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Technical Note

Development of a Standardized Microflow LC Gradient to Enable Sensitive and Long-Term Detection of Synthetic Anabolic-Androgenic Steroids for High-Throughput Doping Controls

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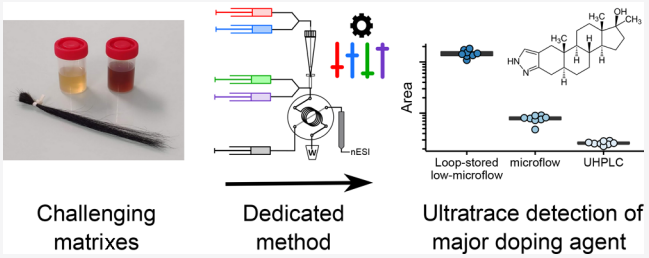
ABSTRACT: Synthetic androgenic anabolic steroids (AAS) are banned compounds and considered as major threats by both racing and sports international authorities. Hence, doping control laboratories are continually looking into analytical improvements to increase their detection capabilities, notably by means of emerging technologies. To enhance analytical performances for the detection of synthetic AAS such as stanozolol, specific chromatographic procedures have been developed using recent quaternary liquid chromatography technology originally designed for high-throughput standardized proteomics connected to mass spectrometry. Applying the newly designed elution procedures described in this paper to the analyses of stanozolol and its metabolites in complex matrixes revealed improved sensitivity compared to previously described high-throughput methods. Indeed, we report the consistent and reliable detection of 16β-hydroxy-stanozolol down to 10 pg/mL in equine urine and being detectable up-to 3 months after a microdosing administration. Furthermore, a five months long elimination of stanozolol and its metabolites could be monitored on horse mane sections after a single dose administration. Our work highlights novel solutions to detect AAS with improved sensitivity. The application of such developments constitutes new landmarks for doping control laboratories and could be extended to other targeted compounds in residue analysis, toxicology, and metabolomics. Based on this work, the developed chromatographic method is now freely available within the Evosep Plus program.



INTRODUCTION

Liquid-chromatography coupled to mass spectrometry (LC-MS) has become an essential tool in doping control laboratories to reliably detect controlled and banned substances in various biological matrixes. To continuously improve their detection capabilities and widen their detection time frames toward major doping agents such as synthetic androgenic anabolic steroids (AAS), doping control laboratories constantly seek analytical enhancements consistent with the processing of several thousand samples per year. These factors limited the application of highly sensitive low-flow based strategies to a few peptide-based targeted applications requiring advanced sensitivity, such as the targeted detection of erythropoietin,[1,2] relaxin,[3] peptide toxins,[4] peptide drugs,[5−7] or proteomics biomarker discovery induced by doping practices[8,9] and unknown protein characterization.[10] To a lower extent, low-flow drug screening methods are reported but are hardly compatible with the processing of tens of thousands samples per year.[11] However, even if peptides and protein-based drugs are raising health and welfare concerns to authorities, these "new analytes"[12] represent fewer adverse analytical findings than anabolic androgenic steroids (AAS) according to Aguilar et al.[13] or a recent world antidoping agency (WADA) report.[14] Thus, there is a critical need to expand the high sensitivity of low-flow LC technologies to additional analytes such as AAS at high throughput.

The recent developments of a standardized proteomics-dedicated LC device[15] brought high robustness and throughput to most proteomics applications and are currently implemented in ultrasensitive proteomics applications such as single cell analysis.[16] However, as the solution proposed by the LC device is intended to standardize gradients for proteomics applications, we created a specialized separation method for more hydrophobic compounds and applied it to detect stanozolol, a performance enhancing synthetic AAS which is still widely misused,[14] and its phase I metabolites in complex matrixes. We

