Journal of Chromatography A, 1464 (2016) 32–41

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fluorescent derivatization combined with aqueous solvent-based dispersive liquid-liquid microextraction for determination of butyrobetaine, L-carnitine and acetyl-L-carnitine in human plasma

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ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 10 August 2016
Accepted 11 August 2016
Available online 22 August 2016

Keywords:
Aqueous solvent-based dispersive liquid-liquid microextraction
L-carnitine
Fluorescence derivatization
Narrow-bore liquid chromatography

ABSTRACT

A novel aqueous solvent-based dispersive liquid-liquid microextraction (AS-DLLME) method was combined with narrow-bore liquid chromatography and fluorescence detection for the determination of hydrophilic compounds. A remover (non-polar solvent) and extractant (aqueous solution) were introduced into the derivatization system (acetonitrile) to obtain a water-in-oil emulsion state that increased the mass transfer of analytes. As a proof of concept, three quaternary ammonium substances, including butyrobetaine, L-carnitine and acetyl-L-carnitine, were also used as analytes and determined in pharmaceuticals, personal care products, food and human plasma. The analytes were derivatized with 4-bromomethylbiphenyl for fluorescence detection and improved retention in the column. The linear response was 10–2000 nM for L-carnitine and acetyl-L-carnitine with a good determination coefficient ($r^2 > 0.998$) in the standard solution. The detection limit for L-carnitine and acetyl-L-carnitine was 4.5 fmol. The method was also successfully applied to a 1 μL sample of human plasma. In the linearity calculations for determining butyrobetaine, L-carnitine and acetyl-L-carnitine in human plasma, the determination coefficients ranged from 0.996 to 0.999. Linear regression exhibited good reproducibility and a relative standard deviation better than 7.50% for the slope and 9.06% for the intercept. To characterize highly hydrophilic compounds in various samples, the proposed method provides good sensitivity for a small sample volume with a low consumption of toxic solvents.

1. Introduction

Dispersive liquid-liquid microextraction (DLLME) was first introduced by Rezaee et al. to provide a simple and miniaturized technique for sample pretreatment [1]. Various modifications of DLLME have been proposed to improve its versatility and applicability. For example, DLLME uses a dispersant to induce the formation of fine droplets for improved liquid-liquid extraction efficiency. The extractant that was used in early applications of DLLME was a small amount of chlorinated solvent with a density higher than that of water. Although the use of high-density solvents increases the convenience of DLLME by enhancing sedimentation, high-density solvents have harmful environmental and human health effects. The proposed solution was low-density DLLME (LD-DLLME), in which a low-density organic solvent is used rather than chlorinated solvents. However, one disadvantage is that the extraction phase forms on top of the aqueous phase in LD-DLLME. To withdrawal the organic phase, various apparatuses have been developed to enable convenient handling of small supernatant volumes [2–7]. The DLLME and LD-DLLME methods are primarily used to extract hydrophobic compounds from water samples and difficult to use for separating polar analytes from aqueous samples. Therefore, an effective method for extracting extremely hydrophilic analytes, such as L-carnitine (2.50 g mL^{-1} in water at 20 °C) [8], from aqueous samples is needed.

In this study, we developed a novel approach for using an aqueous solvent (AS) in DLLME. After using a small volume of water (or AS) as the extractant to obtain the hydrophilic analytes, a derivatization medium (acetonitrile, ACN) was used as a dispersant to assist the formation of a water-in-oil emulsion state. The use of a low-density organic solvent as the continuous phase and remover also improved the phase separation and removed hydrophobic

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http://dx.doi.org/10.1016/j.chroma.2016.08.030
0021-9673/

interferences. Then, the two phases were separated by centrifugation, and the bottom of the aqueous phase was collected for direct analysis. The proposed AS-DLLME method is applicable for analyzing hydrophilic analytes that are difficult to extract from aqueous samples. The method does not require the use of any additional apparatus or the use of hypertoxic organic solvents.

As a proof of concept, three quaternary ammonium substances were selected as analytes including butyrobetaine, L-carnitine and acetyl-L-carnitine. L-carnitine is essential for fatty acid oxidation in the human body [9]. Butyrobetaine is a key precursor of carnitine biosynthesis in the kidney, liver and brain [10]. Acetyl-L-carnitine is an L-carnitine derivative that delays aging-associated degeneration of brain function, cognition and memory [11]. The ratio of acyl-L-carnitine to L-carnitine is considered a useful indicator of the state of health, and L-carnitine depletion has been associated with various diseases. Therefore, an effective method for monitoring the concentrations of L-carnitine and its related compounds is needed. The previously reported L-carnitine determination methods include colorimetry and radioenzymatic assay [12–14], capillary electrophoresis (CE) [15–17], gas chromatography (GC) [18] and high performance liquid chromatography (HPLC) [19–28]. These methods typically require derivatization of butyrobetaine, L-carnitine and acetyl-L-carnitine for several reasons. First, because these compounds are hydrophilic, derivatization may reduce the polarity and improve retention factors in GC and reverse-phase HPLC. Second, providing chromophores or fluorophores for these three analytes can enhance the detection sensitivity during ultraviolet (UV) detection or fluorescence detection (FLD). However, one of the most common methods of clinical analysis of L-carnitine and its related compounds is HPLC combined with tandem mass spectrometry (MS/MS). In this method, butanolic HCl is added to biosamples, such as dried blood (15.2 μL) extractant, plasma, serum, or urine (25–100 μL) [29–36]. After derivatization, the solution is dried and dissolved in a mobile phase for HPLC combined with MS/MS, which is more expensive and more difficult to maintain compared to UV and FLD.

