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Matrix effects in human urine analysis using multi-targeted liquid chromatography–tandem mass spectrometry

Linda Schlittenbauer, Bettina Seiwert, Thorsten Reemtsma*

Helmholtz Centre for Environmental Research – UFZ, Department of Analytical Chemistry, Permoserstrasse 15, 04318 Leipzig, Germany

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ABSTRACT

Different sample preparation methods were tested for human urine for the subsequent analysis with a LC–MS/MS multimethod to quantify 65 micropollutants (pesticides, pharmaceuticals, personal care products, industrial chemicals and metabolites) within the general population. Direct injection of diluted urine revealed highly variable and often severe signal suppression for nearly all analytes at relevant concentration levels during electrospray ionization. Urine samples were highly variable in their total organic carbon (500–10,000 mg L^{-1}) and creatinine (0.35–13 mM) content as well as in electrical conductivity (3–19 mS cm^{-1}) but these differences did not correlate clearly with the strength of matrix effects. Therefore, matrix removal by solid phase extraction was intended and different mixed-mode sorbents were tested. Results showed poor apparent recoveries likely due to insufficient separation of target analytes and matrix compounds for all tested sorbents. The wide variability and high concentration levels of urine constituents strongly affect electrospray ionization as well as recovery during extraction at the sub-microgram per liter level. Especially, the hydrophobic interaction at the hydrophilic–lipophilic-balanced sorbent was affected. It is concluded that the urine matrix is too strong, too diverse and too variable to allow one sample preparation method for the very diverse analytes of a LC–MS/MS multimethod. Instead dedicated methods appear more promising.

1. Introduction

In the past years liquid chromatography–tandem mass spectrometry (LC–MS/MS) multimethods that allow for the quantification of a large number of chemically diverse analytes became increasingly popular. This includes methods for analyzing drinking, surface and waste water [1–3], sewage sludge [4] as well as biological fluids like urine [5,6] and blood [7]. Here, quantification methods by multiple reaction monitoring (MRM)-detection for urine analysis focus on specific analyte classes. Shin et al. [5] compared different quantification approaches for the detection of 35 drugs of abuse and relevant metabolites. In total 204 pesticides were analyzed by Cazorla-Reyes et al. [6] by one single extraction method and detected by a GC–IT-MS/MS (117 analytes) or UHPLC–QqQ-MS/MS method (87 analytes), respectively. The advantage of multimethods is the fast generation of quantitative data for a large number of analytes in one single analysis. This is indeed very useful in biomonitoring studies where often large numbers of samples have to be analyzed, namely in epidemiological studies.

Depending on the sample matrix and the concentration of analytes a specific sample preparation method may be needed before analysis, such as dilution [8], protein precipitation [9] or extraction [10,11]. The latter allow for the enrichment of analytes as well as for the removal of matrix compounds that would otherwise disturb the LC–MS/MS analysis, its sensitivity or robustness. Because endogenous sample constituents may affect the signal of a co-eluting analyte, these matrix effects (ME) have to be checked in method development. For the analysis of biological samples liquid–liquid- (LLE) and solid phase extraction (SPE) are often used sample preparation methods [12,13]. Especially, mixed-mode sorbents are widely used for the enrichment of analytes from complex biological samples [14].

This study aimed at investigating options of sample preparation that are necessary to analyse urine samples by an LC–MS/MS multimethod for polar environmental contaminants. This multimethod comprised different compound classes including pesticides, industrial chemicals, pharmaceuticals and personal care products. Matrix effects after sample dilution were investigated within a set of 20 urine samples and indicated the need for matrix removal. Therefore, one mixed-mode sorbent should be selected suitable for the simultaneous extraction of a large number of chemically diverse analytes from urine samples at a concentration range adequate

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* Corresponding author.
E-mail address: thorsten.reemtsma@ufz.de (T. Reemtsma).

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to biomonitoring of the general population. This extraction should provide good recovery for a large number of analytes and remove sufficient matrix to reduce matrix effects to an acceptable range.

2. Materials and methods

2.1. Chemicals and materials

Analyte standards were purchased of analytical grade (details see Table S1). Methanol, acetonitrile, ultrapure water, ammonium acetate and ammonium formiate (all ULC-MS grade) were obtained from Biosolve B.V. (Valkenswaard, Netherlands) and used for UPLC–MS/MS analysis and sample preparation. Hydrochloric acid suprapure (30%) and ammonium hydroxide p.a. (25%) were purchased from Merck (Darmstadt, Germany). Creatinine, 1-octansulfonic acid sodium, sulfuric acid and methanol for HPLC-UV analysis were obtained from Sigma–Aldrich (Steinheim, Germany). Potassium hydrogen phthalate, potassium nitrate, ammonium sulfate, phosphoric acid and hydrochloric acid for TOC/TN analysis were purchased from Merck (Darmstadt, Germany).

Stock standard solutions of analytes were prepared in methanol, acetonitrile or water/methanol at 1 mg mL^{-1} and stored at −20 °C until use. Stock standard solutions of 5–20 analytes were combined and diluted with ultrapure water to mixed standard solution and afterwards further combined to a mixture including all analytes with concentrations of around 1 μg L^{-1}.

SPE cartridges (30 mg, 1 cc) and 96-well plates (10 mg) were purchased from Waters (Eschborn, Germany).

