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Screening of prohibited substances in human urine by LC-ESI-TOF-MS analysis

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Abstract

This study presents development and validation of an LCMS screening method of small molecules from all categories of prohibited substances. It is based on a single-step liquid-liquid extraction of hydrolyzed urine and the use of a rapid resolution liquid chromatographic system combined with a quadrupole/time-of–flight mass spectrometer acquiring continuous full scan data. Detection criteria were established on the basis of retention time reproducibility and mass accuracy results.

Introduction

The WADA accredited laboratories are based on the use of mass spectrometry (MS) coupled to either gas or liquid chromatography (GCMS or LCMS) for the analysis of prohibited substances of small molecules [1]. Various categories of prohibited substances require different routine screening analytical protocols in order to comply with the WADA specifications for Minimum Required Performance Limits (MRPL). Several laboratories have published LCMS screening protocols which combine in one analytical run different categories of analytes [2-4]. Unification of the screening protocols has become feasible since the increased instrumental sensitivity and the specificity of the tandem or high resolution MS, which anticipates losses from generic sample preparation and mass spectrometric signal acquisition of an extended number of analytes.

This study presents development and validation of an LCMS screening method of small molecules from all categories of prohibited substances. It is based on generic sample

preparation and the use of a rapid resolution LC system combined with a Quadrupole-Time-of –Flight mass spectrometer (QTOFMS) acquiring continuous full scan without the use of the quadrupole mass filter (TOF acquisition mode).

Materials and Methods

Sample preparation: The extraction protocol used in this study is applied at our laboratory for the routine screening analysis of anabolic steroids and corticosteroids. [5] Acetic acid was added at the organic extract before evaporation in order to avoid the loss of volatile stimulants.

LC conditions: An Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies) was used. Chromatographic separation was performed at 35 ^{o}C using Zorbax Eclipse Plus C18 column (100 x 2.1 mm, 1.8 μm). The mobile phase consisted of 5 mM ammonium formate in 0.01 % formic acid (solvent A) and of acetonitrile/water 90/10 (v/v) containing 5 mM ammonium formate and 0.01% formic acid (solvent B). Gradient elution program was used. Analysis run time was 12.5 min and post-run equilibrium time 3.5 min. Injection volume was 5 μL.

Mass spectrometric conditions: The mass spectrometer was an orthogonal accelerator QTOFMS (6520, Agilent Technologies) equipped with orthogonal electrospray ionization source (ESI) and analogue-to-digital converter (ADC) operated at 2 GHz. Ionization was performed in the positive mode. Drying gas flow and temperature were set at 10 L min^{-1} and 350 ^{o}C, respectively and nebulizer gas pressure at 45 psi. The applied capillary voltage was 4000 V. The fragmentor voltage was set at 140 V in order to avoid in-source fragmentation of the analytes' protonated molecules. Full scan mass spectral data were acquired from 100 to 1100 m/z with scan rate 1 scan sec^{-1}. Mass reference correction was used by introducing continuously two reference compounds during acquisition from a second nebulizer.

Method Validation

Extraction recovery: six blank urine samples were fortified with the analytes at the MRPL and extracted together with the same six negative urine samples. The extracts of the non-fortified urine samples were spiked with the analytes into the organic layer before evaporation.

Ion suppression/enhancement: the peak areas of the six urine samples spiked after extraction were compared with a standard solution in mobile phase, corresponding to 100% recovery. The standard deviations of the ion suppression recoveries were also calculated in order to

evaluate variations between different urine matrices.

Identification capability / Limit of detection: Ten different blank urine samples were fortified with the analytes at different concentration levels (0.1, 0.2, 0.5, 2 and 4 times the MRPL) and analysed together with the respective blanks. The LOD was determined as the lowest concentration where a substance could be detected in all samples analyzed (n=10). In order to detect analytes not available as reference materials, urine samples from excretion studies were analyzed. Method's specificity was verified by analyzing twenty different negative urine specimens. Retention time and mass accuracy data were collected for all the analytes.

