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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

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within the nanomolar range. The first one was able to detect 21 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 surgery for at least 3 days. On the day of the experiment, the rat was implanted with the brain microdialysis probe that was inserted into the guide cannula. The probe was perfused with aCSF containing 7 μM of eserine at a constant rate of 2 μL min^{-1} to inhibit acetylcholinesterase. The animal was left in the cage with space for relatively free movement. A bolus dose of a specific ingredient was given by gavage 100 min after starting the experiment and sampling was continued for another 220 min. Microdialysate samples were automatically collected every 20 min directly into vials and frozen at −80 °C until analysis. All studies included a 20 min washout period prior to collecting the dialysates. Six dialysate samples were then collected and defined as basal samples.

Each probe was individually tested in vitro to assess the recovery of the analytes. A solution containing all the compounds in concentrations within the linear dynamic range was prepared. The probe was immersed in the solution and the system was set up to simulate the experimental conditions in the animals. Serial samples were collected every 20 min, analyzed and the recovery calculated. The average recovery for each compound should be higher than 20%. Every probe that did not comply with this requirement was discharged.

In one experiment, the animals were administered with KCl directly into the inflow of the probe as an excitatory compound able to induce neurotransmitter release, especially AcCh [32]. In a second experiment, the animals received a glucose bolus by oral gavage (1 g/kg of body weight).

2.4. Sample preparation

Artificial cerebrospinal fluid is commonly used when sampling brain interstitial fluid. This solution closely matches the electrolyte concentrations of cerebrospinal fluid. It is commercially prepared in high purity water and analytical grade reagents, micro-filtered and sterile. Final ion concentrations in this solution were (in mM): Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; and Cl 155. Samples (typically 40 μL) of microdialysate obtained from in vivo studies were placed in maximum recovery vials. Two microliters of formic acid were added and then mixed for 10 s on a vortex mixer.

2.5. Liquid chromatographic conditions

Chromatographic separation was performed using an UPLC BEH C18 column (2.1 × 100 mm^2, 1.7 μm particle size) from Waters. The standards and samples were separated using a gradient mobile phase consisting of a mixture of water, acetonitrile, HFBA and formic acid in a proportion of 900:100:1:1 (v/v/v/v) as solvent A, and 0.1% (v/v) of formic acid in acetonitrile as solvent B. Gradient conditions were: 0.0–1.0 min, 5–50% B; 1.0–2.0 min, 50–95% B; 2.0–3.0 min, 95% B; and back to 5% B in 1.0 min. Flow rate was 0.4 mL min^{-1}, injection volume 5 μL (in partial loop mode), the column temperature was maintained at 25 °C, the sample temperature at 8 °C and total run time was 4 min. Weak solvent was a mixture of 427.5 mL of water and 72.5 mL of acetonitrile, and strong solvent was pure acetonitrile. These solutions were stable for at least one week at room temperature.

2.6. Mass spectrometric conditions

ESI was performed in positive ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (0.1–1.0 mg L^{-1}). Electrospray ionization spray voltage was 3000 V. Nitrogen was used as a desolvation gas at 800 L min^{-1} and as an auxiliary gas in the cone at 50 L min^{-1}. The temperature of the source was 120 °C and the desolvation temperature was 350 °C. Argon (99.99999% purity) was used as collision gas at an

Table 1
MS parameters for ionization with ESI+ in MRM mode.

Compound	Parent (Da)	Daughter (Da)	Cone (V)	Collision (eV)
[a]*Function 1 (0.0–1.0 min)*				
Bet	118.0	57.9	35	22
		58.9	35	18
Gln	147.0	83.9	18	15
		130.0	18	10
Glu	148.1	83.9	18	15
		130.1	18	10
GABA	104.0	86.9	18	10
		68.9	18	15
PCho	184.0	86.1	30	20
		124.8	30	20
GPCho	258.0	104.1	30	17
		125.0	30	30
CDP-Cho	489.0	184.0	35	35
		264.0	35	25
[a]*Function 2 (0.8–3.0 min)*				
Cho	104.0	44.9	35	15
		59.9	35	15
AcCh	146.0	60.0	23	10
		86.9	23	15
DA	153.9	90.9	20	20
		137.0	20	10
NE	170.0	106.9	12	20
		152.0	12	8
SE	177.0	115.0	15	25
		160.0	15	10
Tyr	182.1	136.0	18	14
		165.0	18	10
E	183.9	107.0	15	22
		166.0	15	10
5-HIAA	192.0	146.1	25	17
		91.1	25	37
Trp	205.1	188.1	18	10
		146.0	18	18

[a] Dwell time (ms):20; and delay time (ms):5.