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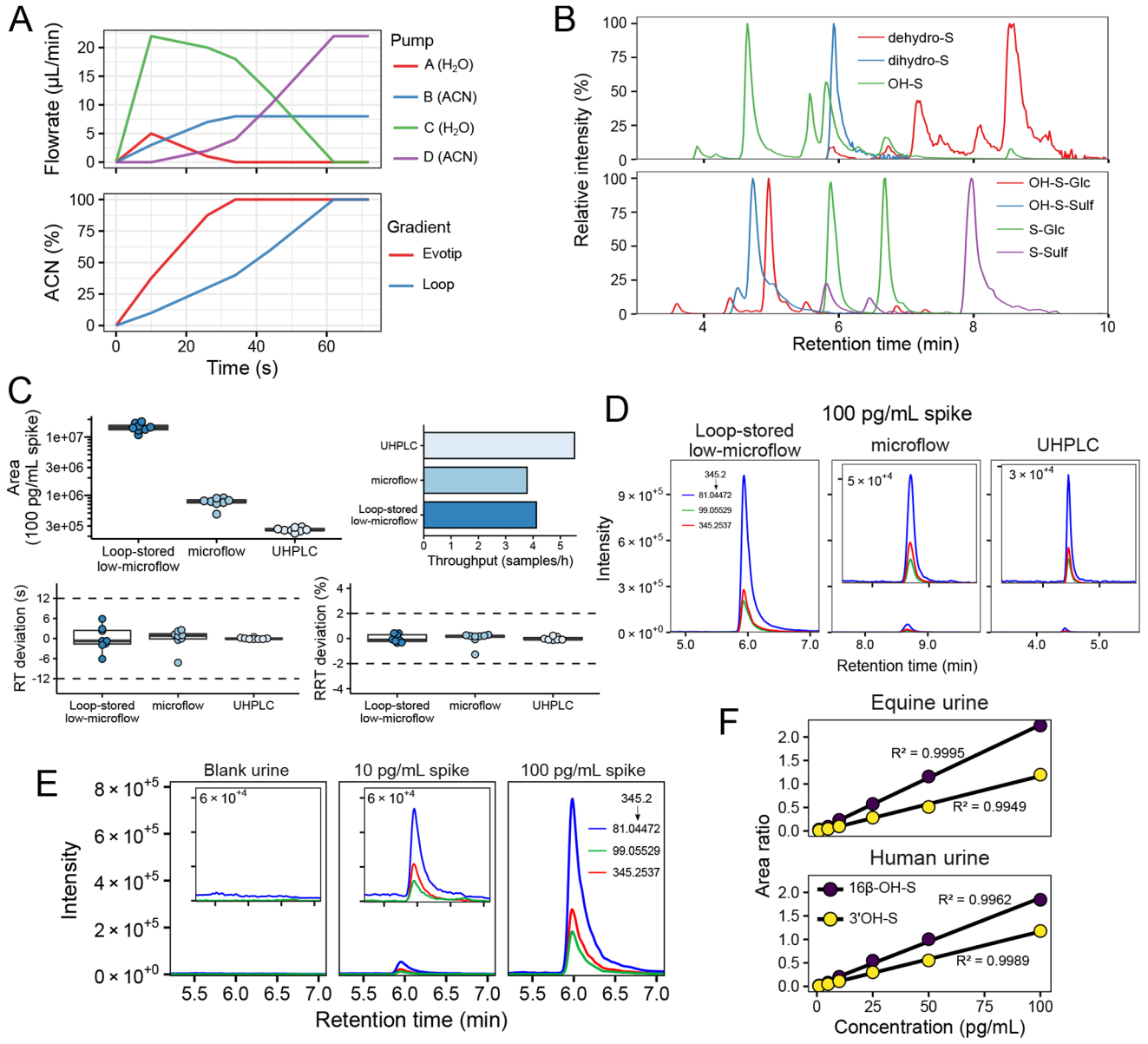

Figure 1. Development of a specialized gradient compatible with the detection of stanozolol metabolites. (A) Flow rates of low-pressure pumps and corresponding gradients in disposable online SPE (Evotip) and analytical column (Loop). (B) Example extracted ion chromatograms of S9 fractions generated stanozolol (S) metabolites $[M + H]^+$ ions. (C) Technological comparison with higher flow rates LC technologies regarding sensitivity, throughput, and robustness for the detection of 16β-hydroxy-stanozolol. (D) Example of 16β-hydroxy-stanozolol product ion chromatograms obtained after injection of 10 μL of the same 100 pg/mL spiked urine extract on three high-throughput LC technologies. (E) Product ion chromatogram of 16β-hydroxy-stanozolol selected transitions in extracted equine urine for method validation. (F) Linearity assessment of the reference metabolites 16β- and 3′-hydroxy-stanozolol in extracted equine and human urine matrixes within the 1−100 pg/mL range.

report significantly improved detection capabilities within the pg/mL and fg/mg levels and several months-long detection time frames for stanozolol and its metabolites in the highly challenging urine and horsehair equine matrixes, respectively. Moreover, we expect that the future adaptation of such a strategy to other hydrophobic compounds and matrixes may open new perspectives to drug testing laboratories at enhanced sensitivities and eventually be applied to high-throughput trace metabolomics and residue analysis.

