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Comparative evaluation of seven different sample treatment approaches for large-scale multiclass sport drug testing in urine by liquid chromatography–mass spectrometry

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ABSTRACT

Sample preparation is a critical step in large-scale multiclass analysis such as sport drug testing. Due to the wide heterogeneity of the analytes and the complexity of the matrix, the selection of a correct sample preparation method is essential, looking for a compromise between good recoveries for most of the analytes and cleanliness of the extract. In the present work, seven sample preparation procedures based on solid-phase extraction (SPE) (with 5 different cartridges), liquid–liquid extraction (LLE) and sorbent-supported liquid extraction (SLE) were evaluated for multiclass sport drug testing in urine. The selected SPE sorbents were polymeric cartridges Agilent PLEXA™ and Oasis HLB™, mixed mode cation and anion exchange cartridges Oasis MAX™ and MCX™, and C18 cartridges. LLE was performed using *tert*-butyl methyl ether and SLE was carried out using Agilent Chem Elut™ cartridges. To evaluate the proposed extraction procedures, a list of 189 compounds were selected as representative from different groups of doping agents, including 34 steroids, 14 glucocorticosteroids, 24 diuretics and masking agents, 11 stimulants, 9 beta-agonist, 16 beta-blockers, 6 Selective Estrogen Receptors Modulators (SERMs), 24 narcotics and 22 other drugs of abuse/sport drugs. Blank urine samples were spiked at two levels of concentration, 2.5 and 25 $\mu g\,L^{-1}$ and extracted with the different extraction protocols ($n = 6$). The analysis of the extracts was carried out by liquid chromatography electrospray time-of-flight mass spectrometry. The use of solid-phase extraction with polymer cartridges provided high recoveries for most of the analytes tested and was found the more suitable method for this type of application given the additional advantages such as low sample and solvent consumption along with increased automation and throughput.

1. Introduction

Sample preparation is a critical step that must be carefully studied in large-scale multiclass analysis such as sport drug testing, which is usually associated with liquid chromatography mass spectrometry-based determination [1]. The wide heterogeneity of the analytes (in terms of polarity, pK_a or size) and the complexity of the matrices, makes the sample preparation step critical. It is essential to obtain a compromise between good recoveries for most of the analytes and cleanliness of the extract. In the case of doping analysis in urine, the sample preparation methods can be grouped into 3 basic methodologies: liquid–liquid extraction (LLE) with different solvents, solid-phase extraction (SPE) using different classes of sorbents, and "dilute-and-shoot" methods [2–19].

LLE has been widely used for the analysis of sport drugs in urine, using different extraction solvents as *tert*-butyl methyl ether [2–4] or diethyl ether [5–10]. This extraction protocols produce clean extracts, but have the inconvenience of the limited number of compounds that are easily extractable, since many of the doping drugs have polar groups in their structure, so this extraction procedure is normally used for the analysis of small group of compounds, specially steroids [6,7,9], corticosteroids [4,5] or β_2-agonists and β-blockers [2,3], although some authors have developed multi-class screening methods using LLE [8,10].

Dilute-and-shoot methods are based on the dilution of the urine sample with an appropriate solvent, reducing the matrix effect [16–19]. This technique has the advantage of the fast and cheap sample preparation, without the use of solid sorbents or expensive solvents, but has some drawbacks such as reduced analyte detectability (because increase of limits of detection due to the dilution factor – and thus the absence of preconcentration step, and also due to remarkable matrix effects if the dilution factor is

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too low), retention time shifts which reduce identification capabilities and also peak shape [16]. The dilution fold varies between 1:1 [11–13,17,19] – usually involving higher matrix effects, and 1:10 [14,18] or even 1:25 [15,16], in order to reduce matrix effects. Most of the dilute-and-shoot methods that work nicely are intended for easily ionizable substances for which the required detection levels in urine are high [16].

SPE presents certain advantages *versus* LLE, as the use of a lower volume of more environmentally friendly solvents, the ease of automation and the commercial availability of different sorbents for general or certain applications. Mixed mode cartridges have been widely used for urine analysis [20–24] since they have a polymeric or C18 sorbent, which is also bonded with ion exchange groups, thus provide a dual retention mechanism. C18 cartridges are used for high throughput methods [25] due to their ease of use because their non-polar retention mechanism, but are not recommended for polar compounds because recovery rates are low for these compounds. This is solved when using polymeric cartridges, that are filled with a polymeric sorbent with a reverse-phase retention mechanism designed for neutral, basic and acidic analytes, obtaining good recoveries for compounds from different groups and even glucuronide-conjugated metabolites [26–28]. Marchi et al. made a comparative study of different extraction procedures with polymeric and mixed-mode ion exchange cartridges [29], but limited to a group of 34 multiclass sport drugs in urine.

