“Detection of prohibited substances by ultra-high performance supercritical fluid chromatography: drawbacks and benefits for anti-doping drug testing”

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Project Overview:

Ultra-high performance supercritical fluid chromatography-mass spectrometry (UHPSFC-MS) could represent in the near future an orthogonal technique to LC-MS(/MS) and GC-MS(/MS) for routine doping analysis.

UHPSFC is a normal phase separation technique that uses carbon dioxide as mobile phase, with the addition of small amounts of methanol as co-solvent. This technology is considered to be a green approach. Using appropriate stationary phase chemistries and dimensions, different pharmaceutical compounds (non-polar and polar) have already been analyzed. However, up to date, only few applications of this technique for the analysis of compounds in biological fluids (urine and blood) exist in scientific literature.
Furthermore, no studies are available for the analysis of forbidden substances in anti-doping area.

In this study, the performance of UHPSFC-MS(/MS) will be evaluated for the analysis of different classes of forbidden substances present in the prohibited list, such as exogenous and endogenous steroids, stimulants, diuretics, narcotics, corticoids, small peptides, etc. Particular attention will be focused to endogenous and exogenous steroids in urine and blood samples as well as to the optimization of analytical conditions (separation and detection) for both screening and confirmation purposes. Results will be evaluated in terms of selectivity, matrix effect, sensitivity and limits of detection in both biological matrices. Benefits and drawbacks of this technique will be discussed and compared to conventional LC-MS(/MS) and GC-MS(/MS) analyses. An improvement of detection capabilities for different forbidden substances hardly detectable by conventional techniques could be expected

Results and Conclusions

Today, LC-MS(/MS) and GC-MS(/MS) remain the most widely used analytical strategies for doping control analysis. The goal of this project was to evaluate the potential of an alternative chromatographic strategy, namely SFC-MS/MS, for the determination of a wide range of doping agents present in the WADA prohibited list. For this purpose, a UHPSFC-MS/MS screening method was developed for 110 stimulants, diuretics and narcotics in urine samples. Then, the same analytical platform was used for the screening of about 100 analytes belonging to the difficult classes of anabolic agents, hormones and

metabolic modulators, synthetic cannabinoids and glucocorticoids in urine samples.

From these two large screening methods, we were able to demonstrate that SFC-MS/MS was extremely reliable and could be considered as an ideal alternative to the widely used LC-MS(/MS) and GC-MS(/MS) platforms for the determination of diverse substances in biological matrices. In this project, the following benefits were noticed in SFC-MS/MS:

1. The retention of SFC is mostly based on polar interactions with the stationary phases (i.e. dipole-dipole and hydrogen bonding), which is the opposite of RPLC, where the retention is driven by hydrophobic interactions. Then, some polar doping agents present in the WADA prohibited list (e.g. methylecgonine, octopamine, oxilofrine, synephrine, phenylephrine…) were sufficiently retained in SFC, while it was not the case in RPLC under generic screening conditions.
2. Because the retention mechanisms in SFC and RPLC are orthogonal, some problematic coelutions in RPLC can be “easily” resolved in SFC and *vice-versa*. This feature was particularly useful for the determination of anabolic agents. In this particular class of compounds, there were indeed a significant number of isobaric substances that deserve special chromatographic attention, since they cannot be resolved by MS/MS detection.
3. In SFC-MS/MS, and contrary to what happen for GC-MS(/MS), the urine samples can be directly analyzed, without the need for a chemical derivatization strategy. Therefore, the sample treatment was simplified and the analytical throughput was strongly enhanced in SFC vs. GC. In view of the workload undergone by a doping control analysis laboratory, such a rapid procedure could be of great value.
4. The instrumental sensitivities achieved in SFC-MS/MS were adequate for screening purposes. Indeed, the LOD achieved were systematically below the MRPLs fixed by the WADA. For the 100 anabolic agents, hormones and metabolic modulators, synthetic cannabinoids and glucocorticoids, there were only one metabolite of each of these substances (norethisterone, turinabol and norbolethone) that did not meet the MRPL criteria among the 100 tested compounds. Through this project, we demonstrated that the sensitivities achieved in SFCMS/MS were comparable and often improved with regard to RPLC-MS/MS and GC-MS/MS, due to the very limited amount of water in the SFC mobile phase.
5. The susceptibility of the developed SFC-MS/MS methods towards matrix effects was evaluated at different concentration levels and the obtained results were compared to that of LC-MS/MS and GC-MS/MS (in some cases). The matrix effects were very low in UHPSFCMS/ MS, and slightly higher in UHPLC-MS/MS, and unacceptable in GC-MS/MS, due to “matrix induced chromatographic response enhancement”, that could jeopardize the reliability of the method.