Results and Discussion

A total of 180 of analytes from all classes of prohibited substances could be analysed within 15 min by employing a single step liquid-liquid extraction and a LC/TOF-MS analysis in positive ESI mode. Furazabol, 16β-hydroxy-furazabol (furazabol MT), 1-methylen-5α-androstan-3α-ol-17-one (methenolone MT), 17α-methyl-5α-androstan-3α,17β-diol, 17α-methyl-5β-androstan-3α,17β-diol, oxymesterone, 13β,17α-diethyl-3α,17β-dihydroxy-5α-gonane, 13β,17α-diethyl-3α,17β-dihydroxy-5β-gonane (norbolethone MTs), 5α-tetrahydronorethisterone (norethisterone MT), letrozole MT and furosemide failed to be detected due to low ionization efficiency. Ritalinic acid, diclorphenamide and octopamine failed to be detected due to low extraction efficiency. Extraction recovery ranged from 25 to 100%. Detected analytes with extraction recovery lower than 10% include chlorothiazide, acetazolamide, benzoylecgonine, synefrine, phenylephrine, ethacrynic acid and amiloride. Ion suppression recovery was between 70-100% for most analytes, except for anabolic steroids where suppression more than 50% was observed with considerable variation between different urine matrices. Most diuretics, stimulants and narcotics were detected at concentrations 0.1 times the MRPL. Retention time (RT) precision was evaluated during method validation and RSD values were lower than 2%. Therefore, RT was accepted as a relevant and reliable detection criterion. Mass accuracy in most cases was within 20 ppm, but for some analytes that produce in source fragments, mass error was greater than 50 ppm. In Figure 1 representative chromatograms of 16-hydroxy stanozolol in a spiked urine sample at 2 ng mL^{-1} (a) and a blank urine sample (b) are presented.

The unification of different screening protocols for this wide range of substances in a single analytical procedure can be cost effective for the laboratory by simplifying sample preparation and reducing analysis time and material consumption. Additional compounds can

be easily introduced to increase the range of screened drugs. Furthermore, full-scan acquisition mode with accurate mass can be a valuable tool in the retrospective evaluation of analyzed samples for new doping agents. Consequently, the preventive role of the anti-doping system against the use of designer drugs could be enhanced by the use of the current method.

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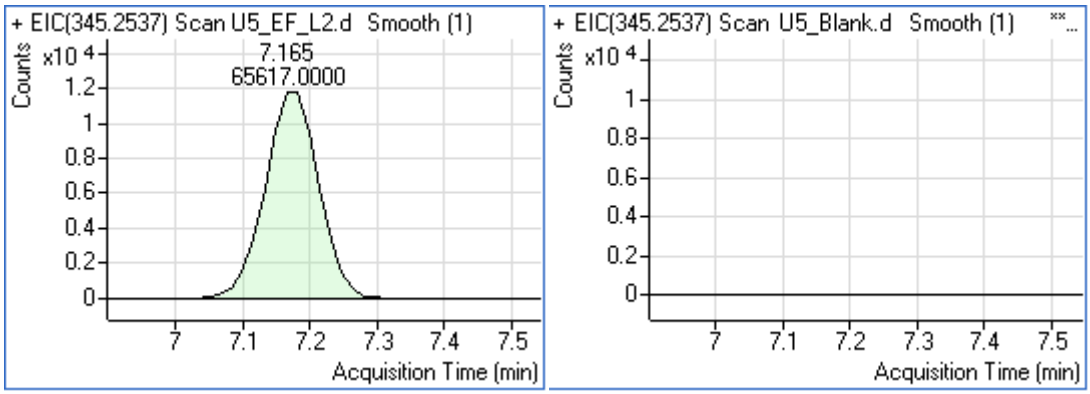

Figure 1: LC/TOF-MS extracted ion chromatograms of a urine sample spiked with 16-hydroxystanozolol at 2 ng mL^{-1} and a blank urine sample.