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Short communication

Determination of carnitine and acylcarnitines in human urine by means of microextraction in packed sorbent and hydrophilic interaction chromatography–ultra-high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A method using semi-automatic microextraction by packed sorbent (eVol®-MEPS) and hydrophilic interaction chromatography–ultra-high-performance liquid chromatography–tandem mass spectrometry (HILIC–UHPLC–MS/MS) was described for the simultaneous determination of carnitine and acylcarnitines in human urine. The optimal conditions of MEPS extraction were obtained using C2 of M1 (C8 + SCX) phase as a sorbent. Chromatographic separation of the analytes was achieved within 2.5 min on Acquity UPLC BEH HILIC column using a gradient elution program with water containing 5 mM ammonium acetate and acetonitrile as the mobile phase. The detection was performed on a triple-quadrupole tandem mass spectrometer in a positive ion mode via electrospray ionization (ESI).

The linearity of the calibration curves for all compounds was found over a range from 0.1 ng/mL to 500 ng/mL. The method afforded satisfactory results in terms of sensitivity, specificity, precision, accuracy, recovery as well as stability of the analyte under various conditions. The method was used successfully for determination of carnitine and acylcarnitines in human urine.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, but their molecular etiology remains not fully understood, in part because they develop slowly as a result of a mixture of genetic and environmental factors. Given the complex nature of CVDs, molecular profiling of processes more "proximal" to the disease than genetic markers may have great promise in revealing both "form" (novel biomarkers with clinical potential) and "function" (mechanisms of disease development) of CVDs [1,2]. Carnitine (L-3-hydroxy-4-N-trimethylaminobutanoic acid) plays an essential role in fatty acids metabolism and is present in tissues and biological fluids in free and esterified forms (acylesters or acylcarnitine) (Fig. S1, Supplementary Material). Acylcarnitines up to C18 are detected in blood, however higher acylcarnitines are found in tissues. Blood profiles of carnitine and acylcarnitines serve as metabolic markers by indicating the performances of respective enzymes in the fatty acid oxidation pathway. Plasma free carnitine is reported to be elevated in patients with congestive heart failure and some kinds of cardiomyopathies, and is associated with poor prognosis [3].

The analytical methods have been described in the literature for the determination of carnitine and some acylcarnitines in biological matrices using high-performance liquid chromatography (HPLC) and various detection techniques such as fluorescence detection (FL) [4] and tandem mass spectrometry (MS/MS) [5–8]. Another separation technique, such as capillary electrophoresis has also been reported [9,10]. However, all these methods use reversed-phase liquid chromatography (RPLC).

Hydrophilic interaction liquid chromatography (HILIC) is an interesting tool for the analysis of polar compounds difficult to be retained in conventional RP systems [11]. In recent years, HILIC has been used to separate e.g. biomarkers [12], fosfomycin [13], methane sulfonic acid [14], drugs and metabolites [15], pteridines [16] and also carnitine and acylcarnitines [17,18]. Up until now, two research papers have described methods for the determination of carnitine and acylcarnitine in plasma, serum, tissue.

The samples preparation has been carried out by means of liquid–liquid extraction (LLE) [10], solid-phase extraction (SPE)

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[5,8,10] and protein precipitation (PP) [6]. However, to the best of our knowledge, none bioanalytical assay was previously developed for the isolation of carnitine and acylcarnitines using microextraction by packed sorbent MEPS as sample preparation procedure.

The aim of this work was to develop a simple, sensitive, rapid and reliable analytical approach for the determination of carnitine and acylcarnitines by means of UHPLC–HILIC–MS/MS. In order to reach the goal a combination of the following approaches was employed: (1) extraction and clean-up of sample by MEPS; (2) efficient UHPLC separation due to sub-2-μm particles; (3) separation selectivity and enhanced ionization due to HILIC conditions; and (4) MS/MS detection using MRM experiment.

To the best of our knowledge, the application of MEPS with HILIC–UHPLC–MS/MS detection for analysis of carnitine and acylcarnitines has not been used yet. Previously, in our study [19] an ultra-performance liquid chromatography method with MS/MS detection was developed for determination only of α-ketoglutaric acid, L-carnitine and acetyl-L-carnitine in human urine samples, however the method was not suitable for the simultaneous determination of carnitine and eight different acylcarnitines. After the application of HILIC it was possible separation of nine carnitines and their determination in human urine. What is more, compared with previously method, the proposed method provides shorter analysis time (2.5 min) and simple MEPS procedure for sample preparation. It can be deduced that the proposed method allows the achievement of quantitative extraction efficiencies for carnitines by using the lowest amount of organic solvent and a reduced sample preparation time. These merits emphasize the fact that the proposed method is highly cost-effective, environmentally friendly and rapid. And what is most importantly, application of HILIC allows to separation a larger numbers of carnitines, as potential biomarkers of cardiac disorders, what may be more useful in clinical studies.