EXPERIMENTAL SECTION

Additional detailed experimental procedures including chemicals and reagents, *in vitro* generation of stanozolol metabolites, UHPLC- and microflow-high resolution tandem mass spectrometry (HRMS/MS) methods for technological comparisons are available in the Supporting Information.

Stanozolol Elimination Studies. Equine urine elimination study was performed according to French guidelines for use and care of animals (February 2009) at the administration and sampling center of "Fédération Nationale des Courses Hippiques" (Coye la Forêt, France). The Equine hair study was conducted in February 2016 at "Station IFCE Chamberet" (IFCE, "Institut Français du Cheval et de l'Equitation") and approved by a local ethics committee. For microdosing administration protocol followed by urine analysis, stanozolol was intramuscularly administered to a 7 year old mare weighing

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481 kg using a total dose of 1.2 mg/kg, which was split into four administrations (300 μg/kg every 4 days), and urine samples were collected over several months, aliquoted, and stored at −20 °C with monitored freeze−thaw cycles. For horsehair analysis, stanozolol was administered using a single 1 mg/kg intramuscular dose to a 3 year old bay mare weighing 441 kg. A 13 cm long × 1 cm cross-section of mane was collected 6 months after administration and stored at room temperature in the dark.

Urine Hydrolysis. Human and equine urines were enzymatically hydrolyzed as described previously.[17] Briefly, 2.5 mL of equine or human urine were buffered using 500 μL of 1 M phosphate buffer pH 5.8, and the final pH was adjusted with acetic acid or KOH to reach pH 6. Urine was then hydrolyzed by adding 25 μL of *Aspergillus melleus* proteinase (500 U/mL) and 25 μL of recombinant *Escherichia coli* K12 β-glucuronidase (166 U/mL) and incubated for 1 h at 55 °C.

Urine Extraction. Hydrolyzed urines were extracted using mixed-mode reversed-phase/strong cation exchange BCX2 96 well plates as described previously[17] with minor modifications. Briefly, 1.6 mL of hydrolyzed urine was loaded on solid phase extraction (SPE) media which was first conditioned with 500 μL of MeOH, 500 μL of ultrapure water, and 500 μL of 0.1 M phosphate buffer. SPE media was washed with 1.5 mL of 1 M acetic acid in ultrapure water followed with 1 mL of MeOH. Analytes were eluted with 1.8 mL of ethyl acetate containing 3% of NH_4OH/H_2O (32% v/v). Extracts were dried and resuspended into 100 μL of H_2O/MeOH 80/20 (v/v).

Equine Hair Extraction. A 13 cm long × 1 cm cross-section portion of hair was cut from a horse mane collected 6 months after stanozolol administration. The hair sample was subjected to three detergent washes using 10 mL of sodium dodecyl sulfate in tap water (0.5 g/L) without further processing of the wash fractions and then rinsed twice using ultrapure water and dried at 37 °C to eliminate any excess of water. Hair was cut into 1 cm long segments using a hair clipper. In total, 100 mg of each segment was collected into polypropylene tubes for further processing. An amount of 10 ng of 3′ hydroxy-stanozolol-d_3 (internal standard) was spiked into each sample. Hair segments were incubated in 5 mL of MeOH/1 M HCl in water (2/3, v/v) at 50 °C for 4 h. Samples were dried under a nitrogen stream at 50 °C until reaching a volume below 1 mL. Then, 5 mL of ultrapure water was added to the extracts, and samples were centrifuged to pellet the remaining particles.

Supernatant was collected and extracted on Oasis MCX cartridges using a GX-274 (Gilson, Middleton, WI). SPE media was conditioned with 4 mL of MeOH, followed by 4 mL of ultrapure water before sample loading. SPE media was sequentially rinsed by 4 mL of ultrapure water, 4 mL of 1 M acetic acid in ultrapure water, and 4 mL of MeOH. Analytes were eluted by 4 mL of ethyl acetate/ACN/32% NH_4OH in H_2O (9/1/0.05, v/v), evaporated to dryness under a nitrogen stream, resuspended into 400 μL of MeOH, and stored in glass vials at 4 °C until analysis.

