Analytica Chimica Acta 809 (2014) 69–81

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Introduction of solid-phase microextraction as a high-throughput sample preparation tool in laboratory analysis of prohibited substances☆

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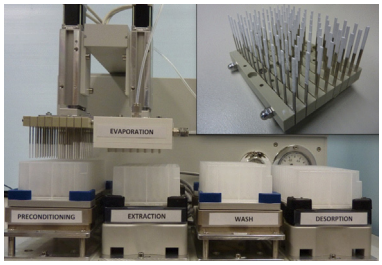
HIGHLIGHTS

- Thin-film SPME–LC–MS protocol offers fully quantitative analysis.
- 110 doping substances and metabolites were quantified.
- C18 coating facilitated extraction of compounds with a wide range of polarities.
- Automated 96-blade system allows high-throughput sample preparation.
- Sample preparation time was 1.7 min/sample.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form
19 November 2013
Accepted 29 November 2013
Available online 7 December 2013

Keywords:
Multiwell high throughput system
Solid phase microextraction
Drugs
Quantitative determinations

ABSTRACT

A fully automated, high-throughput method based on thin-film solid-phase microextraction (SPME) and liquid chromatography-mass spectrometry was developed for simultaneous quantitative analysis of 110 doping compounds, selected from ten classes and varying in physical and chemical properties. Among four tested extraction phases, C18 blades were chosen, as they provided optimum recoveries and the lowest carryover effect. The SPME method was optimized in terms of extraction pH, ionic strength of the sample, washing solution, extraction and desorption times for analysis of urine samples. Chromatographic separation was obtained in reversed-phase model; for detection, two mass spectrometers were used: triple quadrupole and full scan orbitrap. These combinations allowed for selective analysis of targeted compounds, as well as a retrospective study for known and unknown compounds. The developed method was validated according to the Food and Drug Administration (FDA) criteria, taking into account Minimum Required Performance Level (MRPL) values required by the World Anti-Doping Agency (WADA). In addition to analysis of free substances, it was also shown that the proposed method is able to extract the glucuronated forms of the compounds. The developed assay offers fast and reliable analysis of various prohibited substances, an attractive alternative to the standard methods that are currently used in anti-doping laboratories.

☆ Selected Paper presented at the 15th International symposium on Advances In Extraction Sample Preparation for the Omics Age, João Pessoa, PB - BRAZIL, August 2013.

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http://dx.doi.org/10.1016/j.aca.2013.11.056

1. Introduction

The consumption of certain compounds used for performance enhancement can be considered as drug abuse, and thus, a large number of compounds are banned in sport competitions by the World Anti-Doping Agency (WADA), the U.S. Anti-Doping Agency (USADA) and related organizations all over the world. Every year, new compounds and their metabolites are added to the list of prohibited substances, requiring strict control of their consumption by athletes. The diversity of their physico-chemical properties, the necessity for hydrolysis of conjugated forms of analytes, their pharmacology and the required minimum performance limits for determination of those compounds all contribute to make their analysis very challenging [1]. Nowadays, liquid chromatography–mass spectrometry (LC–MS) based analysis methods are increasingly becoming the methods of choice for analysis of these compounds, since most gas chromatography-mass spectrometry (GC–MS) based methods require a derivatization step with hazardous reagents [2–7], in addition to being time consuming. Although high resolution LC–MS and hyphenated liquid chromatography–tandem mass spectrometry (LC–MS/MS) instrumentations provide excellent sensitivity and selectivity, the collected biological samples still require extensive sample preparation before introduction to analytical instruments [8–10]. In effect, simple "dilute and shoot" methods suffer from matrix effects and low sensitivity, providing unreliable results.

The most commonly used sample preparation methods are solid-phase extraction (SPE) [9,11–14] and liquid–liquid extraction (LLE) [15–18]. The definite advantage of SPE, which is frequent method of choice in anti-doping study, is that it can be easily semi-automated and coupled with LC–MS by using 96-well-plates. However, as SPE is considered to be an exhaustive technique, the use of single extraction phase may be limited because of the breakthrough volume of the compound extracted with the lowest efficiency. This situation applies to untargeted screening of doping substances where investigated analytes have a wide range of physico-chemical properties [11]. In such a case different sorbent phases (of different selectivity) may be necessary unless matrix matched calibration is used.