Thanks to this project, UHPSFC was for the first time proven to be applicable for the screening of doping agents in urine samples. The application range that can be managed in SFC-MS/MS was found to be similar and even larger than RPLC-MS/MS. Compared to GC-MS/MS, a much higher throughput can

be achieved, as there was no need for chemical derivatization. The benefits reported in UHPSFC–MS/MS make this approach very attractive and promising for doping control analysis, as a screening strategy or possibly as a confirmatory approach.

Publication / Presentation related to the project

1. L. Novakova, V. Desfontaine, F. Ponzetto, R. Nicoli, M. Saugy, J.-L. Veuthey, D. Guillarme, *Fast and sensitive supercritical fluid chromatography-tandem mass spectrometry multi-class screening method for the determination of doping agents in urine*, Anal. Chim. Acta (2016) 915, 102-110.
2. V. Desfontaine, L. Novakova, F. Ponzetto, R. Nicoli, M. Saugy, J.-L. Veuthey, D. Guillarme, *Ultra high performance liquid chromatography and supercritical fluid chromaography asemerging techniques for the rapid screening of anabolic agents in urine for doping control*, J. Chromatogr. A (2016) accepted, in press.

Oral presentations of results from this project were made during the following meetings:

1. Lucie Novakova, Vincent Desfontaine, Federico Ponzetto, Raul Nicoli, Martial Saugy, Jean-Luc Veuthey, Davy Guillarme, *Can SFC-MS/MS succeed in doping control analysis?* Waters EU User Meeting on SFC, Purification & Related Technologies, 3. -4. December 2015, Prague, CR
2. A. Grand-Guillaume Perrenoud, L. Novakova, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme, *UHPSFC-MS/MS: from hyphenation optimisation to high sensitivity doping agents analysis in urine.* HTC-13 – Bruges, Belgium, January 2014

Poster presentations of results from this project were made during the following meetings:

1. L. Novakova, V. Desfontaine, F. Ponzetto, R. Nicoli, M. Saugy, J.-L. Veuthey, D. Guillarme, *UHPSFC-MS/MS as a viable option in doping control analysis,* HPLC 2016, San Francisco, 2016. 3rd place Winner Best Poster Award.
2. R. Nicoli, L. Novakova, A. Grand-Guillaume Perrenoud, J.-L. Veuthey, D. Guillarme, M. Saugy, *UHPSFC-ESI-MS/MS: a powerful analytical platform for screening of doping agents in urine by 'dilute-and-shoot',* Mandred Donike Workshop, Cologne, February 2016
3. R. Nicoli, V. Desfontaine, L. Novakova, F. Ponzetto, J.-L. Veuthey, D. Guillarme, M. Saugy, *Fast and sensitive screening of anabolic agents in urine samples by UHPSFC-MS/MS and UHPLCMS/ MS: a comparison study,* Mandred Donike Workshop, Cologne, February 2016
4. A. Grand-Guillaume Perrenoud, L. Novakova, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme, *UHPSFC-ESI/MS/MS: a powerful platform for high sensitivity doping agents analysis in urine.* Drug Analysis 2014, Liège, Belgium, June 2014
5. L. Novakova, M. Rentsch, A. Grand-Guillaume Perrenoud, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme, *Optimization and application of UHPSFC-*

MS/MS method for screening of doping agents, IMSC 2014 – Geneva, Switzerland, August 2014

6. A. Grand-Guillaume Perrenoud, L. Novakova, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme, *UHPSFC-ESI/MS/MS: a powerful platform for high sensitivity doping agents analysis in urine.* SFC 2014 - Basel, Switzerland, October 2014