Table 2
Analytical and statistical parameters.

	n	a	b (mL ng^{-1})	R^2 (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})	% P_{lof}
GPCh	5	−32.8	2.482	99.8	5	20	20–5000	22.9
CDP-Cho	5	−25.3	0.770	99.2	50	200	200–5000	98.9
PCho	5	−80.7	2.180	99.8	20	50	50–5000	12.8
Bet	5	−25.7	5.133	99.3	10	20	20–2000	49.1
Cho	5	79.1	31.97	99.1	1	3	3–1000	99.2
AcCh	5	285	283.7	99.8	0.1	0.3	0.3–1000	93.2
DA	5	33.3	3.270	99.7	2	5	5–1000	91.4
SE	5	−16.8	7.711	99.8	2	5	5–1000	93.4
NE	5	9.10	0.502	99.1	20	50	50–1000	51.7
E	5	−28.8	5.759	99.9	5	20	20–1000	62.1
Gln	5	2.60	0.633	99.9	20	50	50–1000	45.4
Glu	5	−14.0	0.405	99.2	20	50	50–1000	31.7
GABA	5	−12.6	1.675	99.5	20	50	50–1000	97.3
Tyr	5	−7.80	1.478	99.4	1	3	3–1500	77.7
5-HIAA	5	−17.0	0.683	99.5	20	50	50–2000	89.4
Trp	5	−34.7	4.365	99.3	3	10	10–1500	91.4

n, calibration levels; *a*, intercept; *b*, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; and $\%P_{lof}$, P-value for lack-of-fit test.

approximate rate of 0.13 mL min^{-1}. Optimized parameters for each compound are listed together with the mass transitions in Table 1. Common parameters in the two functions are InterChannel Delay: 5 ms and InterScan Time: 5 ms.

2.7. Quality parameters and definitions

Several quality parameters of the method have been assessed namely linearity, selectivity, accuracy and sensitivity. The concepts and definitions are as follows:

Linearity: It is defined by the intercept of the calibration curve (a), slope (b) and determination coefficient (R^2) in addition to P-value for lack-of-fit test ($\%P_{lof}$).

Selectivity: The specificity of the method was determined by comparing the blank chromatograms with those corresponding to the samples. Blank samples were aCSF without adding any component to the matrix.

Accuracy: precision and trueness. Due to the lack of certified reference materials, a spike/recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank aCSF samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolating the standard calibration curve. Recoveries (R) were calculated by comparing the interpolated amounts to the theoretical amounts, spiked amounts. To evaluate the precision of the method, the intra- and inter-day precisions (as relative standard deviation, RSD) were assessed at three concentration levels for each compound (see Table 3). Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and were repeated for 5 days to determine inter-day variability.

Sensitivity: Limit of detection (LOD) and limit of quantification (LOQ) are two fundamental aspects that need to be examined in the validation of any analytical method to determine if an analyte

Table 3
Recovery, precision and accuracy of target compounds in microdialysate samples.

