected to produce a glucuronic acid conjugate; however, the origin of the two additional species of identical sum formula was to be clarified and the structure of the 17-O-glucuronidated compound to be verified. Therefore, a urine sample containing predominantly the substances eluting at 5.14 and 5.53 min (3 h post-administration sample) was subjected to enzymatic hydrolysis with  $\beta$ -glucuronidase that reportedly cleaves  $\beta$ -O-glucuronic acid conjugates of steroids.<sup>2</sup> Following hydrolysis, the urine sample was reanalyzed, demonstrating the disappearance of peak 1 (5.14 min), while peak 2 (5.53 min) remained at its initial abundance (Figure 3a, top). Hence it was concluded that peak 1 corresponded to the hydrolysable

17-O-glucuronide of stanozolol while peak 2 was attributed to a nonhydrolysable glucuronic acid conjugate bearing the glucuronide moiety at a nitrogen atom of the pyrazole residue. The product ion mass spectra of the analytes are depicted in Figure 3b and did not reveal significant differences that would allow localization of the conjugation site. However, the putative stanozolol-O-glucuronide (Figure 3b, top) required more collision energy to dissociate (CE = 45 eV) than the corresponding stanozolol-N-glucuronide (Figure 3b, middle) and 17-epistanozolol-N-glucuronide (Figure 3b, bottom), which were collisionally activated with 35 eV only. Under increasing CE values (as shown in the insets measured at CE = 65 eV), both N- and O-conjugated metabolites yielded the diagnostic product ions of stanozolol (e.g.  $m/z$  81.0452).

