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Fast and sensitive supercritical fluid chromatography – tandem mass spectrometry multi-class screening method for the determination of doping agents in urine

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HIGHLIGHTS

- A high throughput UHPSFC-MS/MS method was developed for screening several classes of doping agents in urine.
- Supported liquid extraction in 48-well plate format was successfully applied to extract the doping agents from urine samples.
- Good extraction recoveries and reasonable matrix effects were observed for the whole set of doping agents.
- The method exhibited very low LODs, below the Minimum Required Performance Levels, for most of the 100 compounds.

GRAPHICAL ABSTRACT

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ABSTRACT

This study shows the possibility offered by modern ultra-high performance supercritical fluid chromatography combined with tandem mass spectrometry in doping control analysis. A high throughput screening method was developed for 100 substances belonging to the challenging classes of anabolic agents, hormones and metabolic modulators, synthetic cannabinoids and glucocorticoids, which should be detected at low concentrations in urine. To selectively extract these doping agents from urine, a supported liquid extraction procedure was implemented in a 48-well plate format. At the tested concentration levels ranging from 0.5 to 5 ng/mL, the recoveries were better than 70% for 48–68% of the compounds and higher than 50% for 83–87% of the tested substances. Due to the numerous interferences related to isomers of steroids and ions produced by the loss of water in the electrospray source, the choice of SFC separation conditions was very challenging. After careful optimization, a Diol stationary phase was employed. The total analysis time for the screening assay was only 8 min, and interferences as well as susceptibility to matrix effect (ME) were minimized. With the developed method, about 70% of the compounds had relative ME within the range ±20%, at a concentration of 1 and 5 ng/mL. Finally, limits of detection achieved with the above-described strategy including 5-fold preconcentration were

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below 0.1 ng/mL for the majority of the tested compounds. Therefore, LODs were systematically better than the minimum required performance levels established by the World anti-doping agency, except for very few metabolites.

1. Introduction

The list of prohibited substances published by the World anti-doping agency (WADA) is yearly updated. Today, there are about 250 illicit compounds covering a wide range of physicochemical properties that need to be tracked in the different specimens (urine and blood) collected in-and-out of competition [1]. The declaration of a presence or an absence of a doping agent is a multistep process including an initial screening approach followed by a confirmation procedure, if applicable. To tackle the huge chemical diversity of the banned substances, various analytical methods are implemented in anti-doping laboratories and among them, GC–MS(/MS) and LC-MS/MS are considered as the methods of choice offering high selectivity, sensitivity and fast turnaround. These methods focus on the direct detection of prohibited substances, but also on the determination of their major phase I and phase II metabolites, to further improve the detection windows capabilities in the matrix of interest [2]. To ensure homogeneous performance between laboratories, Minimum Required Performance Levels (MRPLs) for detection of non-threshold substances are established by WADA, and correspond to the minimum concentration levels that should be attained for routine analyses.

To date, supercritical fluid chromatography (SFC) has been scarcely used for doping control purposes, despite some obvious advantages of this separation technique [3]: i) the low fluid viscosity and high diffusion coefficients under SFC conditions provide excellent kinetic performance, ii) the organic solvent consumption is limited compared to LC, despite the fact that a significant proportion of organic modifier (up to 40%) may be used in modern packed column SFC, iii) a wide range of compounds, from relatively polar to highly non-polar, can be analyzed in SFC, with the same mobile phase components (CO_2 and methanol). Still, these benefits were restricted by the poor quality of old-generation SFC instruments providing low sensitivity, unacceptable quantitative performance and above all lack of reliability [4]. However, in the last few years, providers of chromatographic instrumentation have launched new generation of SFC systems which undertake the above-mentioned shortcomings. These new SFC instruments are described as UHPSFC which stands for ultra-high performance supercritical fluid chromatography [5]. UHPSFC systems offer an improved compatibility with modern stationary phases, such as columns packed with fully porous sub-2 μm particles as well as a full compatibility with ESI-MS(/MS) devices thanks to commercial interfaces [6]. Considering all the beneficial features of modern UHPSFC-MS/MS instrumentation, a screening method was previously successfully developed in our laboratory for the determination of 110 doping agents in urine, including diuretics, β-blockers, stimulants and narcotics, using a simple dilute-and-shoot procedure [7,8].

Among the WADA list of banned substances, the determination of the numerous anabolic agents, in particular androgenic steroids, is particularly challenging. Indeed, these substances are excreted in urine with very diverse concentrations, and MRPLs are extremely demanding. In addition, there is a large number of isomers and metabolites, among this particular class, which are difficult to separate and identify in a satisfactory manner [9]. GC–MS(/MS) remains the gold standard for analyzing these substances in anti-doping laboratories, but often requires laborious liquid–liquid extraction procedures as well as time-consuming derivatization steps prior to injection, to make the substances volatile and improve their detectability. Surprisingly, SFC is known to be a reference strategy for the analytical identification and characterization of various types of steroids [10–12], but has rarely been applied so far for the determination of steroids and derivatives in doping control analysis [11], and never in routine analyses.

The goal of this work was to evaluate the performance of a combination of supported liquid extraction (SLE) and UHPSFC-MS/MS analysis, for the high throughput screening of 100 substances (parent compounds and phase I metabolites) in urine. All the selected substances belong to the problematic classes of anabolic androgenic steroids, hormones and metabolic modulators, synthetic cannabinoids and glucocorticoids, for which there are a lot of isobaric compounds that should be detected at very low concentration levels in urine. The SLE recoveries, matrix effects and limits of detection achieved in UHPSFC-MS/MS for the 100 substances will be shown and critically discussed.

2. Experimental method

2.1. Reagents and analytes

All doping agents were kindly provided by the Swiss Laboratory for Doping Analysis (Epalinges, Switzerland). The exhaustive list of these target analytes is reported in Table S-1 and their structure in Fig. S-1. Methanol (MeOH), ethanol (EtOH), isopropanol and acetonitrile (ACN) of ULC/MS grade were provided by Biosolve (Dieuze, France). Ammonium formate (AmF), ammonium acetate, heptane extra dry 99%+ and methyl tert-butyl ether (MtBE) were provided by Sigma–Fluka (Buchs, Switzerland). Diethylether was purchased from Acros Organics (Geel, Belgium). Pressurized gas CO_2 N48 (>99.998%) was purchased from Air Liquide (Dusseldorf, Germany). Ultra-pure water was provided by a Milli-Q system from Millipore (Bedford, MA, USA). β-glucuronidase from *Escherichia coli* was purchased from Roche Diagnostics GmbH (Mannhein, Germany).

2.2. Sample preparation of biological samples

A pool of blank urines was prepared by mixing 6 different urine samples obtained from healthy volunteers. Each urine aliquot of 1 mL was first spiked with doping agent standards in ACN to obtain 5 different levels of concentration, namely 0.1, 0.5, 1, 5 and 10 ng/mL. Then, they were extracted on Isolute SLE+ 48-well plates (Biotage, Uppsala, Sweden). Urine was forced through the plate sorbent using Biotage PRESSURE+ 96 positive pressure manifold at 3 psi. After a waiting time of 5 min, the elution was made by percolating 3 mL of MtBE through the wells into a 48-well collection plate. The SLE wells were dried by operating positive pressure again for few seconds. Then, the extracted samples in the collection plate were evaporated until dryness using UNIVAPO Rotational Vacuum Concentrator 150 ECH (Biolabo Scientific Instruments, Châtel-Saint-Denis, Switzerland) at 1250 rpm, ambient