	Intra-day						Inter-day				
	Spiked (ng mL^{-1})	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	*n*	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	*n*
GPCho	335.3	317.4	16.6	94.7	5.2	3	372.7	43.5	111.2	11.7	15
	670.6	622.9	32.3	92.9	5.2	3	607.5	19.6	90.6	3.2	15
	1342.0	1291.7	90.9	96.3	7.0	3	1174.6	90.8	87.6	7.7	15
CDPCho	420.2	359.9	8.8	85.6	2.4	3	369.7	25.4	88.0	6.9	15
	840.4	801.7	91.2	95.4	11.4	3	715.6	58.9	85.1	8.2	15
	1680.0	1435.5	49.3	85.4	3.4	3	1448.3	55.4	86.2	3.8	15
PCho	264.1	246.9	23.5	93.5	9.5	3	260.7	10.7	98.7	10.7	15
	528.2	499.2	57.2	94.5	11.5	3	496.3	29.5	93.9	5.9	15
	1056.0	975.5	46.5	92.3	4.8	3	1021.0	94.3	96.6	9.2	15
Bet	77.3	77.4	6.6	100.2	8.6	3	86.7	11.4	112.2	13.2	15
	154.6	145.9	18.4	94.4	12.6	3	132.4	9.1	85.7	6.9	15
	309.2	267.1	27.0	86.4	10.1	3	262.8	21.1	85.0	8.0	15
Cho	36.6	33.5	2.0	91.5	5.9	3	36.0	2.3	98.2	6.5	15
	73.2	66.1	4.0	90.2	6.0	3	65.7	6.5	89.6	9.9	15
	146.4	127.9	15.0	87.2	11.7	3	125.9	5.1	85.9	4.0	15
AcCh	157.6	160.5	18.1	101.8	11.3	3	167.7	14.9	106.4	8.9	15
	315.2	306.8	34.8	97.3	11.3	3	336.7	38.1	106.8	11.3	15
	630.4	575.9	28.1	91.3	4.9	3	577.8	36.9	91.6	6.4	15
DA	36.6	36.2	5.3	98.8	14.7	3	35.0	5.0	95.6	14.4	15
	73.2	83.3	6.3	113.6	7.6	3	68.8	9.9	93.9	14.4	15
	146.4	135.6	19.5	92.5	14.4	3	148.5	19.4	101.3	13.0	15
SE	36.6	35.6	4.7	97.2	13.2	3	34.6	4.0	94.4	11.6	15
	73.2	80.1	0.5	109.2	0.6	3	73.0	10.8	99.5	14.8	15
	146.4	139.4	20.1	95.1	14.5	3	166.0	24.0	113.2	14.5	15
NE	77.3	82.8	15.0	107.2	18.2	3	75.5	8.7	97.6	11.6	15
	154.6	163.0	14.5	105.5	8.9	3	147.1	21.0	95.2	14.3	15
	309.2	304.1	13.6	98.4	4.5	3	320.9	34.8	103.8	10.9	15
E	50.7	55.8	4.8	109.9	8.6	3	53.1	6.5	104.8	12.2	15
	101.4	97.0	13.4	95.6	13.8	3	112.0	16.2	110.4	14.5	15
	202.8	203.3	29.0	100.2	14.2	3	218.8	28.6	107.8	13.1	15
Gln	52.4	45.6	0.6	87.1	1.2	3	51.5	6.8	98.2	13.2	15
	104.8	100.5	2.3	95.9	2.2	3	92.5	6.6	88.3	7.2	15
	209.6	191.1	18.7	91.2	9.8	3	184.8	12.8	88.2	6.9	15
Glu	57.1	57.9	5.1	101.3	8.9	3	52.6	5.4	92.1	10.2	15
	114.2	106.8	5.4	93.5	5.0	3	96.9	14.6	84.8	15.0	15
	228.4	228.8	33.5	100.2	14.6	3	201.2	30.8	88.0	15.3	15
GABA	60.6	54.9	6.8	90.6	12.4	3	55.9	6.9	92.2	12.3	15
	121.2	124.9	13.7	103.1	10.9	3	120.6	12.2	99.6	10.1	15
	242.4	254.9	6.8	105.2	2.7	3	242.4	37.0	100.0	15.2	15
Tyr	57.1	65.5	9.5	114.6	14.4	3	54.5	7.5	95.4	13.7	15
	114.2	126.0	13.9	110.3	11.0	3	130.6	18.7	114.3	14.3	15
	228.4	229.5	29.4	100.5	12.8	3	211.0	20.3	92.3	9.6	15
5-HIAA	77.27	73.4	7.5	95.0	10.2	3	68.6	10.1	88.7	14.7	15
	154.5	157.3	28.4	101.8	18.0	3	164.2	14.3	106.2	8.7	15
	309.0	317.2	14.2	102.6	4.5	3	301.375	25.3	97.5	8.4	15
Trp	52.4	59.9	8.7	114.4	14.6	3	54.6	7.3	104.2	13.4	15
	104.8	117.0	13.2	111.7	11.3	3	108.3	10.5	103.4	9.7	15
	209.6	238.3	6.5	113.8	2.7	3	204.0	30.3	97.4	14.9	15

[a] Mean value; SD, standard deviation; *R*, % recovery; RSD, relative standard deviation; and *n*, number of determinations.

is present in the sample. The LOD is the minimum amount of analyte detectable in the sample while the LOQ is the minimum amount that can be quantified. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives. In this work, these parameters were calculated from the signal-to-noise ratio (LODs signal-to-noise ratio = 3, LOQ signal-to-noise ratio = 10) injecting several solutions of decreasing amounts of the compounds of interest spiked into blank aCSF samples.

