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Analysis of multiple quaternary ammonium compounds in the brain using tandem capillary column separation and high resolution mass spectrometric detection

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ABSTRACT

Endogenous quaternary ammonium compounds are involved in various physiological processes in the central nervous system. In the present study, eleven quaternary ammonium compounds, including acetylcholine, choline, carnitine, acetylcarnitine and seven other acylcarnitines of low polarity, were analyzed from brain extracts using a two dimension capillary liquid chromatography–Fourier transform mass spectrometry method. To deal with their large difference in hydrophobicities, tandem coupling between reversed phase and hydrophilic interaction chromatography columns was used to separate all the targeted quaternary ammonium compounds. Using high accuracy mass spectrometry in selected ion monitoring mode, all the compounds could be detected from each brain sample with high selectivity. The developed method was applied for the relative quantification of these quaternary ammonium compounds in three different brain regions of tree shrews: prefrontal cortex, striatum, and hippocampus. The comparative analysis showed that quaternary ammonium compounds were differentially distributed across the three brain areas. The analytical method proved to be highly sensitive and reliable for simultaneous determination of all the targeted analytes from brain samples.

1. Introduction

Endogenous quaternary ammonium compounds (QACs) are broadly distributed in various tissues including the central nervous system, and many QACs have important biochemical functions in brain [1,2]. For example, acetylcholine is a critical neuromodulator that plays a key role in diverse cognitive functions [3,4]. Choline, the product/precursor of acetylcholine, is an important metabolite [5] and can also act as a nicotinic receptor agonist [6]. The acylcarnitines are a class of QACs that are derivates of carnitine [7]. Structurally, all carnitines, acetylcholine and choline share a trimethyl quaternary ammonium group, and in particular, acetylcarnitine bears very close structural resemblance to acetylcholine (see their structures in Fig. 1). There are also a number of documented functional links between carnitines and acetylcholine in brain [1]. For example, acetylcarnitine can act as a source of acetyl moieties and thus provide a precursor for the production of acetylcholine from choline [8–11]. In light of these interesting structural and physiological links, our study targets developing a mass spectrometry (MS)-based relative quantitative method that enables sensitive monitoring of acetylcholine, choline, free carnitine, acetylcarnitine and seven acylcarnitines of low polarity from brain samples. We apply this method to brain samples obtained from tree shrews (*Tupaia belangeri*), a small mammalian species that is a close relative of primates including humans.

MS is a powerful method for the detection of QACs in electrospray mode (ESI) since QACs contain a stably charged quaternary ammonium group and thus exhibit high ionization efficiency [12]. To deal with the complexity of the biological samples, chromatographic separations are required in most cases to obtain high detection capability and high reproducibility in the analysis of QACs. Until now, there is still no analytical method available that allows the separation and detection of the targeted QACs in a single LC–MS analysis. With different side chains, these QACs form a class of molecules with different hydrophobicities, even though they share the structural identity of their ammonium groups. This hydrophobicity diversity poses challenges for the LC–MS analysis. Currently, the separation of QACs of high polarity, such as choline, acetylcholine, carnitine and acetylcarnitine, are commonly conducted using hydrophilic interaction chromatography (HILIC)–MS [13,14], ion-exchange chromatography [15,16], or ion-pairing reversed phase (RP) chromatography [17–19]. To separate carnitines of both low and high polarities, pre-column derivatization has been used to improve their separation on common RP

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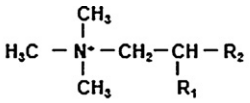

Name	Abbrev.	R_1	R_2	Mass
Choline	Ch	OH	H	104.1070
Acetylcholine	ACh	$OCOCH_3$	H	146.1176
Carnitine	Cart	OH	CH_2COOH	162.1125
Acetylcarnitine	ACart	$OCOCH_3$	CH_2COOH	204.1230
Propionylcarnitine	PropioCart	$OCOCH_2CH_3$	CH_2COOH	218.1387
Hexanoylcarnitine	HexCart	$OCO(CH_2)_4CH_3$	CH_2COOH	260.1856
Octanoylcarnitine	OctCart	$OCO(CH_2)_6CH_3$	CH_2COOH	288.2169
Decanoylcarnitine	DecaCart	$OCO(CH_2)_8CH_3$	CH_2COOH	316.2482
Lauroylcarnitine	LauroCart	$OCO(CH_2)_{10}CH_3$	CH_2COOH	344.2795
Myristoylcarnitine	MyrCart	$OCO(CH_2)_{12}CH_3$	CH_2COOH	372.3108
Palmitoylcarnitine	PalmCart	$OCO(CH_2)_{14}CH_3$	CH_2COOH	400.3421

Fig. 1. Chemical structures of QACs analyzed and their accurate masses.

columns [20]. However, this method is only applicable to carnitines but not to acetylcholine or choline because these two QACs do not have carboxyl group available for such derivatization.

In the present study, we report a sensitive and reliable capillary liquid chromatography–Fourier transform mass spectrometry (LC–FT-MS) method that allows relative quantification of the targeted QACs from brain tissue. The method uses a simple, no-drying sample preparation that facilitates the extraction of all the targeted QACs from brain tissue. To deal with the analytical challenge caused by the diverse hydrophobicities of the QACs, tandem coupling of both RP and HILIC columns was used to separate all the targeted analytes in a single LC–MS analysis. The mobile phase delivered to MS was of high content acetonitrile and ion-pairing reagent free, and thus permitted high detection capability for the QACs. Our relative quantification method was proven to be of high selectivity, sensitivity and reproducibility, allowing profiling each QAC of interest in the different brain areas.

