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Validation of qualitative screening method for doping substances by using UPLC-QTOF system

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Introduction

A list of pharmacological substances prohibited for use in sport is extending rapidly [1]. Therefore, there is a growing need for development of high throughput screening procedures that are cost and labour effective. Such methods are generally based on the use of advanced techniques of chromatographic separation such as UPLC (ultra performance liquid chromatography) or RR-HPLC (rapid resolution high performance liquid chromatography). These in turn are coupled to a mass spectrometer equipped in analyser of high scanning capability and mass accuracy, including TOF (time of flight). To date, several screening procedures employing TOF-based analytical systems have been described [2-4].

This study presents development of a screening method for detection of doping agents of distinct classes in urine, including aromatase inhibitors, β2-agonists, selective estrogen receptor modulators (SERMs), stimulants, and anabolic agents. The sample preparation is a three-step procedure involving solid phase extraction, deconjugation of glucuronides, and liquid-liquid extraction with diethyl ether. Target substances are traced in a full scan mode by an UPLC-QTOF system, and the criteria of detection are based on the reproducibility of retention time and mass accuracy.

Materials and Methods

LC conditions: Chromatographic separation of doping substances was carried out on a Waters Acquity UPLC system with Acquity UPLC columns (BEH C18 Shield 100mm × 2.1mm, 1.7 μm) and an Acquity Van Guard precolumn (BEH C18 5mm × 2.1mm, 1.7 μm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and a step-wise LC gradient was employed at a constant flow rate of 400 μL/min at 40°C. Initial acetonitrile concentration of 15% was constant over 0.5 min and subsequently increased to 95% in 5.5 min. The column was re-equilibrated for 3 min with

5% of solution B. Samples were stored at 4°C in the autosampler prior to analysis and the injection volume was fixed at 15 µL.

MS conditions: Ions of analyzed substances were traced with a Micromass QTOF Premier mass spectrometer (Waters, USA) equipped in an ESI source. Each sample was run twice in order to analyze it for the presence of doping agents in both positive and negative ESI modes as TOF-MS covers only one polarity at a time. The desolvation gas flow was set at 800 L/h at a temperature of 300°C and the source temperature was 100°C. The capillary and cone voltages applied differed in dependence of the ionization polarity and were respectively defined as 3.0 kV and 40V in positive and 2.4 kV and 50V in negative mode. The cone and collision gas flows were set at 20 L/h and 0.2 mL/min, respectively, and were the same in both modes. The system was operated in a wide pass quadrupole mode with the collision energy set at 5eV in order to avoid extensive fragmentation. Data were acquired in a W-optics centroid mode over the range of 100-1000 m/z and with the mass resolution of at least 14,000 FWHM (full-width at half maximum). The scan time was 0.25 s with the interscan delay set at 0.02 s. High accuracy of detected masses was ensured by using the internal lock mass calibrant Leucine enkefalin (2 ng/mL, Sigma-Aldrich).

Sample preparation: An aliquot of urine (1 mL) was spiked with internal standards and studied substances, and then applied on SPE C18 columns for extraction. Compounds of interest were eluted with methanol, and the elution fraction was subsequently evaporated to dryness. The residue was reconstituted in 1 mL of 0.2 M phosphate buffer (pH 7.0), and the samples were then hydrolyzed with β-glucuronidase (*E. Coli*, 1h/50°C). After the incubation, 0.2 mL of 5% K_2CO_3 was added, and the solutions were subjected to a procedure of liquid-liquid extraction with 5 mL of diethyl ether. Afterwards, the organic phase was recovered, evaporated to dryness, and the residue was reconstituted in 100 µL of mobile phase.

Results and Discussion

The developed screening procedure allows for unequivocal detection of 27 active substances prohibited for use in sport at their respective MPRL levels. The experimental data obtained for a urine sample fortified with investigated substances are collected in Table 1. Parent ions of target substances were traced by an UPLC-QTOF system, which was run twice for each sample in positive and negative ESI mode, respectively. The acquisition data were collected in a full scan mode and viewed with a mass tolerance window of 50 ppm.