3. Results and discussion

3.1. Liquid chromatographic analysis

Initial experiments were designed to evaluate chromatographic conditions similar to those described previously in the literature [7–11,14,17,31], but in this case, for an UPLC® instrument. Three liquid chromatography columns were tested: an Acquity UPLC BEH

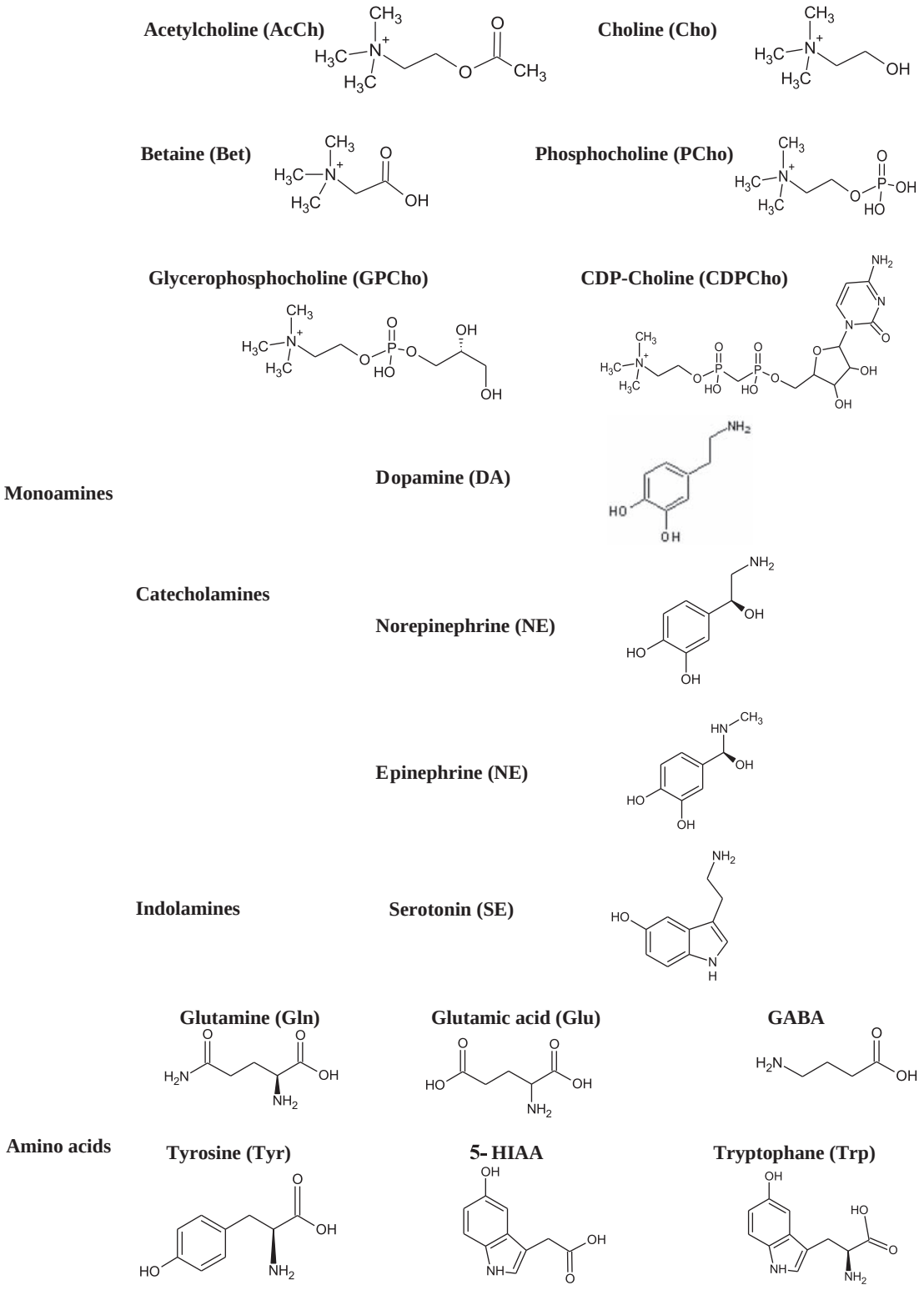

Fig. 1. Chemical structures of the compounds of interest.