Sample Trapping on C18-Evotips. Disposable Evotips (Evosep, Odense Denmark) were prepared according to manufacturer's recommendations with a minor modification regarding the loading procedure (see graphical representation in the Supporting Information). Briefly, Evotips were wetted by immersing the tip-end into isopropanol for 30 s, conditioned with 25 μL of ACN + 0.1% FA, and centrifuged at 800*g* for 1 min. Then, 25 μL of H_2O + 0.1% FA was dispensed and centrifuged. For urine samples, 140 μL of H_2O + 0.1% FA and 80 μL of urine extract were mixed by repeated pipetting in the Evotip. For hair samples, analytes were evaporated to dryness and resuspended with 50 μL of MeOH/H_2O (1/1, v/v) and were mixed with 100 μL of H_2O + 0.1% FA by repeated pipetting in the Evotip. Mixtures were allowed to flow completely through the Evotip by centrifugation at 800*g*. Evotips were rinsed with 200 μL of H_2O + 0.1% FA and centrifuged again (800*g*, 30 s), in order to maintain an aqueous layer on top of the SPE-media to prevent media drying. Evotip racks with trapped samples were placed on the Evosep LC for LC-HRMS/MS analysis.

Dedicated Offset Loop-Stored Gradient Development. A standardized method for detecting stanozolol and associated metabolites was generated based on the 100 samples/day method. However, to account for the significantly more hydrophobic targets, the gradient and gradient offset had to be modified as depicted in Figure 1A. Briefly, we generate a 0−100% ACN gradient with pumps A and B to sequentially elute the analytes of the disposable Evotip which is then diluted and offset using pumps C and D to create a significantly shallower analytical gradient from 0% to 100% ACN. Generation of the analytical gradient (i.e., Evotip gradient + gradient offsetting) is 72 s long. Once the gradient has been generated and stored in the loop, the sample storage loop valve is switched, and its content "pushed" through the analytical column within 11.5 min (1.5 μL/min flow rate). The final analytical gradient was fine tuned to provide the best possible chromatographic performances.

MicroLC-HRMS/MS Analysis. Evosep One (Evosep, Odense Denmark) was equipped with an 8 cm long × 100 μm internal diameter analytical column packed with Dr Maisch C18 AQ 3 μm beads (Evosep/PepSep EV-1064, Evosep, Odense Denmark) and coupled to a hybrid quadrupole-orbitrap (Q Exactive HF, Thermo Scientific, Bremen, Germany) through a NanoFlex ion source (Thermo Scientific) equipped with a microelectrospray M3 multinozzle emitter (Newomics, Berkeley, CA). The multinozzle sheath nitrogen gas flow rate was set to 2.5 L/min, the spray voltage was set to 3.5 kV, and the transfer capillary temperature was set to 320 °C. Data were acquired in positive ESI mode using parallel reaction monitoring (PRM), targeting stanozolol, hydroxy-stanozolol metabolites, and hydroxy-stanozolol-d_3 (internal standard) $[M + H]^+$ species (m/z 329.2, 345.2, and 348.2, respectively) within a 1.2 m/z isolation window. Selected ions were fragmented in the HCD cell with a normalized collision energy of 75. Resolution was set to 120 000 (FWHM at m/z 200), and the automatic gain control (AGC target) was set to 1×10^6 with a maximum injection time of 250 ms.

Data Analysis. For method development and identification of stanozolol metabolite validation, data were analyzed using Thermo Xcalibur 4.1 Qual Browser by extracting the product ions signals within a 5 ppm tolerance window. For elimination studies, data were imported into Skyline-daily[18] using the small-molecules analysis framework.[19] Data were imported into R 4.0.0 for further analysis and visualization.