Table 1. Detailed list of the investigated compounds. (RT) - retention time, (S/N) – signal-to-noise ratio, (MRPL) – Minimum Required Performance Level, (a) – in-source fragment, (b) – two peaks observed in the LC chromatogram are likely to reflect an isomeric nature of the standard used, (c) – the concentration was adjusted for quality control purposes and it is not the MRPL specified for the compound.

Compound	Ion traced	MRPL [ng/ml]	RT [min]	S/N	ESI mode
Anabolic agents					
Clenbuterol	277.08690	2	1.67	20	+
	203.0137[a]			74	
Gestrinone	309.18491	10	4.08	28	+
17-epioxandrolone	307.2273	10	4.13	10	+
Methyltrienolone	285.18491	10	3.73	48	+
3'-hydroxystanozolol	345.25366	2	3.06	67	+
4α-hydroxystanozolol	345.25366	10	3.18	44	+
16β-hydroxystanozolol	345.25366	10	3.11	155	+
Zearalenone	317.1389	10	4.52	25	-
Alpha-Zearalanol	321.1702	10	4.15	740	-
β2-agonists					
Bambuterol	368.21800	100	1.87	8275	+
Fenoterol	304.15434	100	0.86	113	+
Formoterol	343.16633	100	1.70	280	-
Salbutamol	240.15942	100	0.60	115	+
Salmeterol	416.27954	100	2.83	354	+
Terbutaline	226.1443	100	0.63	193	+
Hormone antagonists and modulators					
Aminogluthetimide	233.12845	50	1.03	51	+
Anastrozole	294.17132	50	3.25	1348	+
Letrozole	284.09417	50	3.34	710	-
Testolactone	301.1804	50	3.10	171	+
Clomiphene	406.19322	50	3.45	78	+
Raloxifene	474.17336	50	2.71	11131	+
Tamoxifen	372.23219	50	3.48	329	+
3-Hydroxy-4-methoxytamoxifen (1)[b]	418.2382	50	2.94	120	+
3-Hydroxy-4-methoxytamoxifen (2)[b]			3.21	577	+
Toremifene	406.19322	50	3.44	538	+
Stimulants					
Benzylecgonine	290.1392	500	1.53	19	+
Cocaine	304.15434	500	1.77	6412	+
Pemoline	175.0508	500	1.66	145	-
Internal standards					
Methyltestosterone	303.2324	100[c]	4.14	2352	+
Mefruside	381.0346	150[c]	3.46	3284	-

The design of TOF instruments allows for detection of unlimited number of analytes in a single run, and TOF-based procedures testing for the presence of 100 and more substances in urine have been published recently [2-4]. Importantly, the addition of new entries to the list of traced substances do not compromise the specificity and sensitivity of the method being modified. Thus, the scope of the presented screening can be further extended simply by introduction of new compounds from the *WADA Prohibited List*. In fact, this method is rather treated as a preliminary approach for developing a more general screening procedure covering the majority of the small molecular substances banned in sport. Additionally, the development of a pre-confirmatory QTOF-MS/MS procedure is also considered as an effort to significantly lower the level of false positive results of screening testing [5].

References

[1] World Anti-Doping Agency. The 2010 Prohibited List. International Standard, Montreal (2010) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf (access date 23.08.2010)

[2] Kolmonen M, Leinonen A, Kuuranne T, Pelander A, Ojanperä I. (2009) Generic sample preparation and dual polarity liquid chromatography-time-of-flight mass spectrometry for high-throughput screening in doping analysis. Drug Test Anal. 1(6):250-66.

[3] Badoud F, Grata E, Perrenoud L, Avois L, Saugy M, Rudaz S, Veuthey JL. (2009) Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry I. Screening analysis. J Chromatogr A. 1216(20):4423-33.

[4] Vonaparti A, Lyris E, Angelis YS, Panderi I, Koupparis M, Tsantili-Kakoulidou A, Peters RJ, Nielen MW, Georgakopoulos C. (2010) Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometry. Rapid Commun Mass Spectrom. 24(11):1595-609.

[5] Badoud F, Grata E, Perrenoud L, Saugy M, Rudaz S, Veuthey JL. (2010) Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry. II: Confirmatory analysis. J Chromatogr A. 1217(25):4109-19.