C18, an Acquity UPLC BEH HILIC, and an Acquity UPLC HSS T3. All three columns provided good resolution for the analytes. However, the BEH C18 column provided the best resolution in the shortest amount of time. Therefore, this column was selected.

Different mobile phases were also studied in order to optimize the separation and peak shapes. With that objective in mind, two organic solvents (methanol and acetonitrile) commonly used in reversed-phase liquid chromatography were evaluated. Acetonitrile

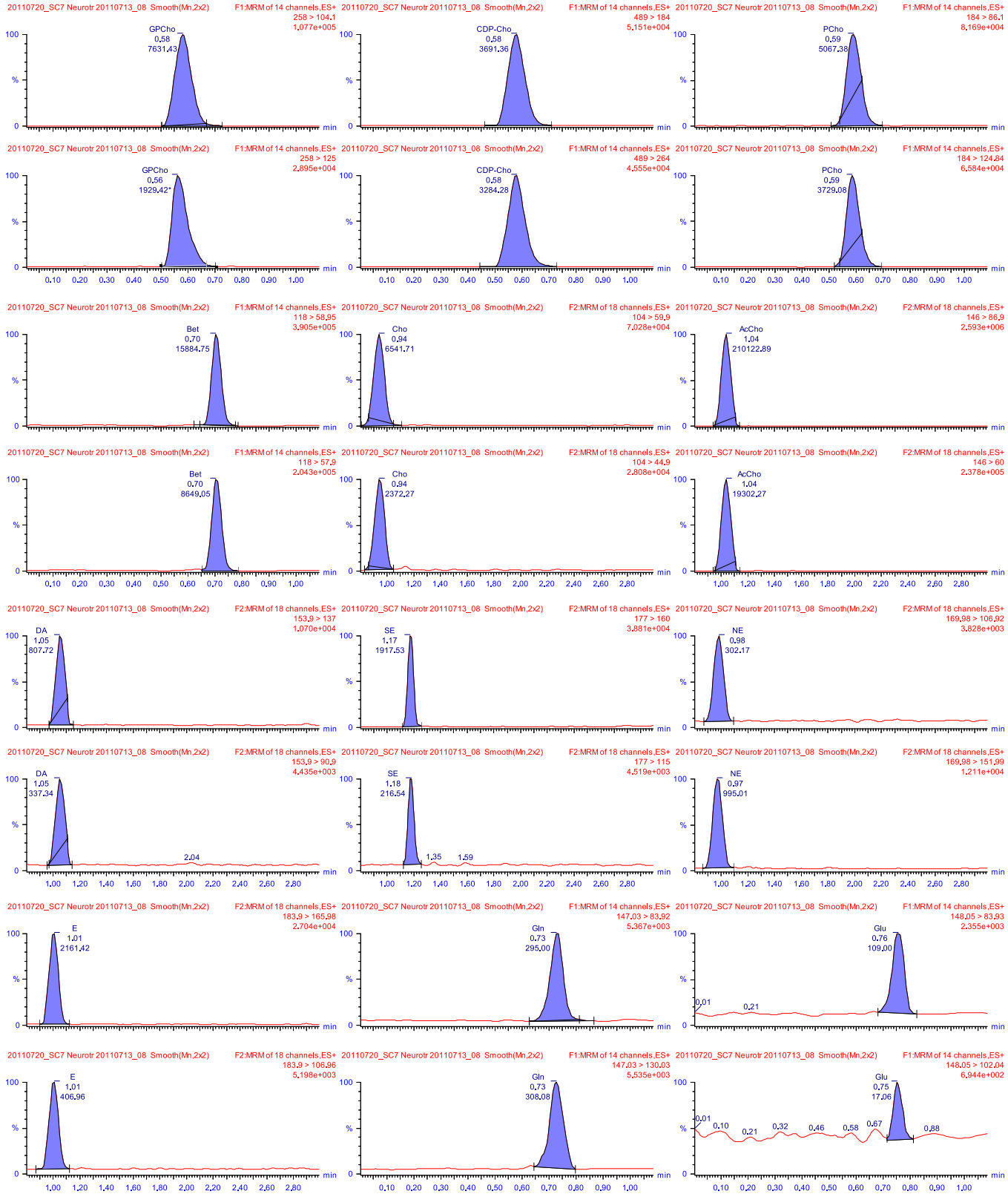

