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Determination of non-neuronal acetylcholine in human peripheral blood mononuclear cells by use of hydrophilic interaction ultra-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Background: Cholinergic components in non-neuronal tissues and cells are proposed as important in maintaining cellular proliferation and immune homeostasis. However, direct quantification of non-neuronal acetylcholine (ACh) in cells has been inefficient. Therefore, we developed a stable method for determination of intracellular ACh.
Methods: We used ultra performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) with Waters CORTECS chromatographic columns to measure the intracellular ACh in human peripheral blood mononuclear cells (PBMCs). The presence of ACh was validated by RT-qPCR of mRNAs of cholinergic components.
Results: This method successfully separated ACh from iso-ACh with highly sensitive precursor/product ion transitions and allowed a faster chromatography run time within 3.5 min. The detection limit of intracellular ACh was 0.005 ng/10^6 PBMCs. Intra-assay and inter-assay coefficient variations were all <7.0%. Mean recoveries in the samples were between 93.20% and 104.73%. Here, intracellular ACh was stable under multiple storage conditions, partly attributable to mutually stable relationships among cholinergic components. This method was successfully applied in a stroke study and revealed activation of the stroke-induced cholinergic anti-inflammatory pathway.
Conclusions: This method allows direct determination of intracellular ACh in various tissues. Evidences of cholinergic activity linking both the nervous system and non-neuronal system can be profiled by assessing ACh.

1. Introduction

Acetylcholine (ACh) is a classical cholinergic neurotransmitter of crucial electrical activity in the nervous system. Nevertheless, accumulating evidence suggests that ACh is also synthesized by a diversity of non-neuronal tissues [1], such as those bearing immune cells and epithelial cells [2–5]. The participation of non-neuronal ACh is indispensible for cell proliferation, gland secretion and immune regulation [6,7]. However, few studies have established a valid and stable method for objectively quantifying ACh in these cells.

In early investigations, radiochemical assays were used for identifying ACh in blood and plasma. However, such radiologic assays, which were indirect and radiological, lacked sufficient specificity [8]. Further, it is still debatable whether ACh is actually present in human plasma, because ACh degrading enzymes are so abundant that considerable effort is required to detect even traces of ACh. Chromatography has also been applied for this purpose, i.e., gas chromatography-mass spectrometry, high performance liquid chromatography with electrochemical detection and high performance liquid chromatography with mass spectrometry. These methods are commonly used in micro-dialysates of animal brain but are laborious, time consuming, or inadequate because of low limits of detection, insufficient separation sensitivity and requisite usage of derivatization [9–15]. The difficulty is instability of ACh under conditions of enzymatic and chemical degradation in the cerebral spinal fluid. The property hampered delicate sample preparation that may require specific precautions, including an indirect method of electrochemical detection. Recently, the enzyme-linked immune-sorbent assay (ELISA) provided an

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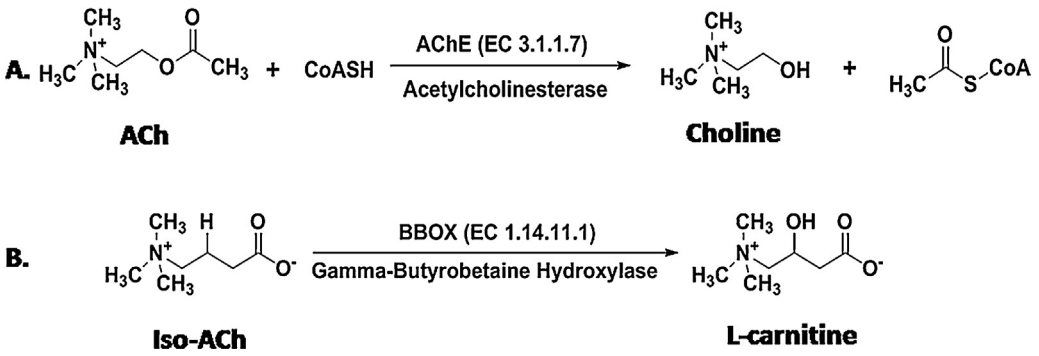

Fig. 1. ACh and iso-ACh have similar chemical structures and hydrophilic natures. (A) ACh can be degraded by AChE into choline and acetyl-CoA. (B) Iso-ACh is hydroxylated by BBOX into L-carnitine, the last step in the L-carnitine biosynthesis pathway.