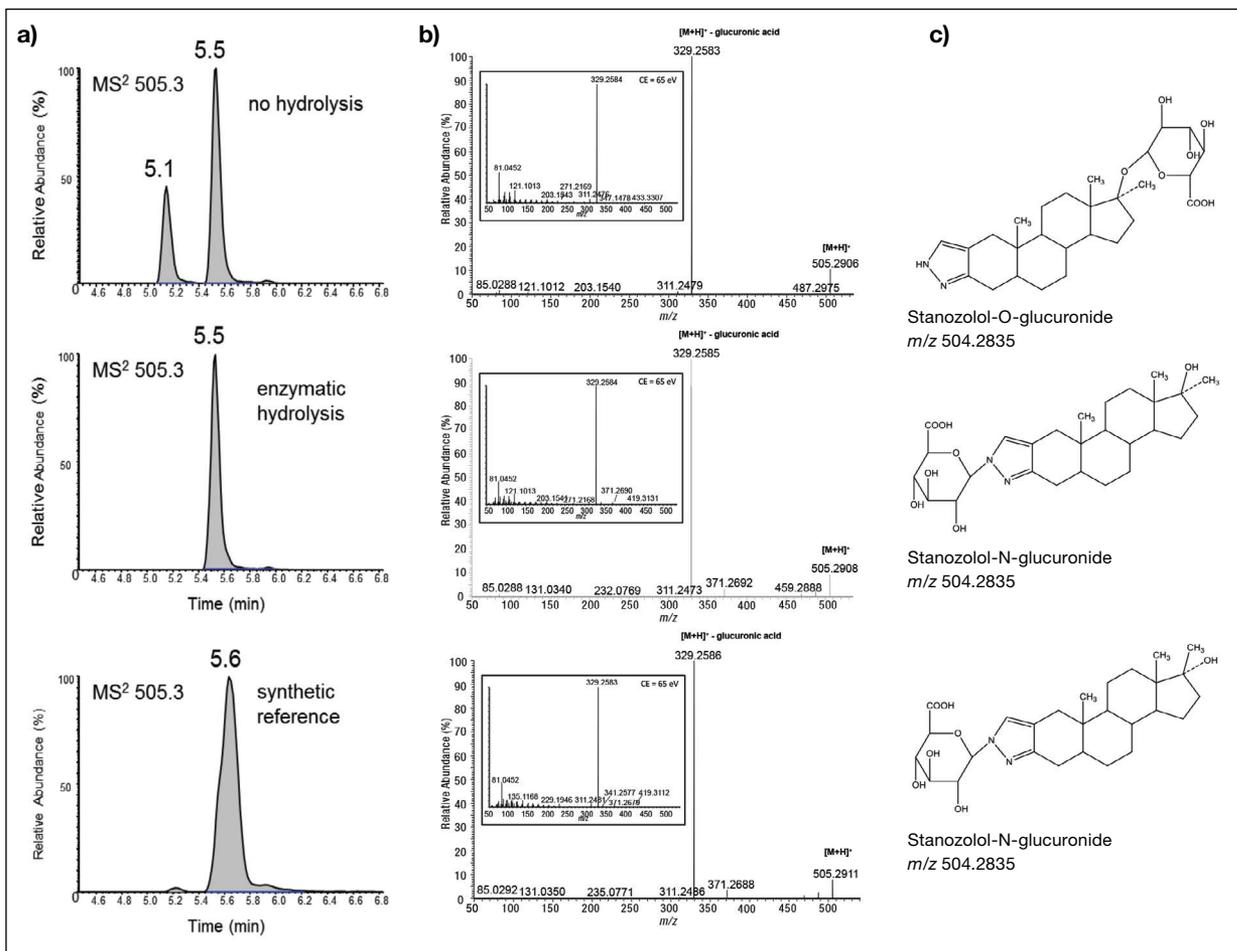


Figure 3. a) Extracted ion chromatograms of a post-administration sample (3 hours after application of 5 mg of stanazolol), indicating the presence of stanazolol-O-glucuronide (at 5.1 min) and stanazolol-N-glucuronide (at 5.5 min), b) product ion mass spectra of the analytes, c) structures

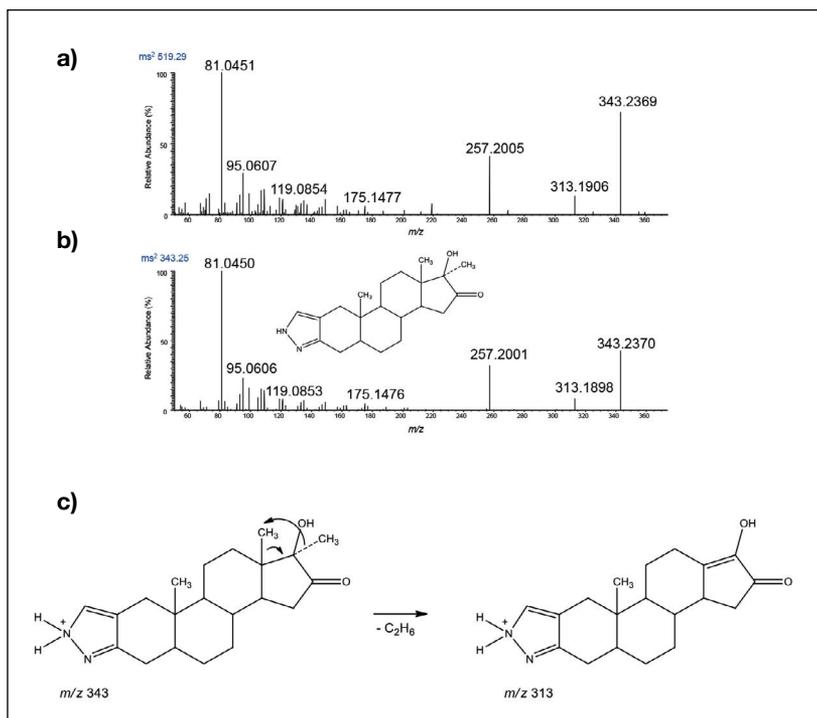


Figure 4. Product ion mass spectra of the protonated molecules [M+H]<sup>+</sup> of a) glucuronic acid conjugate of a metabolite observed in administration study urine samples attributed to 16-oxo-stanozolol-glucuronide at m/z 519, and b) reference standard of 16-oxo-stanozolol. The elimination of 30 Da resulting from the loss of C<sub>2</sub>H<sub>6</sub> is suggested to include the methyl residues at C-15 and C-17 as illustrated under c).