Fig. 2. Chromatogram of each analyte standard in aCSF (two transitions). The first transition was used for quantification and the second for confirmation. GPCho (2908 ng mL^{-1}); CDPCho (4202 ng mL^{-1}); PCho (2641 ng mL^{-1}); Bet (772.7 ng mL^{-1}); Cho (366.5 ng mL^{-1}); AcCh (825.5 ng mL^{-1}); DA (412.5 ng mL^{-1}); SE (431.6 ng mL^{-1}); NE (572.7 ng mL^{-1}); E (507.3 ng mL^{-1}); Gln (523.7 ng mL^{-1}); Glu (544.76 ng mL^{-1}); GABA (605.88 ng mL^{-1}); Tyr (571.2 ng mL^{-1}); 5-HIAA (772.7 ng mL^{-1}) and Trp (523.7 ng mL^{-1}).

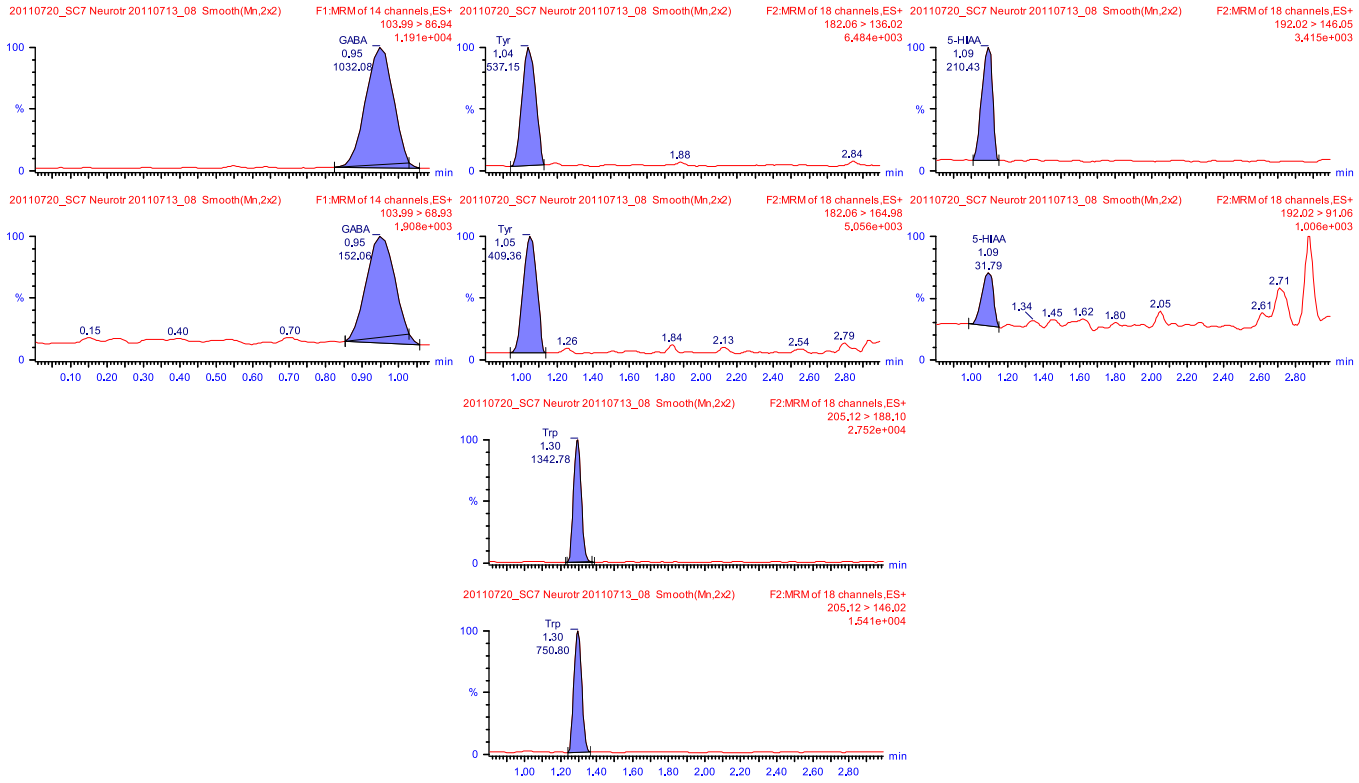

Fig. 2. Continued.