RESULTS AND DISCUSSION

Method Development and Confirmatory Analysis Validation. To accommodate hydrophobic analytes such as stanozolol and its metabolites, tuning of quaternary flow rates had to be performed (Figure 1A). Indeed, the A/B and C/D pump flow rates were kept complementary (disposable trap column and gradient offsetting, respectively). Additionally, loop overfill which would result in the loss of the most early eluted compounds was avoided. Finally, the method had to be

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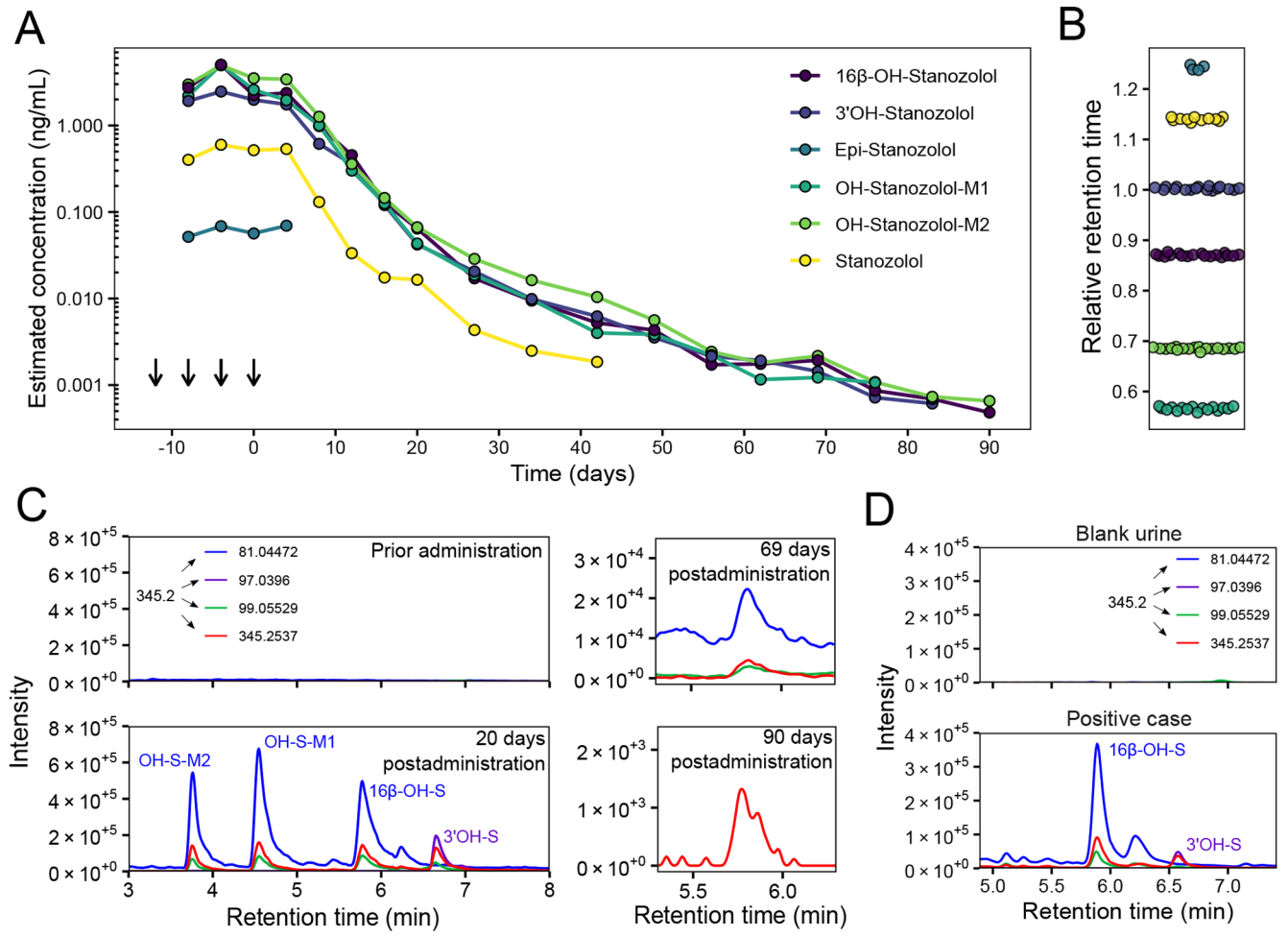

Figure 2. Long-term and ultratrace detection of stanozolol metabolites *in vivo*. (A) Wide time frame elimination study in equine urine after 4-fold split 1.2 mg/kg intramuscular administrations (represented by arrows). (B) Relative retention time reproducibility among the elimination study. (C) Example chromatograms obtained along the *in vivo* study. (D) Low-concentration detection of stanozolol metabolites in a postrace equine urine sample.

compatible with the detection of targeted compounds with sufficient chromatographic resolution in a 11.5 min LC-MS run.