Recent findings of long-term metabolites generated from 17-methyl-17-hydroxy-steroids such as metandienone, oxandrolone, dehydrochloromethyltestosterone, and oxymetholone, fueled the search for analogous metabolites in the case of stanazolol. A common feature of the aforementioned long-term metabolites under ESI-MS/MS conditions is the elimination of formaldehyde (30 Da), which also served as indicator in the present study. In the product ion mass spectrum of the precursor ion [M+H]<sup>+</sup> at m/z 519, which was attributed to a hydroxylated and glucuronidated analog of 17-hydroxymethyl,17-methyl-18-norstanazolol, the ion of the aglycon was observed at m/z 343.2369 with the experimentally determined elemental composition of C<sub>21</sub>H<sub>30</sub>O<sub>2</sub>N<sub>2</sub>. In addition, a product ion at m/z 313.1899 (-30 mass units, Figure 4a) was present. However, the accurate masses revealed the difference of C<sub>2</sub>H<sub>6</sub> rather than CH<sub>2</sub>O between m/z 343 and 313, demonstrating that the analyzed species was not 17-hydroxymethyl,17-methyl-18-norstanazolol but 16-oxo-stanozolol comprising the same elemental composition as corroborated by the analysis of the respective reference substance (Figure 4b). The peculiar loss of ethane (30 Da) was suggested to originate from the steroidal D-ring including C-18 and C-20 and the introduction of a C-13 – C-17 double bond as shown schematically in Figure 4c. Here, deuterium labeling of either C-18 or C-20 would provide further insights and will be subject of future studies.

## Excretion Study Urine Samples

In order to estimate the utility of the newly identified metabolites to prolong and/or improve the detection of stanozolol abuse, the traceability of stanozolol-*O*-glucuronide, stanozolol-*N*-glucuronide, 17-epistanozolol-*N*-glucuronide, 16 $\beta$ -OH-stanozolol-*O*-glucuronide, 4 $\beta$ -OH-stanozolol-*O*-glucuronide, and 3'-OH-stanozolol-*O*-glucuronide by the above mentioned screening method was assessed in administration study urine samples. In Figure 5, the intensities (log scale) of analyte signals were plotted against the time points of urine sampling, demonstrating considerably longer visibility of 17-epistanozolol-*N*-glucuronide, which was detected up to 672 h (28 days) post-administration.

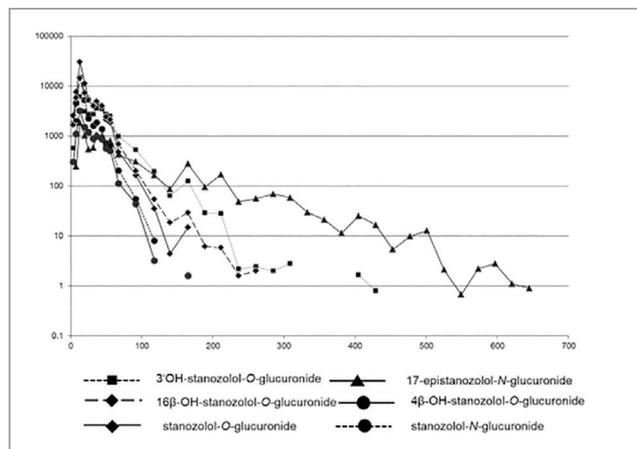


Figure 5. Pharmacokinetics of six metabolites monitored in the administration study urine samples collected after application of 5 mg of stanozolol. The *N*-glucuronide of 17-epistanozolol was detected up to 28 days.

## Methods Characterization / Validation

Before using 3'-OH-stanozolol glucuronide and the metabolites for doping control purposes, the fit-for-purpose of initial testing and confirmation approaches were determined using typical methods. The resulting method characteristics and validated parameters are summarized in Table 1.

