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Liquid chromatography and supercritical fluid chromatography as alternative techniques to gas chromatography for the rapid screening of anabolic agents in urine

Vincent Desfontaine[a], Lucie Nováková[b], Federico Ponzetto[c], Raul Nicoli[c], Martial Saugy[c], Jean-Luc Veuthey[a], Davy Guillarme[a,*]

[a] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland
[b] Department of Analytical Chemistry, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 50005 Hradec Králové, Czech Republic
[c] Swiss Laboratory for Doping Analyses, University Center of Legal Medicine Lausanne-Geneva, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Chemin des Croisettes 22, 1066 Epalinges, Switzerland

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ABSTRACT

This work describes the development of two methods involving supported liquid extraction (SLE) sample treatment followed by ultra-high performance liquid chromatography or ultra-high performance supercritical fluid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS and UHPSFC–MS/MS) for the screening of 43 anabolic agents in human urine. After evaluating different stationary phases, a polar-embedded C18 and a diol columns were selected for UHPLC–MS/MS and UHPSFC-MS/MS, respectively. Sample preparation, mobile phases and MS conditions were also finely tuned to achieve highest selectivity, chromatographic resolution and sensitivity.

Then, the performance of these two methods was compared to the reference routine procedure for steroid analyses in anti-doping laboratories, which combines liquid–liquid extraction (LLE) followed by gas chromatography coupled to tandem mass spectrometry (GC–MS/MS). For this purpose, urine samples spiked with the compounds of interest at five different concentrations were analyzed using the three analytical platforms. The retention and selectivity of the three techniques were very different, ensuring a good complementarity. However, the two new methods displayed numerous advantages. The overall procedure was much faster thanks to high throughput SLE sample treatment using 48-well plates and faster chromatographic analysis. Moreover, the highest sensitivity was attained using UHPLC–MS/MS with 98% of the doping agents detected at the lowest concentration level (0.1 ng/mL), against 76% for UHPSFC–MS/MS and only 14% for GC–MS/MS. Finally, the weakest matrix effects were obtained with UHPSFC–MS/MS with 76% of the analytes displaying relative matrix effect between −20 and 20%, while the GC–MS/MS reference method displayed very strong matrix effects (over 100%) for all of the anabolic agents.

1. Introduction

The list of prohibited substances in the practice of sport is yearly updated by the World Anti-Doping Agency (WADA). This list includes different categories of doping agents such as anabolic agents, hormone and metabolic modulators, peptide hormones, β2-agonists, diuretics, stimulants, narcotics and glucocorticoids [1]. Among them, the class of anabolic agents (S1), including exogenous and endogenous anabolic androgenic steroids (AAS), is certainly one of the most widely used by athletes to increase their sport performance. Indeed, as documented in 2014 WADA statistics, 48% of reported adverse analytical findings were due to this class of compounds [2]. Moreover, from an analytical point of view, they are also one of the most challenging groups to analyze because: i) steroids share very similar backbone structures leading to the presence of numerous isomers, and, since their differences are related to the ring saturation and the presence and position of additional functional groups, their specific measurements constitute an analytical challenge in terms of selectivity, ii) they are metabolized through different metabolic pathways in human body, and often generate numerous phase I and phase II metabolites at very low concentrations in biological fluids [3]. In consequence,

* Corresponding author.
E-mail address: davy.guillarme@unige.ch (D. Guillarme).

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WADA has set very challenging minimum required performance levels (MRPL) for the detection of these compounds in urine, necessitating highly selective and sensitive analytical methods to satisfy these requirements.

Historically, gas chromatography coupled to mass spectrometry (GC–MS) and more recently gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) have been used as reference methods for the analysis of AAS in anti-doping laboratories. Numerous methods involving the use of GC–MS and GC–MS/MS have been reported in the literature [4,5]. The main issue of those methods is the time-consuming sample preparation, which generally includes an enzymatic or chemical hydrolysis step to remove the glucuronide and/or sulfate groups from the phase II metabolites, predominantly present in urine, followed by a liquid–liquid extraction (LLE) and chemical derivatization to ensure sufficient purification, pre-concentration and volatility prior to GC analysis. Some potential drawbacks caused by these chemical reactions are incomplete deconjugation or derivatization, leading to sensitivity decrease or incorrect detection and identification [6]. Moreover, LLE does not allow for easy automation and traditional GC methods can be lengthy.