The aim of the present work is to evaluate the suitability, usefulness and performance of seven different extraction protocols based on SPE (with different sorbents), LLE and solid-supported liquid extraction (SLE) for large-scale multiclass sport drug testing in urine. The efficiency of the different extraction procedures in terms of recovery/extraction efficiency and accuracy was tested for up to 189 doping agents, which were selected as representative from different groups including 34 steroids and related metabolites, 14 glucocorticosteroids and related metabolites, 24 diuretics and masking agents, 11 stimulants and related metabolites, 9 beta-agonists, 16 beta-blockers, 6 Selective Estrogen Receptors Modulators (SERMs), 24 narcotics, cannabinoids and metabolites, and 22 related multiclass drugs of abuse/sport drugs.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile, methanol and methyl *tert*-butyl ether (MTBE) were obtained from Sigma–Aldrich (Madrid, Spain). Drug analytical standards of the 189 species included in the study were purchased from Cerilliant (Round Rock, TX), Dr. Ehrenstorfer (Madrid, Spain), European Pharmacopeia, National Measurement Institute (Australia) and Sigma–Aldrich (Madrid, Spain). Individual stock solutions were prepared in methanol and were stored at −18 °C. Formic acid, hydrochloric acid, ammonium hydroxide, sodium acetate, acetic acid, sodium chloride and sodium hydroxide were purchased from Fluka (Madrid, Spain). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC water used during the analyses and to prepare all the solutions.

Bond Elut PLEXA™ SPE cartridges (200 mg, 6 mL) and Chem-Elut™ SLE cartridges (6 mL, unbuffered) were purchased from Agilent Technologies (Santa Clara, CA). Oasis HLB® (200 mg, 6 mL), Oasis MAX™ (150 mg, 6 mL) and Oasis MCX™ (150 mg, 6 mL) SPE cartridges were acquired from Waters (Millford, MA). Discovery DSC-18™ SPE cartridges (500 mg, 6 mL) were purchased from Supelco (Bellefonte, PA). A Supelco (Bellefonte, PA) Visiprep™ SPE vacuum system was used for SPE experiments and a TurboVap LV nitrogen evaporator from Zymark (Hopkinton, MA) was used for solvent evaporation.

2.2. LC–TOFMS analysis

The separation of the analytes from the urine extract was carried out using an ultra-high-performance pressure liquid chromatography (UHPLC) system (consisting of vacuum degasser, auto sampler and a binary pump) (Agilent Infinity 1290, Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase XDB-C18 rapid resolution analytical column of 4.6 mm × 50 mm and 1.8 μm particle size (Agilent Technologies, Santa Clara, CA). 20 μL of the extract were injected in each run. Mobile phases A and B were water with 0.1% formic acid and acetonitrile. The chromatographic method held the initial mobile phase composition (10% B) constant for 3 min, followed by a linear gradient to 100% B up to 15 min and kept for 3 min at 100% B. The flow rate used was 0.5 mL min^{-1}. The UHPLC system was connected to a time-of-flight mass spectrometer Agilent 6220 accurate mass TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating either in positive or negative ion mode, using the following operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas flow rate, 9.0 L min^{-1}; gas temperature, 325 °C; skimmer voltage, 65 V; octapole 1 rf, 250 V; fragmentor voltage (in-source CID fragmentation) 190 V. Although the use of fast polarity switching mode is enabled in this type of instrumentation, a dedicated run was performed in each ionization mode. LC–MS accurate mass spectra were recorded across the range of *m/z* 50–1000 in positive ion mode, and *m/z* 50–1100 in negative ion mode. The instrument performed an internal calibration using the reference mixture provided by the manufacturer over the range 50–1100 *m/z* using a second sprayer with a reference solution containing the masses purine (*m/z* 121.0509) and hexakis-(1H,1H,3H-tetrafluoropropoxy)phosphazine (HP-921) ($C_{18}H_{18}O_6N_3P_3F_{24}$, *m/z* 922.0098). The instrument was operated in the 4 GHz-high-resolution mode, providing a typical resolution of *ca.* 19,000 at *m/z* 922. The full scan data were recorded with Agilent Mass Hunter Data Acquisition software (version B.04.00) and processed with Agilent Mass Hunter Qualitative Analysis software (version B.04.00). The details of the identification parameters used for the 189 species (elemental composition, accurate mass, ionization mode and retention time) are detailed in Table S-1 (Suppl. Data).

2.3. Sample preparation procedures

2.3.1. SPE Bond Elut PLEXA polymeric cartridges

The cartridges were preconditioned with 4 mL of MeOH/MeCN (1:1) and 4 mL of HPLC grade MilliQ water. After the conditioning step, 2 mL of spiked urine buffered with 2 mL of formic acid/formate (50 mM) at pH 2.6 were passed through the SPE cartridge. 4 mL of 5% MeOH in MilliQ water were then added to rinse the cartridge prior to elution. The cartridges were dried under vacuum in order to remove the excess of water. The analytes were finally eluted with 4 mL of MeOH/MeCN (1:1 (v/v)). The extracts were evaporated to near dryness using a Turbo Vap LV from Zymark (Hopkinton, MA), with a water bath temperature of 37 °C and a N_2 pressure of 15 psi. The samples were then taken up with 0.5 mL of MeOH/water (10:90 (v/v)) (preconcentration factor: 4:1). The reconstituted extracts were filtered through a 0.45 μm syringe filter and then transferred to a 2-mL glass vial prior to LC–MS analysis.

2.3.2. SPE Oasis HLB and SPE Discovery DSC-18 cartridges

4 mL of MeOH and 4 mL of HPLC grade MilliQ water were added to the cartridges for conditioning. After the conditioning step, 2 mL of spiked urine buffered with 2 mL of formic acid/formate (50 mM) at pH 2.6 were passed through the SPE cartridge. The cartridges were then rinsed with 4 mL of 5% MeOH in MilliQ water and dried under vacuum. The analytes were eluted with 4 mL of MeOH. The