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Quantitative determination of neurotransmitters, metabolites and derivates in microdialysates by UHPLC–tandem mass spectrometry

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ABSTRACT

The main objective of the present work is to study the time-course of rat brain neurotransmitters in vivo after an oral challenge with a nutritional ingredient or an external stimulus, such as a chemical agent. An ultrahigh performance liquid chromatography–tandem mass spectrometry method for the identification and quantification of neurotransmitters, metabolites and derivates in microdialysates from rat brain was previously developed. Betaine, glutamine, glutamic acid, gamma-aminobutyric acid, phosphocholine, glycerophosphocholine, cytidine 5'-diphosphocholine, choline, acetylcholine, dopamine, norepinephrine, serotonin, tyrosine, epinephrine, tryptophan and 5-hydroxyindoleacetic acid were selected as analytes. The method involves direct injection of samples of microdialysates from rat brain onto the chromatographic equipment and quantification with a triple quadrupole mass spectrometer detector using an electrospray ionization interface in positive mode. The limits of detection ranged from 0.1 to 50 ng mL^{-1} and the limits of quantification from 0.3 to 200 ng mL^{-1}. The inter- and intra-day variability were lower than 15%. Recovery rates ranged from 85% to 115%.

1. Introduction

Synaptic transmission between neurons is the mechanism by which information is transmitted in the central nervous system. Most of this transmission occurs through the release and activity of neurotransmitters, which can be classified according to their chemical structure and activity into: (i) monoamines, (ii) amino acids, and (iii) peptides and hormones. Other compounds that can also work in neuronal communication, such as nitric oxide, ATP or adenosine, do not belong to any of the above chemical groups.

Microdialysis is a widely used technique in neuroscience and is one of the few that permits quantification of neurotransmitters, peptides, and hormones in the behaving animal [1] in response to different stimuli: performance of task, administration of drugs systemically or locally, and intake of a nutrient. The microdialysis technique requires the insertion of a small microdialysis probe into an area of the brain. The probe is designed to mimic a blood capillary and consists of a shaft with a semipermeable hollow fiber membrane at its tip, which is connected to inlet and outlet tubing. This technique enables the continuous measurement of small-molecular-weight substances like most neurotransmitters from the interstitial space.

Sensitive analytical methods are needed for the separation and quantification of neurotransmitters in microdialysates. First, the concentration of most neurochemicals in the extracellular space is very low. In addition, the temporal resolution of a microdialysate analyte is inversely related to the volume. Therefore, the analytical methods should provide detection limits below the lowest concentration expected in the dialysate and should require less sample volume than the one used in the microdialysis protocol. Moreover, pipetting or sample clean-up techniques are often impossible. Finally, the perfusion medium itself contains inorganic ions that may interfere with the quantification method employed [2].

The classical methods for the measurement of neurotransmitters depended on their chemical structure, for example, liquid chromatography with electrochemical detection for catecholamines or liquid chromatography with fluorescent detection for amino acids [2]. This fact limited the simultaneous determination of different neurotransmitters in microdialysates due to the low sample volume. Perry and Kennedy published a five-year review of analytical techniques for the determination of neurotransmitters, and also grouped the methods by chemical structure [3]. They included a special section on 'multiplexing' or multi-analyte monitoring and highlighted the opportunity of observing interactions of neurotransmitter systems and detecting changes that were not anticipated by the original hypothesis. They concluded that only a limited number of methods detect analytes from different categories of neurotransmitters. Only two out of six methods detected more than four compounds of different families within the nanomolar range. The first one was able to detect 21

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analytes related to the metabolism of tyrosine, tryptophan and glutamic acid [4]. The second one detected six compounds in extracellular brain fluid [5]. A more recent article reported a method to profile the neurologically related metabolites of multiple principal transmitter pathways in the rat brain [6].

A number of methods have been developed previously for the determination of these compounds in biological matrices using a wide range of techniques, reagents, additives, derivatization procedures, equipments, and detectors as mass spectrometry in several modes, among other detectors. Liquid chromatography–tandem mass spectrometry with electrospray ionization in positive or negative mode (HPLC–ESI-MS/MS) [7,9,11,12,15,17,18,20,23,24,26,28,29]; HPLC coupled to atmospheric pressure chemical ionization mass spectrometry (HPLC–APcI-MS) [8,22,25]; HPLC coupled to thermospray-mass spectroscopy (HPLC–TS/MS) [10]; HPLC with electrochemical detection (HPLC/ECD) [10,16,19,21,27]; ultrahigh performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) [13,14]; HPLC with fluorescence detector [25,30]; HPLC coupled to single-quadrupole mass spectrometer (HPLC–MS) [31]; HPLC with ultraviolet-visible detector (HPLC-UV) [31] and gas chromatography with mass spectroscopy (GC/MS) [10], have been used. However, the previous methods have several shortcomings in comparison to the proposed method. Those methods are limited by the number of compounds that can be analyzed simultaneously, and they are not applicable to the matrices for which the proposed method was developed, and they are restricted by higher limits of detection (LODs). Recent methods that rely on modern analytical techniques like liquid chromatography with tandem mass spectrometry generally have lower LODs, but are restricted to only a few analytes [11,13,14]. Attempts to improve LODs by derivatization have been made, but the procedures are tedious and time consuming [13].