Because of the limitations mentioned above, various analytical alternatives to GC–MS(/MS) have emerged during the last decade and, among them, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is certainly the most contemplated one. Despite the fact that most anabolic agents have low proton affinities, leading to poor ionization in electrospray (ESI), more and more sensitive methods have been reported for their detection by LC–MS/MS [7–13]. The main advantage of this technique is the direct analysis of such compounds using simpler sample treatment (without derivatization) and faster analytical runs. In addition, the possibility of analyzing intact phase I and phase II metabolites represents a powerful tool to investigate steroids metabolism pathways more deeply [8,13].

On the contrary, supercritical fluid chromatography (SFC) has been scarcely evaluated for doping control analysis up to now. However, this technique has made a remarkable comeback among the analytical chemistry community, thanks to the recent development of new state-of-the-art instruments [14] allowing the use of highly efficient columns (i.e. fully porous sub-2 μm and sub-3 μm superficially porous particles) [15–17]. The performance of such instruments competes with ultra-high performance liquid chromatography (UHPLC), which is the current gold standard for chromatographic analysis [18]. In addition, ultra-high performance supercritical fluid chromatography (UHPSFC), which can be coupled to MS and MS/MS analyzers, has shown to display orthogonal selectivity to UHPLC and can be considered as a complementary technique [19,20]. In this context, SFC may be a suitable strategy for AAS screening in doping control analysis. Indeed, the good solubility of steroids in supercritical CO_2 has turned them into good probe analytes to explore this technique [21,22]. In 1991 already, Berger et al. have used steroids in their evaluation of mobile and stationary phases in SFC [23]. More recently, some studies have dealt with the evaluation of UHPSFC as an improvement or complementary technique to LC and GC for doping control of β2-agonists, stimulants, diuretics and narcotics [24,25], and of glucuronidated and sulfated steroids [26] in urine.

In the present study, two new methods (UHPLC–MS/MS and UHPSFC-MS/MS) were developed for the rapid screening of 43 anabolic agents in human urine. This included the development of a new and semi-automated extraction procedure using supported liquid extraction (SLE) instead of the conventional LLE. The limits of detection (LOD) and matrix effects (ME) were systematically evaluated and compared with the reference method consisting of a LLE followed by GC–MS/MS analysis.

2. Material and methods

2.1. Chemicals and reagents

All substances were kindly provided by the Swiss Laboratory for Doping Analyses (Epalinges, Switzerland). The exhaustive list of 43 target analytes is reported in Table 1 and their structures 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, formic acid, heptane extra dry 99%+, methyl *tert*-butyl ether (MtBE), ethanethiol and ammonium iodide (NH_4I) 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), while helium (99.999%) from Carbagas (Gümligen, Switzerland). Ultra-pure water was provided by a Milli-Q system from Millipore (Bedford, MA, USA). β-glucuronidase from *E. Coli* was purchased from Roche Diagnostics GmbH (Mannhein, Germany). Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium hydrogen carbonate ($NaHCO_3$) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany) whereas sodium carbonate (Na_2CO_3) and sodium hydroxide (NaOH) from VWR Chemicals (Leuven, Belgium). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Duren, Germany).

Phosphate buffer 0.8 M at pH 7.0 was prepared by mixing 57.6 g KH_2PO_4 and 53.5 g K_2HPO_4 in 800 mL of ultra-pure water. If needed, pH was adjusted to 7.0 with NaOH or HCl and the volume was completed to 1000 mL with water. Solid carbonate buffer was prepared by mixing Na_2CO_3 and $NaHCO_3$ 1:10 (w/w).

Derivatizing solution for GC–MS/MS analysis was prepared by mixing 5 mL MSTFA, 100 mg of NH_4I and 150 μL of ethanethiol. This solution was heated at 60 °C and vortexed until complete dissolution. 1 mL of this solution was then added to 9 mL MSTFA to obtain the final derivatizing solution. The components of this mixture lead to the formation of trimethylsilyl iodide (TMSI) which is able to react with hydroxyl groups from the analytes, thus increasing their volatility.

2.2. UHPLC–MS/MS and UHPSFC–MS/MS analyses

2.2.1. Supported liquid extraction

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 water to obtain five different levels of concentration, namely 0.1, 0.5, 1, 5 and 10 ng/mL. 0.2 mL phosphate buffer 0.8 M was added to fix the pH at 7.0 before hydrolysis with 30 μL β-glucuronidase at 50 °C for 1 h. Then, all samples 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 × 1 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 temperature and 30 Torr for 45 min. Then, the samples were reconstituted in 200 μL of a mixture of water and ACN (1:1). After 5 min of agitation, the contents of each well were finally transferred into total recovery vials (Waters, Milford, MA, USA) for injection into UHPLC–MS/MS and UHPSFC–MS/MS.

In addition, post-spiked blank urine extracts were generated using the same procedure to evaluate matrix effects. After SLE