2. Experimental

2.1. Reagents

Choline (Ch) chloride, acetylcholine (ACh) chloride, carnitine (Cart) hydrochloride and acetylcarnitine (ACart) hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Propionylcarnitine (PropioCart), hexanoylcarnitine (HexCart), octanoylcarnitine (OctCart), decanoylcarnitine (DecaCart), lauroylcarnitine (LauroCart), myristoylcarnitine (MyrCart) and palmitoylcarnitine (PalmCart) chloride were obtained from Tocris Bioscience (Ellisville, MO, USA). Choline-d9 chloride (Sigma–Aldrich), acetylcholine-d4 chloride (Medical Isotopes Inc., Pelham, NH, USA), carnitine-d3 hydrochloride, acetylcarnitine-d9 hydrochloride, octanoylcarnitine-d3 hydrochloride and palmitoylcarnitine-d3 hydrochloride (Cambridge Isotope Laboratories Inc., Andover, MA, USA) were used as internal standards (ISs). LC–MS grade formic acid, ammonium hydroxide solution, methanol and acetonitrile were supplied by Sigma–Aldrich. Water was obtained from a Gen-Pure water system (TKA, Niederelbert, Germany).

2.2. Preparation of standard and internal standard solutions

Individual standard and internal standard stock solutions (1 mg/mL) were prepared in 20% methanol containing 0.1% formic acid and stored at −20 °C. These stock solutions were diluted to obtain a mixed standard and internal standard working solution in 25% acetonitrile containing 0.3% formic acid that was used to characterize the analytical performances of our tandem column HPLC–MS system (each analyte and IS at 50 nM except for choline-d9 at 1 μM). A mixed internal standard spiking solution in 20% acetonitrile containing 0.1% formic acid was also prepared from the stock solutions (acetylcholine-d4, carnitine-d3, acetylcarnitine-d9, octanoylcarnitine-d3, palmitoylcarnitine-d3 at 1 μM and choline-d9 at 20 μM).

2.3. Animals and sample preparation

Tree shrews (*T. belangeri*) were used in this study ($n = 3$). The animals were housed under constant temperature and humidity with free access to food and water. The handling of the animals and the experimental procedures were approved by the veterinary office of Fribourg, Switzerland. The tree shrews were sacrificed by decapitation after anesthetization with ketamine (100 mg/kg, Streuli Pharma AG, Uznach, Switzerland). The head was immediately heated up to 80 °C in 16 s using microwave irradiation [21]. The brains were rapidly removed from the cranium and three areas (prefrontal cortex, striatum and hippocampus) were dissected. The tissues of each area were collected from three different animal brains and then pooled together, homogenized by the automated Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) and divided in three samples that were processed separately. The striatum and hippocampus tissues (each 30 mg) were successively spiked with 20 μL of the mixed internal standard spiking solution. The prefrontal cortex tissues (each 20 mg) were spiked with 13.3 μL of the mixed internal standard spiking solution. The tissues were then homogenized in the Precellys 24 homogenizer, using ice-cold acetonitrile containing 0.3% formic acid (100 μL for striatum and hippocampus samples and 66.7 μL for prefrontal cortex samples). The homogenates were centrifuged at 22,000 × *g* for 20 min at 4 °C. The supernatants were collected and filtered by 0.20 μm filter membranes (Millex-LG, Millipore, Billerica, MA, USA). The filtered supernatants were diluted four times with water containing 0.3% formic acid before the analysis. The final concentrations of acetylcholine-d4, choline-d9, carnitine-d3, acetylcarnitine-d9, octanoylcarnitine-d3 and palmitoylcarnitine-d3 in each sample was 50 nM, 1000 nM, 50 nM, 50 nM, 50 nM and 50 nM, respectively.

2.4. Tandem column FT-MS analysis

In this study, a NanoLC-2D system (Eksigent, Dublin, CA, USA) coupled to a LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used. The two independent binary gradient pumps of our LC arrangement allowed achieving a two-dimensional separation and efficient online solvent mixing without the use of a second HPLC system. A capillary RP column and a HILIC column were coupled in a tandem mode through a nano T-piece. One of the ports of the T-piece was connected to the capillary pump (Channel 1) in the Eksigent LC. The inlet line of the RP column was connected to the autosampler, which was coupled with the nanoflow pump (Channel 2) in the Eksigent LC. The outlet line of the HILIC column was coupled with the ESI-MS source. For the primary separation, a C18 column (100 mm × 150 μm I.D.) packed with 5 μm particles (ReproSil-Pur C18 AQ, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was used and the secondary separation was performed with an HILIC column (250 mm × 200 μm I.D.) with 5 μm particles (Polyhydroxyethyl Aspartamide, PolyLC, Columbia, MD, USA). The RPLC mobile phase was composed of 0.2% formic acid in water (A) and acetonitrile (B) using the following gradient program at a flow rate of 0.4 μL/min: 0–10 min, linear gradient 2–30% (B); 10–25 min, linear gradient 30–70% (B); 25–35 min, linear gradient 70–90% (B); 35–40 min, returning linear gradient 90–2% (B); 40–45 min,