In the present work, an ultrahigh performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the quantification of compounds of different families of neurotransmitters within the ultratrace range was developed and validated. The method consists of a multi-analyte approach for the measurement of neuromediators with chemical structures related to amino acids, including tyrosine (Tyr), glutamine (Gln), glutamic acid (Glu), gamma-aminobutyric acid (GABA) and tryptophan (Trp); monoamines, including acetylcholine (AcCh); metabolites such as choline (Cho), glycerophosphocholine (GPCho), cytidine 5'-diphosphocholine (CDPCho), phosphocholine (PCho), betaine (Bet); catecolamines such as dopamine (DA), epinephrine (E), norepinephrine (NE); and indolamines such as serotonin (SE), and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). This novel method of multi-analyte detection has the advantage of measuring a higher number of compounds at the same time with acceptable LODs and higher selectivity than the methods based on LC with electrochemical, ultraviolet or fluorescence detection. The method was subsequently applied to microdialysis experiments for testing the influence of a stimulant compound injected locally or a carbohydrate given orally.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Bet, Gln, Glu, GABA, PCho, GPCho, CDPCho, Cho, AcCh, DA, NE, SE, Tyr, E, 5-HIAA and eserine were supplied by Sigma-Aldrich (Madrid, Spain). Methanol, acetonitrile, sodium thiosulfate, ammonium hydroxide, ammonium acetate, ammonium formate, acetic acid, heptafluorobutyric acid (HFBA) and formic acid (LC–MS grade) were purchased from Scharlab (Barcelona, Spain). Artificial cerebrospinal fluid (aCSF) or Ringer solution was purchased from Harvard Apparatus (Holliston, MA, USA).

A stock solution was prepared by weighing 0.01 g of each compound into a 10 mL flask, except CDPCho and PCho, for which 0.1 g were weighed. Then, 1 mL of concentrated formic acid (98–100%, v/v) and water up to the final volume were added. The solution remained stable for at least one month at 4 °C. An intermediate solution (N° 1) was prepared by diluting 50 μL of the stock solution to 10 mL in water. A second intermediate solution (N° 2) was prepared by diluting 100 μL of solution N° 1 to a final volume of 2 mL with aCSF.

Work standards for calibration purposes, named WS1, WS2, WS3, WS4, WS5 and WS6, respectively, were prepared by taking 3 μL, 5 μL, 40 μL, 200 μL, 400 μL and 800 μL from intermediate solution N° 2 and diluting to a final volume of 1 mL with aCSF. Each vial received 20 μL of formic acid before the final volume. Two more solutions were prepared from intermediate solution N° 1 by diluting 100 μL and 250 μL to a final volume of 1 mL with aCSF, and also adding 20 μL of concentrated formic acid to each one; they were named, WS7 and WS8, respectively. The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS4) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment.

2.2. Apparatus and software

An UPLC® Acquity from Waters (Milford, MA, USA) equipped with a binary pump, a vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector were used. The chromatograph was connected on-line to a triple quadrupole mass spectrometer detector (TQD) with electrospray ionization (ESI) interface. The following chromatographic columns were tested: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 μm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 μm particle size), and Acquity UPLC HSS T3 (2.1 mm × 100 mm i.d., 1.8 μm particle size) from Waters. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis.

Auxiliary apparatuses were: analytical balance with a precision of 0.1 mg, vortex-mixer, maximum recovery LC vials, screw caps from Waters, and eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom).

2.3. Animal manipulation

Sprague-Dawley rats (Charles River, France) weighing 400–500 g were used. The animals were kept at constant room temperature (22 ± 2 °C) and 45–55% humidity under a regular 12-h light/dark schedule. Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 2101–2005, 86/609/CEE).

Rats were anesthetized intraperitoneally with a mixture of ketamine–rompun (2:1, v/v) and commercially supplied probes (4 mm long membrane, PAN. 30 kDa MW, BASI, USA) were stereotaxically inserted into the left lateral hippocampus area (−5.6 mm anterior bregma, 4.4 mm lateral, and −7.5 mm from the dura mater) according to the coordinates described in a stereotaxic atlas for rats. The animal was allowed to recover from