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Sample preparation of urine samples for liquid chromatography–tandem mass spectrometry using functionalized ferromagnetic beads

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Abstract

Several classes of doping substances such as corticosteroids, diuretics, stimulants, anabolic steroids, narcotics, selective androgen receptor modulators (SARMs), peroxisome proliferator–activated receptor agonists (PPARs) were investigated by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using different methods of sample preparation. A new extraction procedure for small molecules from urine was proposed. This method is based on using functionalized ferromagnetic beads (FFB) with a C18-modified surface. Comparison of FFB with traditional extraction methods e.g. solid phase extraction (SPE) and liquid-liquid extraction (LLE) under optimal conditions showed that FFB was superior for extraction of different doping substances from complex sample matrices like urine because of lower ion-suppression effects and higher recoveries. Additional advantages of FFB are simplicity, rapidity (5 minutes per sample) and possibility of automation.

Introduction

LC–MS/MS has opened new horizons for doping control, and this technology allows the straightforward development of versatile routine methods. Some steps for sample clean-up prior to LC–MS/MS analysis are mandatory. Ion-suppression effects lead to a significant decrease in method sensitivity. SPE or LLE are techniques typically used for analyte concentration and elimination of matrix background. These traditional methods have common disadvantages such as slowness of sample preparation, difficulty of automation (LLE) and low recovery and selectivity. LLE requires large quantity of organic solvents, and SPE cartridges require complex mechanical and/or pneumatic handling.

An alternative extraction method of doping substances from urine could be a magnetic separation method. This method is based on using functionalized ferromagnetic beads. These micro-particles with defined surface properties acting as solid phase for extraction are ideally suited for automation since this "solid phase" can be manipulated as a liquid [1]. Methods for DNA purification based on FFB have been extensively used in clinical laboratories. Today this principle of extraction is applied for peptide isolation and concentration in automated immunoassay system. Extraction protocols based on the use of FFB have successfully been automated for serum protein profiling in a non-quantifying approach for MALDI–TOF [2-3]. So far, however, FFB have not been applied for sample preparation for small molecules extracted from complex urine matrices.

Therefore the aim of our study was to investigate the applicability of FFB for doping control purposes and to compare it with "traditional" means of sample preparation (LLE, SPE).

Materials and Methods

LLE procedure: 10 µL of internal standards solution (fluoxymesterone 10 µg/mL, mefruside 10 µg/mL, methyltestosterone 10 µg/mL) was added to 1 mL urine followed by addition of 100 mg $K_2CO_3/KHCO_3$ (2:1) to adjust pH to 9.5. LLE was performed for 10 min by rolling with 3 mL diethyl ether in presence of 0.5 g ammonium sulphate. After centrifugation (3000 rpm) the organic layer was evaporated to dryness. The remaining residue was reconstituted in 100 µL of the initial mobile phase.

SPE procedure: 1mL of urine, added with 10 µL of internal standards solution were passed through a Bond Elute-Certify, 130 mg × 3 mL, Varian (previously activated by 3mL of MeOH and 3mL of H_2O) and then eluted, after washing with 3mL of H_2O, with 3mL of MTBE. The eluate was evaporated to dryness under a stream of N_2 at T=40ºC. The residue was reconstituted in 100 µL of initial mobile phase.

FFB procedure: Urine samples were extracted using a C18-functionalized ferromagnetic micro-particles (Dynal Dynabeads RPC18, Invitrogen, Norway; 12.5 mg/mL; mean particle diameter 1 µm). The protocol for the preparation of urine samples was as follows: 10 µL of an internal standard solution was pipetted into 1.5 mL reaction tube, and 1 mL of urine was added. Then 200 µL of the magnetic particle working suspension ([c]=1.25 µg/µL) was added; after mixing analyte adsorption to the extraction material was allowed for two minutes. The sample matrix was then removed with a pipette tip after again inserting the

magnet into the separator device. The magnet was removed, 500 µL of H_2O was added for washing, and after magnetic separation of the particles the H_2O was discarded. For analyte elution, 100 µL of mixture CH_3OH/H_2O (50/50 v/v) was added; particles were re-suspended and finally separated again to allow the transfer of the extract into LC vials.

LC–MS/MS analysis of the extracts was performed using a TSQ Quantum Ultra instrument with a heated ESI ion source connected. *Test for ion suppression effects.* 50 µL of the solution of the mixture of analytes was evaporated to dryness in a nitrogen flow. The dry residue was dissolved in 100 µL of initial mobile phase or in 100 µL of an extract obtained by LLE, SPE or FFB procedure from urine. The samples were analyzed and the intensities of the respective characteristic ions were compared.

Results and Discussion

For the first time a new extraction procedure using ferromagnetic beads with a C18-modified surface was proposed for detection of small molecules in urine matrix. During optimization of the FFB procedure good extraction recovery was observed when small quantities of the magnetic particles were applied. While evaluating the optimal condition for LLE, diethyl ether was found the best choice in combination with ammonium sulphate at pH=9.5. After evaluation of SPE cartridges and conditions the optimal selection was Bond Elute-Certify cartridges (130 mg × 3mL, Varian) and elution using MTBE.

Comparison of FFB with extraction methods such as SPE and LLE after their optimization showed that FFB was best way for extraction of different classes of doping substances from a complex matrix like urine due to lower ion-suppression effects and higher recoveries (Table 1). Probably this is due to the intensive interaction of the extraction material which was dispersed into sample in case FFB, in contrast to a weaker interaction within packed extraction cartridges.

The basic advantages of magnetic separation are following: minimized handling of solid consumables, minimized volumes of extraction fluids, and no technically demanding application of vacuum or pressure. In that way we have demonstrated that FFB can be used for highly efficient extraction of small molecule analytes from urine for analysis by LC–MS/MS.

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«Investigation of a new doping substances and development of the methodology for their determination using state-of-the-art analytical instrumentation».

Table 1. Comparison of different sample preparation methods.

Subtances	Recovery, %				Ion-suppression effects, %		
	LLE		SPE	FFB	LLE	SPE	FFB
Corticosteroids	68–81		74–88	85–92	17–38	11–24	4–9
	Exeptions: Metabolite of fluticasone propionate	43					
Diuretics	62–77		79–84	83–91	8–37	8–29	5–15
	Exeptions:						
	Acetazolamide	23					
	Amiloride	21					
Stimulants	58–82		65–84	77–89	13–25	11–17	7–11
	Exeptions:						
	Heptaminol	13					
	Etilefrine	17					
	Norfenephrine	15					
	Prenylamine	13					
	Amiphenazole	26					
	Oxilofrine	17					
	Etilefrine	17					
Anabolic steroids	72–88		81–92	91–95	4–15	5–11	4–7
SARMs	67–76		83–87	95–97	11–32	3–8	3–5
PPARs	73–78		82–87	95–98	9–12	3–7	1–5
Narcotics	60–83		76–89	88–94	3–17	5–9	3–7

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