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High throughput quantification of prohibited substances in plasma using thin film solid phase microextraction☆

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ABSTRACT

Simple, fast and efficient sample preparation approaches that allow high-throughput isolation of various compounds from complex matrices are highly desired in bioanalysis. Particularly sought are methods that can, without sacrificing time, easily remove matrix interferences capable of inducing ionization suppression/enhancement, or causing detrimental effects in instrumental performance. In this work, an automated high-throughput sample preparation method using thin film solid phase microextraction (SPME) for the analysis of multiple prohibited substances in plasma is proposed. A biocompatible SPME extraction phase made of hydrophilic–lipophilic balance particles immobilized with polyacrylonitrile (PAN) demonstrated satisfactory extraction capabilities for 25 compounds of a wide range of polarities (log *P* from −2 to 6.8). Due to the well-known biocompatible characteristics of PAN-based SPME coatings, minimum sample handling was required. Experimental conditions for pre-conditioning, extraction, wash and desorption were carefully optimized for the proposed method. By taking full advantage of the 96 thin film handling capability of the automated system, a preparation time of approximately 1.5 min per sample can be achieved. Satisfactory results in terms of absolute matrix effects were found for the majority of the studied analytes, given that 24 out of 25 compounds exhibited values in the range of 100 and 120%. The method was validated in terms of linearity ($R^2 > 0.99$), inter and intra-day accuracy (85–130%) and precision (<20%) and limits of quantitation (0.25–10 ng mL^{-1} for most compounds).

1. Introduction

One of the ultimate goals in doping control is the development of a simple, fast, reliable and comprehensive analytical methods for biological matrices such as blood and urine. Due to the complexity of such matrices, as well as the diversity of prohibited substances listed by the World Antidoping Agency (WADA), sample preparation is often a challenging task [1]. Currently, WADA has stipulated minimum required performance levels only for the detection and identification of prohibited substances in urine samples [2]. However, analytical determinations in blood (plasma/serum) as a means to obtain complementary information to urinalysis results have been garnering a wealth of interest [3–7]. Some advantages of blood analysis in doping control include finding intact unknown doping substances, determining temporal information regarding drugs prohibited in-competition only, and detecting if an athlete is participating in blood doping practices [3,6,7]. Sample preparation procedures reported for analysis of prohibited substances in blood, serum, and plasma include protein precipitation, solid phase extraction (SPE) and more recently, dried blood spot (DBS) [3,8–11]. Several studies using these sample preparation methods for comprehensive screening of human and equine plasma/blood have been reported in recent years [4,5,12–18]. Despite these approaches being effective, they can be time-consuming, unsuitable in some cases for automation, and prone to ion suppression/enhancement effects. Regarding instrumentation, undoubtedly, liquid chromatography coupled to mass spectrometry (LC–MS) has become the preferred method in sports drug testing due to its flexibility compared to gas chromatography–mass spectrometry (GC–MS) and to immunological assays [7,19–21]. For these reasons, simple and effective sample preparation protocols suitable for automation and compatible with LC–MS based methods are highly desired. Recently, the introduction of thin film solid phase microextraction (SPME) in an automated configuration has opened up a new alternative in sample preparation for bioanalysis. Biocompatible SPME coatings prepared by immobilizing various sorbents with polyacrylonitrile have demonstrated great performance in the extraction of drugs from complex matrices.

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By taking advantage of this technology, a comprehensive protocol for automated quantitative urinalysis of doping agents was recently introduced [22]. In that work, more than 100 compounds of different classes and polarities were simultaneously extracted from urine samples using a C18-polyacrylonitrile (PAN) extraction phase and the Concept 96 automated sample preparation station. By using the 96 SPME thin films that the aforementioned system can handle, the optimized method was able to provide throughput of less than 2 min per sample. Furthermore, the proposed automated SPME method allowed satisfactory sample clean-up since negligible absolute matrix effects were observed for the majority of the compounds. Considering the good performance of the proposed method, and by taking advantage of the biocompatibility of the SPME extraction phases, a new high throughput SPME-based protocol for plasma analysis is introduced herein. Twenty-five compounds of a wide range of polarities (log *P* from −2 to 6.8), including different prohibited drug classes and some metabolites such as benzoylecgonine, morphine-3β, and 6β glucuronide, were selected for this study. Given that SPME only extracts an amount of analyte proportional to its free concentration, the method was carefully optimized, taking into account the binding that some compounds might experience due to the high protein content of plasma. For this purpose, simultaneous SPME pre-conditioning and sample pre-incubation under a controlled temperature were enabled by modifying the software of the Concept-96 autosampler. With the aim of covering a broad range of compounds in a single extraction, a thin film SPME coating made of hydrophilic–lipophilic balance (HLB) Oasis particles immobilized with polyacrylonitrile (PAN) was chosen. To the best of our knowledge, this is the first time that such SPME coating is used in a multi-residue bioanalytical application.

