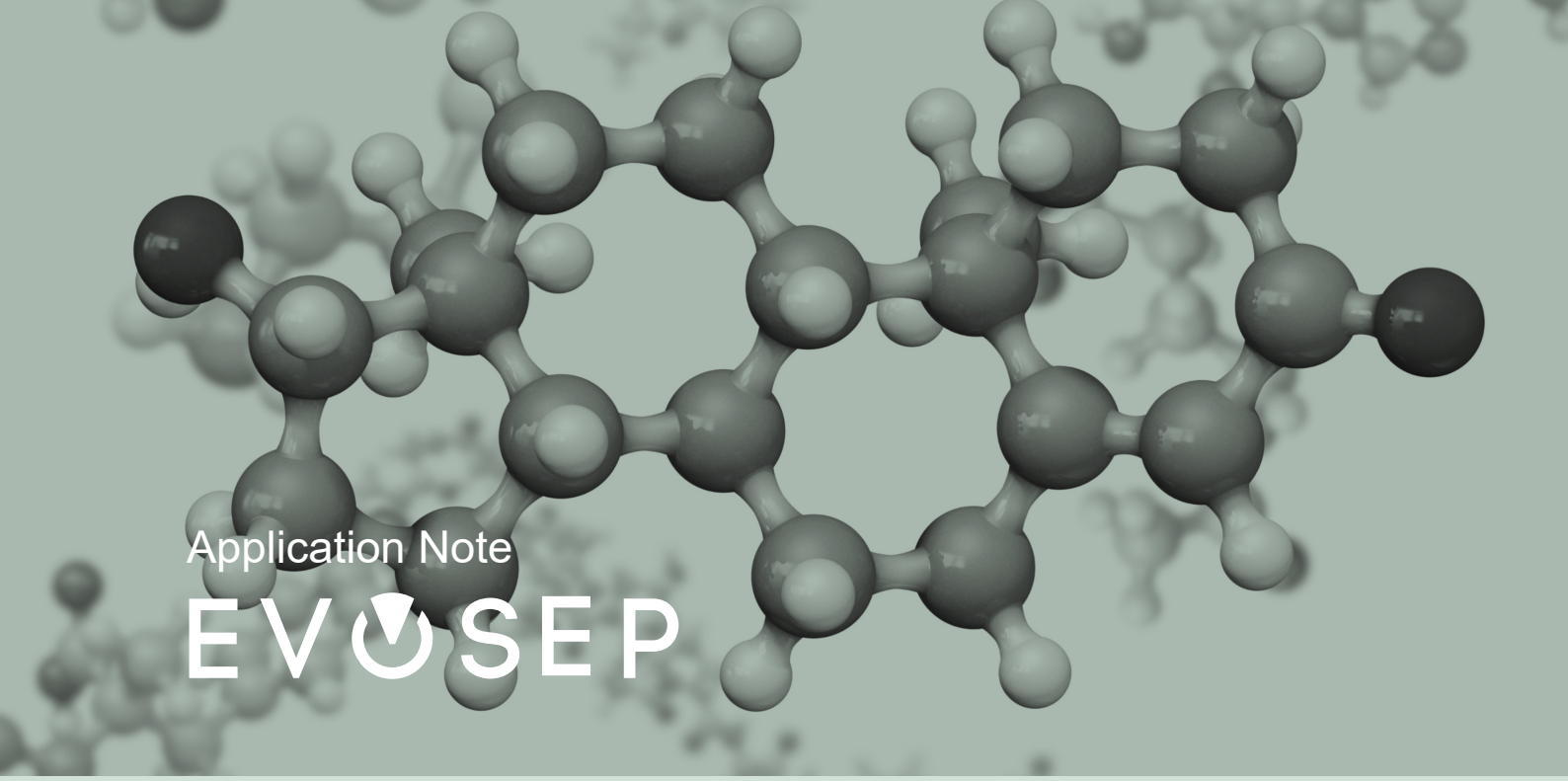

Application Note

EVOSEP

Extended detection of anabolics misuse in high-throughput doping controls with the Evosep One

1. Introduction

Doping control laboratories constantly look for technological improvements providing additional sensitivity, while maintaining the needed high throughput to perform several thousands of samples per year. This is particularly relevant to ensure the accurate identification of banned compounds such as synthetic anabolic androgenic steroids (AAS) at lower limits of detection as well as extending the period after usage, where the compound is detectable in the analysis. We have previously described the sensitive detection of protein-based banned substances such as EPO with the Evosep One[1,2]. Since AAS are significantly more hydrophobic than the majority of tryptic peptides, we generated a new Evosep method (High Organic method) to efficiently detect stanozolol, a notorious synthetic AAS. This method allows the sequential elution of analytes within a nearly linear 0 – 100 % ACN gradient with a throughput of 100 samples per day.

2. Methods

Detailed experimental procedures are available in the article published in Analytical Chemistry[3] describing the method development and performances in full.