Table 1. Method characteristics and validated parameters

Dilute-and-Inject Assay			Confirmation Assay								
LOD (pg/mL)	Specificity	Ion Suppression	LOD (pg/mL)	Specificity	Ion Suppression	Recovery (%)	Calibration Curve	Intraday Precision (n=30)		Interday Precision	
								(n=30+30+30)	CV (%)	Concentration (pg/mL)	CV (%)
20	No Interference (10+10)	3–45%	5	No Interference (10+10)	15–84%	106	25–150 pg/mL	25	15	25	16
							Linear (r <sup>2</sup> = 0.994)	100	9	100	10
								250	7	250	7

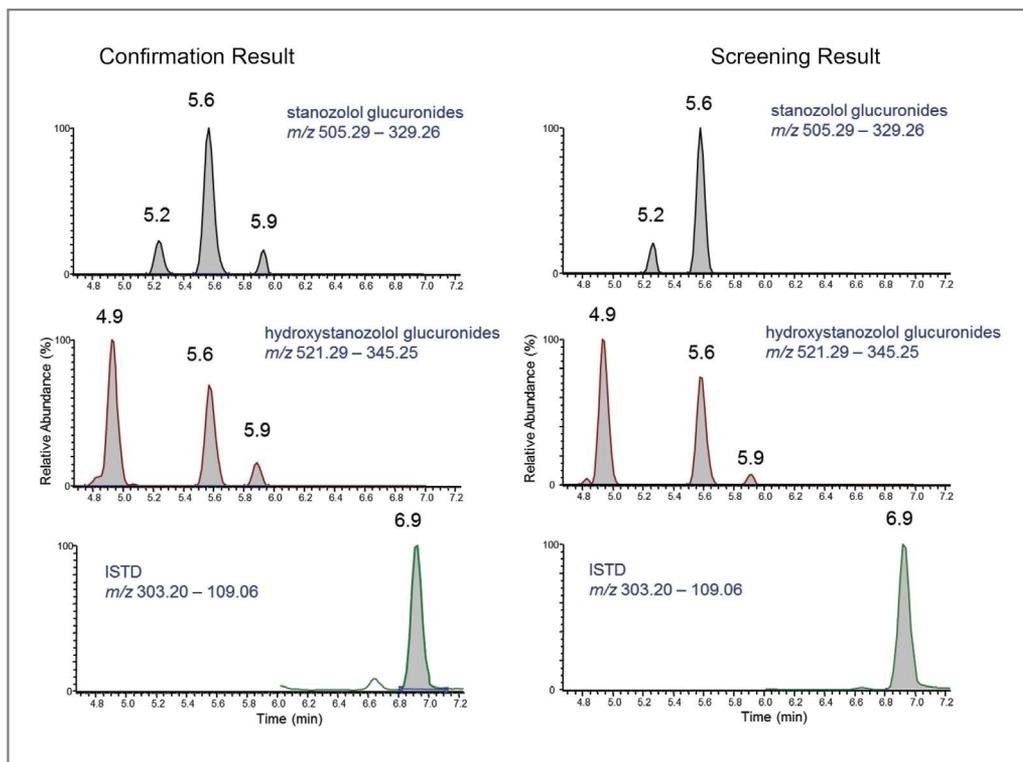


Figure 6. Chromatograms of an authentic doping control routine sample representing an adverse analytical finding for stanozolol in both screening and confirmation assays.

## Conclusion

In this study, the utility of direct dilute-and-shoot analysis of glucuronic acid conjugates of stanozolol by means of liquid chromatography and high-resolution, accurate-mass mass spectrometry in sports antidoping testing was assessed and demonstrated. Characterized and validated by means of commercially available 3'-OH-stanozolol glucuronide, dilute-and-inject and confirmation methods were established, which allowed for the unambiguous identification of stanozolol misuse in routine doping control samples. Additionally, new long-term metabolites for the detection of stanozolol abuse were observed in administration study urine samples. These new target analytes, assigned as stanozolol-*N*-glucuronide and 17-epistanozolol-*N*-glucuronide, were characterized by mass spectrometry, and hydrolysis experiments. Both proved particularly useful as target compounds, enabling the determination of drug abuse for up to 28 days post-administration of 5 mg of stanozolol. Since high-resolution, accurate-mass mass spectrometry has been found to be essential for the successful identification of lowest amounts of stanozolol metabolites in human urine, expansion of its use in doping control is encouraged.

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