As highlighted in Figure 1B, this strategy was successfully adapted for the detection of *in vitro* generated phase I/II metabolites of stanozolol, a misused synthetic anabolic agent. Indeed, the elution of stanozolol metabolites was contained in the 3.5−9 min range which is nearly optimal gradient usage, considering the dead-volume and the 100% ACN wash. Additionally, the gradient provided higher resolution, narrower peaks, and thus increased intensity compared to the initial preset gradient (Figure S1).

The separation technology was compared with high-throughput technologies running at higher flow rates (i.e., UHPLC at 800 μL/min), which are routinely employed to detect stanozolol and its respective metabolites by analyzing equal volumes of multiple spiked urine extracts. Furthermore, this comparison is in line with recent microflow development using higher flow rates LC (80 μL/min) as competitive alternatives to nanoflow chromatography for robust and high throughput proteome characterization.[20−22] As depicted in Figure 1C, the loop-stored low-flow microLC (1.5 μL/min) provides improved sensitivity performances with minimal compromise on robustness compared to high flow rates regimen LC with minor RT and RRT fluctuations and compatible with positive case reporting (Figure 1C, dashed lines). Extracted product ion chromatograms obtained after analysis of the same extract on three different technologies exhibited over an order of magnitude of intensity gain using the same mass spectrometer (Figure 1D). These observations confirm a general LC-MS paradigm with low flow rate conferring improved sensitivity while high flow rate LC strategies grants unmatched robustness and throughput. Even if the sensitivity limitation of UHPLC and microflow strategies could be overcome by injecting greater quantities of extracts as reported in recent proteomics developments,[23] this would require extensive quantities of biological matrix which is often hardly applicable in a doping control context.

In order to enable the use of such optimized sample analysis in a doping control context and to exploit the sensitivity gain, identification of the most prevalent equine stanozolol metabolite 16β-hydroxy-stanozolol[24,25] was validated for qualitative confirmatory analysis according to recommended guidelines.[26] For this purpose, extractions were performed on different negatively tested equine urines (n = 10) and 16β-hydroxy-stanozolol spikes (5 × 10 pg/mL and 5 × 100 pg/mL). To achieve detection at lower sensitivity magnitudes, deep inspection of stanozolol and its 16β-hydroxy-metabolite fragmentation spectra was performed. Thus, m/z 99.05529 ($C_4H_7N_2O^+$) was defined as a

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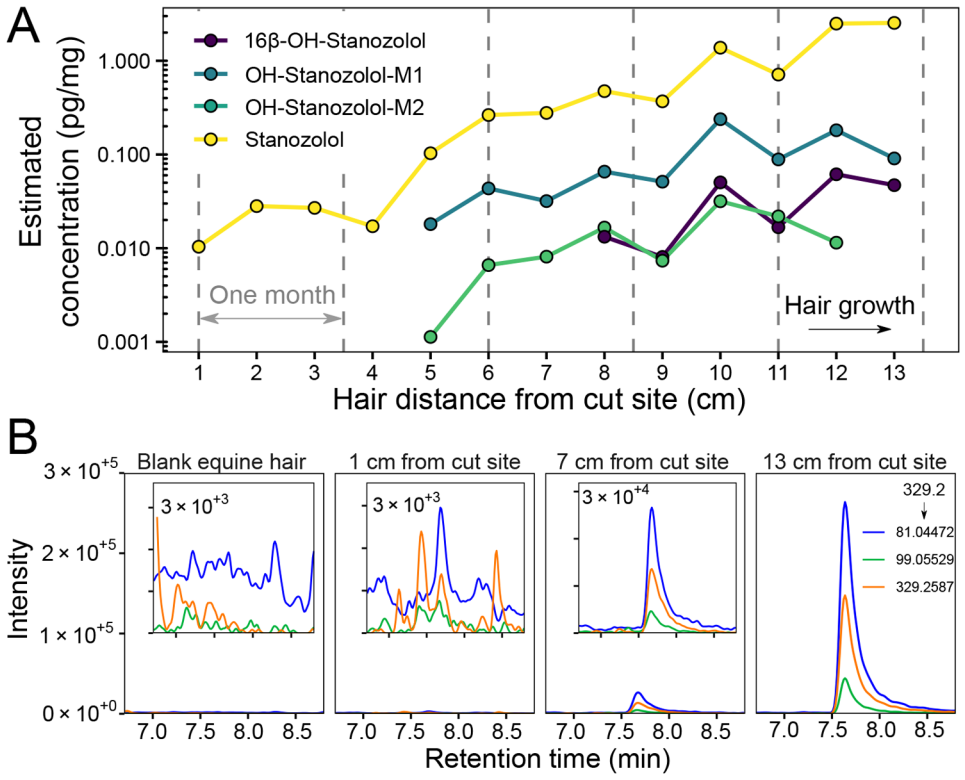