Briefly, equine and human urines were enzymatically hydrolyzed and extracted using mixed-mode reversed phase/strong cation exchange BCX2 96 well plates (UCT) as described previously[4] with adapted elution procedures employing ethyl acetate containing 3 % of NH_4OH/H_2O (32 %v/v). Extracts were dried, resuspended in 20 % methanol and loaded on Evotips. Briefly for hair analysis, the hair cross-section was washed using aqueous SDS, rinsed with water, dried at 37 °C and cut into 1 cm length segments using a hair clipper. 100 mg of each segment were collected and incubated in MeOH/1M HCl in water (2/3, v/v) at 50 °C for 4 h. Samples were resuspended with 5 mL of H_2O, and the supernatant was extract-

ed on mixed-mode reversed-phase/strong cation exchange Oasis MCX cartridges (Waters). Extracts were dried, resuspended in methanol and loaded on Evotips.
The High Organic Method was used for separation of analytes in combination with our EV1064 Endurance column. Briefly this method generates a 0-100% acetonitrile gradient over the Evotip suited for significantly more hydrophobic targets. Data were acquired using parallel reaction monitoring (PRM) on a Q Exactive HF mass spectrometer and selected ions were fragmented in the HCD cell with a normalized collision energy of 75.

3. Urine analysis

No specific signal was detected with the targeted product ions in blank urine samples, whereas 10 pg/mL and 100 pg/mL spiked urine extracts provided unambiguous identification of 16β-hydroxy-stanozolol (Figure 1) in accordance with AORC guidelines. These results demonstrate the reliability of the Evosep One for targeted analysis of stanozolol metabolites in extracted equine urine. Furthermore, detection linearity was assessed. Equine and human urines were spiked with two reference stanozolol metabolites at six different concentrations ranging from 1-100 pg/ml. The analysis resulted in standard curves with linear signals, indicating that this approach is viable for quantitative analysis (Figure 1).

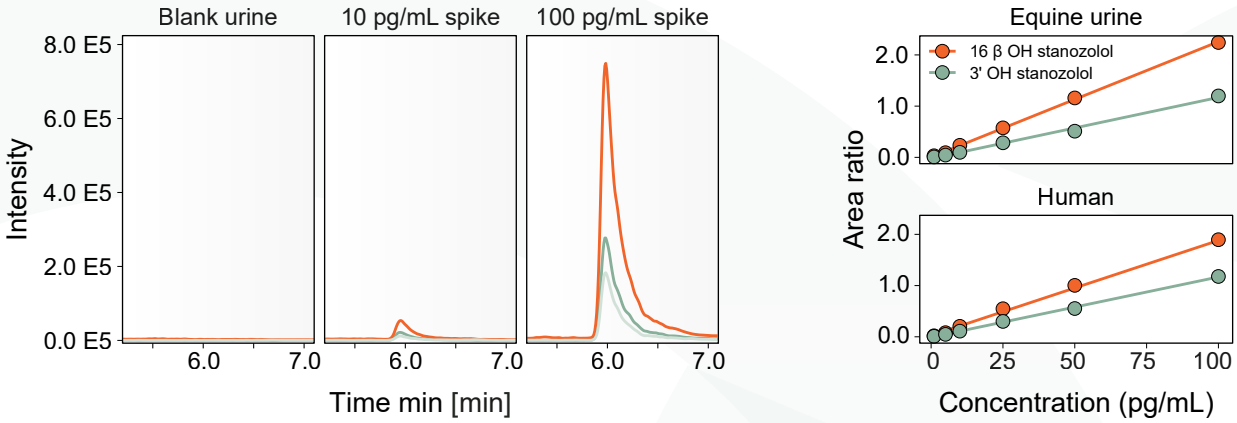

Figure 1: XIC of 16β-hydroxy-stanozolol obtained after analysis of equine urine samples (left). Linear regression analysis of 16β- and 3'-hydroxy-stanozolol in equine and human urine (right).

4. Long-term detection in urine

These results were completed with the analysis of samples collected after a split-dose *in-vivo* study with long-term urine monitoring. Numerous stanozolol metabolites including 16β-, 3'-hydroxy-stanozolol and stanozolol could be detected up-to three months (Figure 2). Furthermore, the High Organic Method

exhibited high reproducibility of retention times. Finally, the High Organic Method appears to be well suited to efficiently separate closely related molecules such as isomers with the example of stanozolol and epi-stanozolol being separated by approximatively 30 seconds.