Liquid–liquid extraction methods, in turn, are also widely used because of their great simplicity. In addition, novel multi-well-plate formats allow automation and high-throughput sample preparation. Recently, an automated method based on LLE was reported for detection of 72 doping substances in urine [17]. By using this method the authors were able to process 96 samples in 17 h. However, most LLE methods reported up to date are not automated which makes them time consuming and laborious [15]. Additionally, it has been reported that LLE is characterized by high recovery variability [18]. The authors also stated that LLE is not the best choice for multi-residue analyses of substances varying in physicochemical properties, particularly because of the low extraction efficiency of hydrophilic compounds.

Solid-phase microextraction (SPME) is a well-established sample preparation technique. The extraction mechanism is either based on the distribution coefficient, when equilibrium between the extraction phase and the sample matrix is established, or on a mass transfer rate at pre-equilibrium. SPME is suitable for automation, it requires low consumption of organic solvents, and is a simple, relatively less expensive method, considering the reusability of the sampling devices [19]. However, in LC-based applications carryover on fiber can be an issue and it should be evaluated carefully for reusability of the same fiber. Although the overall protocol of SPME is easy to follow, the method development requires more careful evaluation comparing to standard methods based on exhaustive extraction. As mentioned before, the SPME coating equilibrates with the free fraction of the analytes and therefore any adsorption of the hydrophobic or permanently charged compounds to glass, tubing or other surfaces can affect quantitation by giving false negative results. Additionally, the unconventional calibration approaches used mainly during pre-equilibrium extraction need to be carefully selected. These features contribute to make SPME an ideal alternative to classic exhaustive-extraction procedures for sample preparation; the feasibility of SPME for bioanalytical applications has been demonstrated in the analysis of various drugs and banned compounds from biological samples. For example, Walles and co-workers successfully used restricted access materials-based SPME coatings made of alkyldiol-silica for direct extraction of benzodiazepines from blood samples [20]. Vuckovic et al. prepared biocompatible SPME coatings using octadecyl, polar-embedded and cyano particles; these coatings were then used for the extraction of carbamazepine, propranolol, pseudoephedrine, ranitidine and diazepam from plasma and urine samples [21], without the requirement of a sample pre-treatment step. In another study, various particles considered as solid-phase extraction media were used by the same authors in the preparation of SPME coatings, and evaluated in terms of extraction of a wide polarity range of compounds (log P range of −7.9 to 7.4) from biological matrices [22].

Control of prohibited substances in biological fluids during sport competitions generates numerous samples that must be analyzed in a short time; as such, a high-throughput analysis in doping control is of major importance. Recently, a LC-focused high-throughput SPME system was introduced [23–26], combining the advantages of thin-film geometry: improved surface area, and simultaneous extraction of 96 individual samples. The thin-film geometry of the SPME device allows for high recovery extraction from biological matrices without the risk of clogging, due to the open bed format of the technology [26]. SPME extraction takes place in a 96-well-plate, and full automation can be achieved when a new SPME robotic station is used [26,27].

There have been studies published that demonstrate the applicability of SPME for analysis of doping compounds [28–32]; however, up until the publication of this study, the selection of target compounds for analysis had been limited to few substances. Recently, Zhang et al. used laboratory prepared SPME for the determination of 3α-hydroxy-5α-androstane-17-one, dihydrotestosterone, androstenedione and methyltestosterone in pig urine [33]. Another demonstration of the successful application of SPME for analysis of doping compounds, by Aresta et al., was the determination of the beta-adrenergic drug clenbuterol in urine and serum, using a polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated fiber, followed by LC-UV detection [29].

Since there are a number of compounds that can be potentially used as performance enhancers by sportsmen, the chosen method should be able to screen as many drugs of interest as possible in one single analysis, without sacrificing sensitivity or time length of analysis. In this study, an automated thin-film microextraction method was developed and validated for the quantification of more than 100 compounds from 10 different classes of compounds banned by WADA. Four coatings were evaluated in terms of extraction efficiency and carryover. The feasibility of the method for determination of both conjugated and free forms of compounds from urine is demonstrated, with analytical figures of merits that comply with both the requirements of WADA, and the FDA.

2. Experimental

2.1. Chemicals and materials

Standards of target doping substances, metabolites, glucuronated standards and deuterated internal standards were