2. Material and methods

2.1. Chemicals and reagents

L-Carnitine hydrochloride (C0), *O*-acetyl-L-carnitine (C2) and mildronate (MLD) (IS, internal standard) were purchased from Sigma–Aldrich (St. Louis, MO, USA). (±)-Propionylcarnitine chloride (C3), (±)-hexanoylcarnitine chloride (C6), (±)-octanoylcarnitine chloride (C8), (±)-decanoylcarnitine chloride (C10), (±)-lauroylcarnitine chloride (C12), (±)-myristoylcarnitine chloride (C14), (±)-palmitoylcarnitine chloride (C16) were supplied by Tocris Bioscience, Tocris House, IO Centre (Bristol, UK). Chemical reagents, including acetonitrile of LC–MS grade and water of LC grade were obtained from Merck (Darmstadt, Germany). Ammonium acetate was supplied from Sigma–Aldrich (St. Louis, MO, USA). Analytical-grade methanol, acetonitrile and formic acid were purchased from POCH S.A. (Gliwice, Poland).

2.2. Standard solutions, calibration standards and quality control (QC) sample

The primary stock solutions of the analytes and IS (1 mg/mL) were prepared in mixture of water:methanol (1:1; v/v) and stored at 4 °C and were brought to room temperature before use. For the preparation of the calibration standards, working solutions in the range from 1 to 5000 ng/mL were used. The working solutions were prepared by diluting the stock solutions of all analytes in water. A working standard solution of IS (10 μg/mL) was prepared by diluting the stock solution with water and stored at 4 °C.

Carnitine and acylcarnitines are endogenous compounds. It is hard to get blank urine to prepare QC samples. In this study to facilitate the determination of carnitine and acylcarnitines in urine, calibration standards (CS) and quality control (QC) samples were prepared by spiking the proper amounts of each working solution into synthetic urine during validation [19]. For studies of applications of developed procedure was used real samples of human urine.

To obtain calibration standards (CS), the working solutions were added to control synthetic urine. In this way, calibration standards in the range from 0.1 to 500 ng/mL for carnitine and acylcarnitines were prepared. Quality control (QC) samples were prepared in the same way as the calibration samples, at four concentrations: 0.1 ng/mL (lower limit of quantitation, LLOQ), 1 ng/mL (low, LQC), 200 ng/mL (middle, MQC), and 400 ng/mL (high, HQC).

2.3. Chromatography and mass spectrometry

The chromatographic system was a Dionex UPLC system (Dionex Corporation, Sunnyvale, CA, USA) supported with UltiMate 3000 RS (Rapid Separation) pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment with a thermostable column area and an UltiMate 3000 variable wavelength detector. All of which were operated using Dionex Chromeleon™ 6.8 software. The analytes were separated in an Acquity UPLC BEH HILIC column (75 mm × 2.1 mm, 1.7 μm) (Waters, USA) at 30 °C with a mobile phase consisting of 5 mM ammonium acetate in water (pH 6.4) (solvent A) and acetonitrile (solvent B). The gradient elution with a total run time of 2.5 min was set as follows: initial, 15% A (flow rate 0.5 mL/min) and increased to 50% A in 2.5 min (flow rate 1.0 mL/min). The injection volume was 3 μL.

Mass spectrometric analyses were performed using an AB SCIEX 4000 Q TRAP triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source in positive mode. The source temperature (TEM) was maintained at 500 °C, and the ionization voltage (ISV) was set at 3000 V. The nebulizer gas (GS1), heater gas (GS2), curtain gas (CUR) and collision activated dissociation gas (CAD) were set at 90, 80, 30 and 6 psi, respectively. The dwell time was kept at 50 ms for each analyte. The detection of the ions was performed in the multiple reaction monitoring (MRM) mode. Tuning and optimization of the compound-dependent parameters declustering potential (DP), collision energy (CE), entrance potential (EP), collision exit potential (CXP) were performed by the direct infusion of a standard solution into the ion source using a Harvard syringe pump (Table 1).

2.4. Sample preparation

The MEPS procedure was carried out with eVol® semi-automatic syringe (SGE Analytical Science, Melbourne, Australia), loaded with a 500 μL gas-tight syringe with a removable needle. The syringe was fitted with a BIN (barrel insert needle) containing 4 mg of the sorbent materials (C2 (ethyl-silica), C8 (octyl-silica), C18 (octadecyl-silica), M1 (80% C8 and 20% strong cationic exchange)). The eVol® is a digitally controlled dispensing system that enables rapid method development for miniaturized SPE sample preparation and clean up. MEPS experiments were conducted using 4 mg of C2 solid-phase (for extraction of myristoylcarnitine and palmitoylcarnitine) and 4 mg of M1 (C8 + SCX) (for extraction of L-carnitine, *O*-acetyl-L-carnitine, propionylcarnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, lauroylcarnitine) materials. Before being used for the first time, the MEPS sorbents were activated with 200 μL of methanol followed by 200 μL of 0.1% formic acid in water.

Human urine (100 μL) was diluted with 90 μL of deionized water in a glass tube and 10 μL of IS solution (20 ng/mL) was added. The sample was slightly vortex-mixed for 30 s. Then, urine sample containing the IS (1.00 ng/mL) has passed through the C2/C8 + SCX