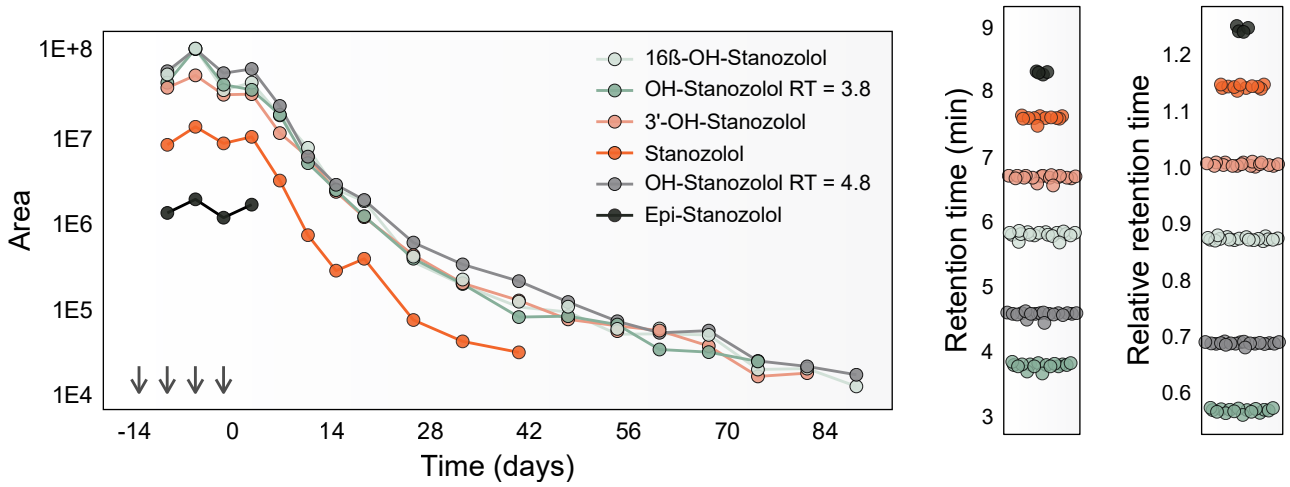

Figure 2: Long term detection of stanozolol and its metabolites in equine urine (left). Retention time and relative retention time reproducibility (right).

5. Horsehair analysis

Hair has high retrospective power[5] due to its molecule-trapping capabilities and this matrix was evaluated on samples collected several months after in-vivo study. As depicted on Figure 3, stanozolol as well as some hydroxylated metabolites were observed in multiple hair segments, with decreasing concentration consistent with 5 months hair-growth. In addition, most samples exhibited sub pg/mg estimated concentrations, confirming the capabilities of using the Evosep One for the analysis of low abundance analytes.

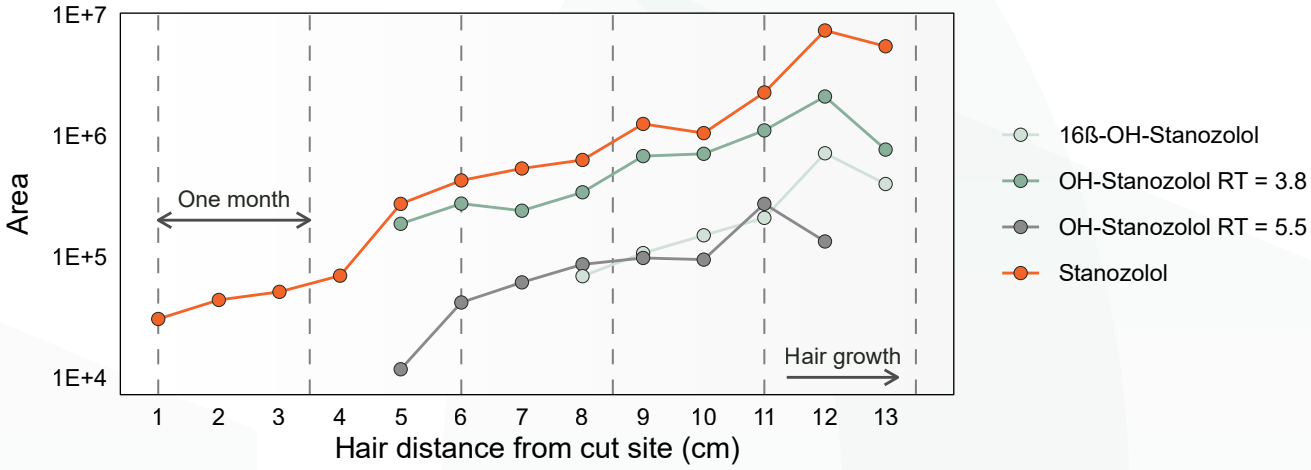

Figure 3: Long term detection of stanozolol and its metabolites in equine hair.

6. Conclusion

The High Organic Method setup on the Evosep One provides an additional standardized tool for drug testing laboratories with the detection of AAS in doping control analyses. It was applied to the detection of stanozolol and its metabolites demonstrating robustness and sensitivity with a throughput of 100 samples per day. Furthermore, analysis from horsehair revealed enhanced retrospective power. This provides additional detection time frames to drug testing laboratories and could be useful to detect other threats. Moreover, we expect that this approach could be adapted to other trace-level compounds and matrices in other related fields such as forensics or metabolomics.

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Authors

Vivian Delcourt[1], Patrice Garcia[1], Isabelle Pottier[1], Nasrine Mansoibou[1], Nicolai Bache[2], Yohan Glavieux[1], Benjamin Chabot[1], Isabelle Perot[1], Francois André[1], Benoit Loup[1], Agnés Barnabé[1], Marie-Agnés Popot[1] & Ludovic Bailly-Chouriberry[1]

[1] LCH, GIE-Laboratorie des Courses Hippiques, Verriéres-le-Buisson, France
[2] Evosep Biosystems, Odense, Denmark