Table 1
Instrument parameters for UPLC–MS/MS analysis of ACh, iso-ACh and D9-ACh.

Analyte	Parent ions (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
ACh	146.10	60.01	20.00	10.00	1.91
	146.10	86.98	20.00	13.00	
iso-ACh	146.10	60.01	20.00	10.00	2.19
	146.10	86.98	20.00	13.00	
D9-ACh	154.99	69.03	20.00	13.00	1.91
	154.99	87.14	20.00	10.00	

ultrasensitive method for measuring the release of ACh by using acetylcholinesterase (AChE, EC 3.1.1.7) for the conversion of ACh to choline(Fig. 1A) [16]. But as is known, choline is not only the precursor of ACh formed by choline *O*-acetyltransferase (ChAT, EC 2.1.3.6) but is almost always present in various cells for the synthesis of phospholipids. Therefore, the ELISA method may not exclude the interference of endogenous choline present in cells and body fluids [17].

Another urgent problem to be solved in detecting ACh is its differentiation from the natural isomer of ACh (iso-ACh), butyrobetaine (BB, (3-carboxypropyl)trimethylammonium) (Fig. 1B). This intrinsic precursor of carnitine has the same molecular weight as but minor structural differences from ACh. In cell metabolism, iso-ACh is an essential material in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix. So the possible interference of iso-ACh should not be overlooked while attempting to determine ACh content in cells [18,19].

Here, a rapid and selective ultra performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for the simultaneous quantification of ACh and iso-ACh in human peripheral blood mononuclear cells (PBMCs). Compared to a previous study of ACh in liver tissue [18], our method provided a much shorter time for analysis and freedom from endogenous matrix interference with an isotope internal standard. The efficiency was obviously improved for preparing batch samples. Subsequently, we successfully applied this method to a clinical study of stroke patients.

2. Materials and methods

2.1. Chemicals and reagents

Acetylcholine chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard acetylcholine-D9 chloride was from C/D/N (Quebec, Canada). The butyrobetaine was purchased from TCI (Tokyo, Japan). Physostigmine salicylate was from Sigma-Aldrich. The 99% ammonium formate, 98% formic acid (LC–MS grade, Sigma-Aldrich, St Louis. Mo, USA) and acetonitrile were obtained from Merck Chemicals (HPLC grade, Merck KGaA, Darmstadt, Germany).

2.2. Liquid chromatographic conditions

The analysis was performed using a Waters Acquity ultra-performance liquid chromatography (Waters Corporation, Milford, MA, USA) consisting of a binary pump, an automatic sampler and a column compartment. The analytes were separated on the Waters Acquity CORTECS hydrophilic interaction liquid chromatography (HILIC) column (100 mm × 2.1 mm; 1.6 μm).The column temperature was set to 40 °C. The flow rate was 0.4 mL/min. The solvents were 50 mmol/L ammonium formate (solvent A, pH adjusted to 3 with formic acid) and acetonitrile (solvent B) with a gradient over the 3.5 min run time as follows: 20% A (initial), 20–50% A (0–0.75 min),50–70% A (0.75–1.25 min), 70–70% A (1.25–1.7 min), 70–20% A (1.7–2.1 min),20–20% A (2.1–3.5 min). All gradient steps were linear. The sample injection volume was 5 μL.

2.3. Mass spectrometric conditions

Identification and quantification of the target analytes and their internal standard were performed by a triple quadrupole mass spectrometer (Xevo-TQ-S mass spectrometer, Waters Corporation, Milford, MA, USA) using a positive electrospray ionization (ESI) mode. The source temperature was 150 °C, capillary voltage was 0.40 kV, desolvation gas (N_2) temperature was 350 °C at a flow rate of 700 L/h, and the cone gas (N_2) flow rate was 50 L/h. Collision gas (Ar) flow was maintained at 0.30 L/h. Individual cone and collision energy voltages, as well as multiple reaction monitoring (MRM) mass transitions are summarized in Table 1.

2.4. Preparation of standards

Stock solutions of acetylcholine chloride (50 mg/50 mL) and acetylcholine-D9 chloride (50 mg/50 mL) were prepared in acetonitrile-water (50:50 v/v) and stored in a refrigerator at 4 °C. Standard solutions were prepared by diluting the ACh stock solution with acetonitrile-water (50:50 v/v). Each calibration standard