Figure 3. Long-term detection of stanozolol in horsehair after a single 1 mg/kg controlled administration. (A) Elimination curve of stanozolol and its hydroxylated metabolites in segmented horsehair. (B) Example chromatograms obtained for stanozolol after analysis of blank and postadministration hair samples.

novel MS/MS product ion in addition to 81.04472 ($C_4H_5N_2^+$) as well as the use of the parent ion for our detection strategy. Indeed, the presence of nitrogen-containing product ions in HRMS/MS spectra significantly improves detection selectivity compared to CxHyOz product ions. Moreover, the exceptional stability of stanozolol and its hydroxy-metabolite precursor ions at high collision energies is a rare aspect which is beneficial for unambiguous identification, as previously employed elsewhere.[27] Analysis of blank samples revealed the absence of chromatographic peak for the selected product ions, granting analysis specificity, whereas 10 pg/mL and 100 pg/mL spiked urine extracts demonstrated efficient detection of 16β-hydroxy-stanozolol (Figure 1E). Furthermore, analysis results were in accordance with the Association of Official Racing Chemists (AORC) analytical guidelines for unambiguous MS-based identification of 16β-hydroxy-stanozolol, such as MS/MS ion ratios reproducibility with sufficient S/N (>3), relative retention time stability (<2% deviation), and mass accuracy (<5 ppm) (Table S1).[26] These results demonstrate that the detection strategy is suitable for confirmatory analysis and positive case reports.

In doping control analyses, qualitative detection of stanozolol and related metabolites is usually performed because of its synthetic nature. However, to assess the compatibility with quantitative analysis, we performed linear regression analysis of equine and human spiked urine samples for the two reference stanozolol metabolites within the 1−100 pg/mL range. As depicted in Figure 1D, the signal of 16β-hydroxy- and 3′-hydroxy-stanozolol in extracted equine and human urine are linear, indicating that this approach could be adapted to a quantitative analysis. Furthermore, the potential sample saturation which may occur on the disposable sample trap does not affect later detection, and signals remain linear with area ratios (Figure 1F, $R^2 > 0.99$) or raw areas (Figure S2, $R^2 > 0.97$) within the tested concentration range.

Postadministration Equine Urine Analyses. To confirm these analytical improvements, we performed the analysis of urine extracts obtained after a controlled multiple-dose administration of stanozolol to a horse, mimicking a microdosing doping scheme, and where urine samples were collected for a long period. Strikingly, multiple hydroxy-stanozolol metabolites including 16β- and 3′- hydroxy-stanozolol were detectable up-to 69 days after the last microdose administration with three transitions and high relative retention time stability (Figure 2A−C). Moreover, a chromatographic peak extracted from the precursor m/z ion could be detected in the PRM scans up to 90 days with the S/N > 3 (Figure 2C) and estimated at 0.48 pg/mL. While this can be considered as a wide detection time frame, it cannot be fairly compared to previous stanozolol-related published studies on liquid biological fluids because of differences in administration protocols,[24] matrix,[28] or species.[29] Finally, the developed gradient provided high relative retention time stability (Figure 2B), confirming the highly robust nature of this low-flow chromatography method.