2. Experimental

2.1. Materials and supplies

Amphetamine, methamphetamine, 17-α-trenbolone, morphine, benzoylecgonine, codeine, codeine-d_3, oxycodone-d_3, cannabidiol-d_3, methadone-d_3, stanozolol, (±)11-nor-9-carboxy-Δ^9-THC (THCCOOH), (±)11-nor-9-carboxy-Δ^9-THC-d_3 (THCCOOH-d_3), cortisol-d_4, morphine-3β-glucuronide, morphine-6β-glucuronide, morphine-3β-glucuronide-d_3, (±)11-nor-9-carboxy-Δ^9-THC glucuronide (THCCOOH-glu), and (±)11-nor-9-carboxy-Δ^9-THC glucuronide-d_3 (THCCOOH-glu-d_3) standards were purchased from Cerilliant Corporation (Round Rock, TX, USA). Nikethamide, propanolol, metoprolol, clenbuterol, exemestane, bisoprolol, budenoside, dexamethasone, furosemide, salbutamol, prednisolone, strychnine and testosterone-d_3 were purchased from Sigma–Aldrich (Oakville, ON, Canada). Salbutamol-d_3 was purchased from CDN isotopes (Pointe-Claire, Quebec, Canada). Toremifene and GW501516 were purchased from Toronto Research Chemical (Toronto, ON, Canada).

Sodium chloride, potassium chloride, potassium phosphate monobasic, sodium phosphate dibasic, formic acid, and polyacrylonitrile (PAN) were also purchased from Sigma–Aldrich (Oakville, ON, Canada). N,N-dimethyl formamide (DMF) was purchased from Caledon Labs (Georgetown, ON, Canada). Modified polystyrene divinylbenzene (PS-DVB) was obtained from Macherey-Nagel (Düren, Germany), Oasis hydrophilic–lipophilic balance 30 μm sorbent particles (HLB) were obtained from Waters (Milford, MA, USA), and Discovery silica-based C18 5 μm particles were obtained from Supelco (Bellefonte, PA, USA). Polypropylene Nunc U96 deep well plates were purchased from VWR international (Mississauga, ON, Canada) and bare stainless steel blades were obtained from Professional Analytical System (PAS) Technology (Magdala, Germany). LC–MS grade acetonitrile, methanol, and water were obtained from Fischer Scientific.

2.2. Working solutions

A stock methanolic solution (20 μg mL^{-1}) containing all analytes was prepared and further dilutions were done as required. A stock solution (8 μg mL^{-1}) containing multiple deuterated compounds as internal standards was prepared in methanol.

2.3. Plasma samples

Different lots of potassium (K_2) ethylenediaminetetraacetic acid (EDTA) pooled human plasma from healthy donors were purchased from Lampire Biological Laboratories (Pipersville, PA, USA). A phosphate-buffered saline solution (pH 7.4) was prepared according to the procedure described in the supplementary information.

2.4. Thin film SPME devices

Various coating chemistries in thin film SPME format were prepared by immobilizing different SPE sorbents (C18, PS-DVB and HLB) with a PAN-DMF solution according to the procedure already reported by Mirnaghi et al. [23]. The only exception to the original protocol was the curing temperature, which was decreased from 180 to 150 °C.

2.5. Sample preparation: automated Concept 96-blade SPME system

Automated SPME extractions were carried out using the Concept-96 system (Professional Analytical Systems (PAS) Technology, Magdala, Germany). This robotic sample preparation unit has been described in detail elsewhere [24,25]. A typical SPME protocol using this automated station involves preconditioning, extraction, washing and desorption steps. In this work, a simple modification of the controlling software allowed for the simultaneous pre-incubation of samples at a given temperature (extraction station) and pre-conditioning of SPME devices (preconditioning station).

The SPME method was developed and optimized in terms of coating selection, pH control, extraction time and temperature, type of desorption solvent used, and desorption duration. A recent work on doping control using SPME demonstrated the suitability of C18 coatings for the extraction of a wide range of doping substances from urine samples [22]. Due to the high protein content in plasma, and consequently the decrease of free concentrations of drugs that typically exhibit high protein binding, it was critical to evaluate the performance of different coating chemistries at such conditions. For optimization of the SPME method, both plasma and PBS standards were prepared by spiking analytes from stock solutions, keeping the organic solvent content constant at 1%. Spiked plasma aliquots were pre-incubated in the fridge overnight to allow complete binding before extraction. Sample preparation involved mixing 1080 μL spiked plasma aliquots with 10 μL internal standard solution and 120 μL of 1 M phosphate buffer (pH = 7). Subsequently, samples were homogenized in the 96-well plate at constant agitation for 30 min before starting the SPME procedure. Optimum SPME conditions were set as follows: pre-conditioning of SPME devices in 1:1 methanol:water (1500 μL) for 30 min and simultaneous plasma samples pre-incubation at 30 °C, then 90 min extraction at 30 °C, 10 s washing step in deionized water (1500 μL), and 20 min desorption in 4:1 methanol:acetonitrile with 0.1% formic acid (1200 μL). Agitation rate was set at 1500 rpm. It is worth emphasizing that the total incubation time of plasma aliquots after adding buffer and internal standard solution was 1 h.