Urine spot samples were obtained from healthy volunteers and stored at −20 °C until analysis. For preparation of urine mixture samples were thawed at room temperature, centrifuged (10 min at 4000 rpm) and equal aliquots were combined. The urine mixture was stored at −20 °C and used for all extraction experiments.

2.2. UPLC–MS/MS analysis

Chromatographic separation was performed on an UPLC™ system (Acquity i-Class, Waters) consisting of a binary solvent manager, a column manager with preheater and a sample manager equipped with a 250 μL extension loop and a 500 μL sample syringe. During all analysis 100 μL of sample were injected on an Acquity UPLC HSS T3 column (100 mm × 1.8 mm, 1.7 μm) equipped with a 0.2 μm pre-column filter at a column temperature of 60 °C and a flow rate of 600 μL min^{-1}. The mobile phase was ultrapure water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The gradient was as follows: 0 min 0.5% B, 2 min 0.5% B, 7.21 min 99.9% B, 10.21 min 99.9% B, 10.31 min 0.5% B, 12 min 0.5% B. The LC system was coupled to a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Cooperation) equipped with an electrospray ionization (ESI) source. The mobile phase was diverted to waste for the first 2.5 min of the MS acquisition in order to prevent salt deposition in the ion source. Analytes were detected by multi-reaction monitoring (MRM) mode with positive and negative ESI switching within one run. Capillary voltage was set to 2 kV for positive and negative ionization mode. Desolvation gas (nitrogen) was delivered at 1000 L h^{-1} and 600 °C. Flow rate of cone gas was set to 150 L h^{-1} and source temperature to 120 °C. For each analyte two MRM-transitions were selected, where one was used for quantification (Q) and one for confirmation (q) (Table S1). Analytes were positively confirmed if both MRM-transitions were present and the ion ratios (Q/q) were between 80 and 120% compared to the standard in ultrapure water. For LC–MS/MS analysis data acquisition, data handling and instrument control were performed with MassLynx® software (Waters).

2.3. Limits of quantification (LOQ)

Seven point calibrations were performed with concentrations ranging from 1.2 to 300 ng L^{-1} in ultrapure water and from 6 to 1500 ng L^{-1} in human urine of all analytes. For calculation of LOQs in urine the samples from three individuals with varying creatinine concentration were used and diluted with ultrapure water (1:5, v:v) prior analysis. Lowest acceptable calibration point was used as LOQs. Calibration points were accepted in which the response factor did not deviate by more than ±25% from the average response factor of that analyte.

2.4. Matrix effects by UPLC–MS/MS

Matrix effects of urine samples (20 human urine samples) were calculated relative to ultrapure water according to Eq. (1) (adopted from [2]). Response factor (RF) in urine samples were obtained from addition of analytes at a level of 1 μg L^{-1} and 1:5-dilution with ultrapure water (dilution factor [DF] = 5) before analysis. For RF_{water} average response factor from a seven point calibration between 10 and 1000 ng L^{-1} were used.

$$\text{ME} = \left(1 - \frac{\text{RF}_{\text{urine}} \times \text{DF}}{\text{RF}_{\text{water}}}\right) \times 100 \tag{1}$$

2.5. Creatinine concentration by HPLC-UV

The creatinine concentration was determined by ion pair-LC using UV detection on a HPLC 1100 Series coupled to a diode array detector (both Agilent Technologies). For sample dilution and eluent preparation 10 mM 1-octansulfonic acid (IPR) was prepared in ultrapure water. During all analysis 5 μL of sample were injected on a Zorbax Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 μm). Column temperature was 37 °C and a flow rate of 750 μL min^{-1} was used. Total run time was set to 15 min in isocratic mode. The mobile phase consisted of IPR:methanol (70:30, v:v) and was adjusted to pH 3.2 by sulfuric acid. Detection wavelength was 215 nm and urine samples were diluted 1:10 or 1:100 with IPR after centrifugation (10 min at 5000 rpm). For quantification a 15 point calibrations were performed with concentrations ranging from 0.5 to 200 μM.

2.6. Total organic carbon (TOC) and total nitrogen (TN)

Total organic carbon and nitrogen were analyzed on a Multi NC 3000 (Analytik Jena) analyzer after appropriate sample dilution.

2.7. Solid phase extraction

For SPE OASIS® cartridges (30 mg) and OASIS® 96-well plates (10 mg) were used. For matrix effect evaluation OASIS® MCX, WAX, MAX and WCX sorbent were compared. Generic OASIS 2 × 4 method was slightly modified.

Procedure for 30 mg cartridge: sorbents were conditioned with 1000 μL methanol, dried 10 min at vacuum and equilibrated with 1000 μL ultrapure water. After sample loading the cartridges were washed with either 1000 μL ultrapure water containing 2% formic acid (MCX and WAX) or 1000 μL ultrapure water containing 5% ammonium hydroxide (MAX and WCX). First elution step was performed with 1000 μL methanol and the second elution with 1000 μL methanol containing 2% formic acid (MAX and WCX) or 1000 μL methanol containing 5% ammonium hydroxide (MCX and WAX). Eluates were separately collected, dried and reconstituted in 500 μL of methanol:water (5:95, v:v) for LC–MS/MS analysis.

Procedure for 10 mg 96-well plates: sorbents were conditioned by 500 μL methanol, vacuum-dried for 10 min and equilibrated with 500 μL ultrapure water. After sample loading the cartridges