Interestingly, stanozolol could be identified in the urine extract as previously reported,[24] which supports that stanozolol can be directly metabolized into phase II N- and/or O-glucuronides conjugates by the equine enzymatic machinery. These phase II metabolites have been observed in humans[29] and dogs[30] and *in vitro* using horse S9 liver fractions.[31]

In addition, we performed the analysis of a positive equine urine sample which was previously confirmed using an accredited confirmatory analysis method and estimated at 50 pg/mL for 16β-hydroxy-stanozolol. As illustrated by Figure 2D,

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16β-hydroxy- as well as 3′-hydroxy-stanozolol were clearly detected in the reported equine urine sample, confirming the method capabilities to detect low-concentrations of stanozolol metabolites in postrace samples.

Postadministration Horsehair Analyses. Considering the promising results obtained on equine and human urine, the approach was next evaluated to another chemically challenging matrix such as horsehair (mane). Indeed, hair is considered as an excessively valuable matrix for doping controls and toxicology because of its molecule-trapping capabilities over time,[32] especially for doping control analyses of major doping agents. For this, we processed 1 cm long sections (100 mg input material) of hair obtained on a horse that had been subjected to a single administration several months beforehand. Remarkably, elimination of stanozolol as well as some hydroxylated metabolites could be precisely observed in multiple hair segments (Figure 3A,B) with decreasing estimated concentrations along the hair segments. In addition, most samples exhibited subpg/mg estimated concentrations, confirming the capabilities of such liquid-chromatography separation approaches for the analysis of low abundance analytes. Furthermore, stanozolol in proximal hair segments was estimated within the 10−100 fg/mg range, suggesting a significant improvement compared to recent related literature with a reported LOD of 200 fg/mg.[33]

As the estimated growth-rate of equine hair is between 2 and 3 cm per month, stanozolol administration was observed in hair sections for at least 5 months. This is consistent with the hair sampling period (i.e., 6 months after administration) and size of the hair sample (i.e., 13 cm). Considering that hair analysis provides enhanced retrospective power due to the drug incorporation in the hair matrix from the bloodstream, it is believed that analytes are trapped longer in hair than in biofluids (i.e., urine and plasma).[32] Thus, the analysis of extracted hair by means of dedicated low-flow LC-HRMS/MS may provide additional detection time frames to drug testing laboratories.

Analysis Throughput and Versatility. Approximatively 200 samples were acquired for this manuscript, including gradient optimization, confirmatory analysis validation, and postadministration studies in urine and hair. The analytical setup is able to perform 4.12 samples/hour with 3.1 min of sample-to-sample overhead, totalizing nearly 80% of MS use and achieving approximately 100 samples per day throughput at improved sensitivity. This brings high-sensitivity low-flow technologies closer to the more conventional high-throughput liquid-chromatography approaches such as UHPLC, even though these technologies still provide the highest throughput and robustness. Finally, our report demonstrates that the technology, which was first developed exclusively for high-throughput proteomics, can be adapted to broader analytical strategies such as synthetic AAS detection in doping control analysis, providing versatility to this technology. As the developed approach brings significant improvement toward AAS detection, the method could be considered as a novel standardized approach by microLC for the analysis of stanozolol and eventually the detection of other AAS.

CONCLUSION

In this Technical Note, we created a dedicated and specialized method to adapt a novel separation technology and expand its initial capability to the detection of AAS in trace-level doping control analyses. After gradient development, we demonstrated that the approach provides robustness, improved sensitivity, and significantly enhanced detection time frames at a high throughput and is compatible with positive case reporting. Furthermore, the methodology was successfully applied to the detection of stanozolol and its metabolites over several months long time frames in highly complex matrixes such as equine urine and hair. The application of similar developments to other major doping agents will reinforce the analytical capabilities of doping control laboratories and could potentially highlight more doping practices at the ultratrace level in the future. Moreover, the analytical approach may be adapted to other endogenous or exogenous metabolites in the growing field of metabolomics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c03392.

Additional materials and methods including chemicals and reagents, experimental procedures for *in vitro* generation of stanozolol metabolites, Evotip preparation flowchart, LC-HRMS analysis of stanozolol metabolites, LC-HRMS/MS methods (UHPLC and MicroflowLC) for technological comparison, and supplementary figures and a table (PDF)

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Notes

The authors declare the following competing financial interest(s): Nicolai Bache (co-author) is currently an employee of Evosep, a liquid chromatograph manufacturer.

A dedicated "high organic" method is freely available within the Evosep Plus program.

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