The objectives of this study were as follows: (1) to develop a rapid, sensitive and reliable analytical process that combines derivatization and narrow-bore LC with FLD for increased analytical sensitivity, (2) to use butyrobetaine, L-carnitine and acetyl-L-carnitine to demonstrate that AS-DLLME increases the extractability of water-soluble analytes in water samples and (3) to validate the application of the proposed method for use with pharmaceuticals, personal care products, food and human plasma.

2. Materials and methods

2.1. Chemicals, reagents and materials

Acetonitrile, methanol (MeOH), dimethyl sulfoxide (DMSO), acetone, toluene, *n*-hexane, ethyl acetate (EA), ammonium acetate (NH_4OAC), ammonium chloride (NH_4Cl), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), ammonium bicarbonate (NH_4HCO_3), sodium bicarbonate ($NaHCO_3$), potassium hydroxide (KOH), potassium carbonate (K_2CO_3), potassium bicarbonate ($KHCO_3$), 25% ammonia solution (NH_4OH), formic acid (FA), and acetic acid (AA) were purchased from Merck (Darmstadt, Germany). L-carnitine, 4-bromomethylbiphenyl (Br-MBP), and 9-aminoacridine (internal standard, IS) were purchased from the Tokyo Chemical Industry (Tokyo, Japan). Butyrobetaine hydrochloride and acetyl-L-carnitine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was obtained from a Millipore Milli-Q system (Bedford, USA).

The stock solutions of butyrobetaine, L-carnitine, and acetyl-L-carnitine were prepared by dissolution in ACN at concentrations of 100 μM and water at concentrations of 200 μM for standard and human plasma analysis, respectively. The different concentrations of the working solution were obtained by diluting the stock solution. The Br-MBP solution was freshly prepared in ACN. KOH, K_2CO_3, and $KHCO_3$ were prepared in ACN, and IS was prepared in MeOH. All of the solutions were stored at 4 °C prior to use.

L-carnitine chewable tablets and injections (CARNITENE®) that were purchased from Harvest & Health CO., LTD. (Taiwan) were analyzed. Personal care products and food samples containing L-carnitine were purchased from local markets and included capsules, candy, nutritional drinks, coffee, gels, shampoos, and creams. The samples were maintained in their original containers prior to analysis. The plasma samples from three healthy blood donors were collected in heparin tubes. The experiments were approved by the Institutional Review Board of Kaohsiung Medical University Chung-Ho Memorial Hospital (KMUH-IRB-20130135).

2.2. Instruments

The narrow-bore LC-FLD system consisted of an Agilent 1200 LC system (Santa Clara, CA, USA) with binary pump, degasser, autosampler and 1260 fluorescence detector. The analytical column was a Chromolith® PerformanceRP-18e column (100 mm × 2 mm), which was obtained from Merck.

The nano LC–MS/MS system consisted of a Waters nano ACQUITYUPLC system (Milford, MA, USA) and LTQ Orbitrap Discovery hybrid Fourier Transform Mass Spectrometer (Thermo Fisher Scientific, Inc. Bremen, Germany) with a nano spray source and a resolution of 30000. The desalting column (Symmetry C18; 180 μm × 20 mm, 5 μm) and analytical column (BEH C18; 75 μm × 100 mm, 1.7 μm) were obtained from Waters (Milford, MA, USA).

2.3. Sample preparation for pharmaceuticals, personal care products, and food items

The analysis included fourteen samples (i.e., pharmaceuticals, personal care products and food items) of varying formulations. Depending on the complexity of their content, different sample preparations were used. For tablets, 10 units were ground and homogenized. Then, a 0.5 mg sample of the powder was diluted in ACN up to the calibration range. For personal care products and food samples, 10 mg samples were diluted in ACN up to the calibration range. For injection formulas, 10 units were mixed, and 10 μL of the injection formula were diluted in ACN up to the calibration range. All of the ACN solutions were filtered through 0.45 μm filters. Finally, 20 μL of the solution was derivatized with Br-MBP for all of the samples.

2.4. Sample preparation for human plasma

A 1 μL aliquot of human plasma was pipetted into PCR tubes containing 20 μL of analytes in varying amounts of the aqueous solution (0, 5, 25, 100, 200 pmol). The protein precipitating reagent (85 μL volume of acetone) was vortexed with the sample solution for 2 min and centrifuged for 10 min at 14800 rpm. Then, 100 μL of the supernatant was removed and dried with a centrifugal evaporator at 37 °C. The residue was used in subsequent derivatization steps.

2.5. Derivatization methods

The derivatization steps for the standard and pharmaceutical, personal care product, and food product samples were as follows: First, 20 μL of the ACN solution described in Section 2.3 was transferred to PCR tubes. Next, 5 μL of the Br-MBP solution (15 mM in