gave better results than methanol in terms of resolution and peak shape. Moreover, based on some of the previous methods mentioned above, several additives were evaluated (ammonium hydroxide, ammonium acetate, ammonium formate, acetic acid, formic acid, and HFBA) in order to improve the analytical signal and the resolution of the chromatographic peaks. The best separation was obtained using a mobile phase composed of water, acetonitrile, HFBA and concentrated formic acid in a proportion of 900:100:1:1 (v/v/v/v) as solvent A and 0.1% (v/v) of formic acid in acetonitrile as solvent B. The linear gradient described previously was used.

To improve the sensitivity of the method, a study was performed to evaluate the possibility of increasing the injection volume and to evaluate different modes of injection. Injection volumes ranging from 1 to 10 μL were studied. An extra broadening of the peaks was observed at volumes higher than 5 μL, therefore, this volume was selected. An investigation of injection mode showed that full loop required a greater amount of sample compared to partial loop. Therefore, partial loop was chosen.

The stability of the standards was also studied, which included an investigation of different additives to extend the expiration. Sodium thiosulfate, acetic acid, formic acid and several mixtures of acetonitrile, methanol and water were tested [15]. The results showed that a 10% (v/v) solution of formic acid in water was the most appropriate additive to prepare the stock solution. Microdialysate samples were prepared with 7 μM of eserine, to prevent the degradation of AcCh by inhibiting acetylcholinesterase. There is no agreement on the literature about the use of acetylcholinase inhibitors or the type of compound used [16,28]. Nonetheless, we preferred to prevent degradation as no interferences were evident in treated samples (Fig. 3).

3.2. Mass spectrometric analysis

The MS/MS detection was set up by direct infusion of each individual compound to optimize the response of the precursor ion. ESI and combined electrospray and atmospheric pressure ionization source (ESCI) interfaces in positive and negative modes were evaluated. ESI interface in positive mode was selected because it showed higher sensitivity for all compounds of interest. The response of two daughter ions (two reactions), one for quantification and the other for identification or confirmation, were monitored. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperature, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1. Additionally, Fig. 2 shows chromatograms of a standard mixture of compounds.

3.3. Analytical performance

A calibration curve was obtained for each compound by injecting 5 μL of different standard solutions (prepared in aCSF) at concentrations ranging from 20 to 5000 ng mL^{-1} for GPCho, 200 to 5000 ng mL^{-1} for CDPCho, 50 to 5000 ng mL^{-1} for PCho, 20 to 2000 ng mL^{-1} for Bet, 3 to 1000 ng mL^{-1} for Cho, 0.3 to 1000 ng mL^{-1} for AcCh, 5 to 1000 ng mL^{-1} for DA and SE, 50 to 1000 ng mL^{-1} for NE, Gln, Glu and GABA, 20 to 1000 ng mL^{-1} for E, 3 to 1500 ng mL^{-1} for Tyr, 50 to 2000 ng mL^{-1} for 5-HIAA and 10 to 1500 ng mL^{-1} for Trp. Analytical performance was evaluated according to the recommendations of Analytical Methods Committee [33]. The *lack-of-fit* test was applied to two replicates and three injections of each standard (five concentration levels). The results are summarized in Table 2.

A quality control standard (WS4) was injected after every 20 injections to assure the validity of the calibration curve. The predicted value expected to not exceed ± 15% of the theoretical value.

3.4. Method validation

Validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity, was performed according to the US Food

and Drugs Administration (FDA) guidelines for the bioanalytical assay validation [34].

Linearity: The values obtained for R^2 ranged from 99.1% for Cho to 99.9% for Gln, and $\%P_{lof}$ values were higher than 5% in all cases. This indicated a good linearity within the stated ranges.

Selectivity: No interferences from endogenous substances were observed at the retention times of each respective analyte (Fig. 2), which eluted at 0.55 min, 0.57 min, 0.58 min, 0.70 min, 0.73 min, 0.75 min, 0.92 min, 0.94 min, 0.97 min, 1.01 min, 1.04 min, 1.04 min, 1.05 min, 1.09 min, 1.17 min and 1.30 min for GPCho,

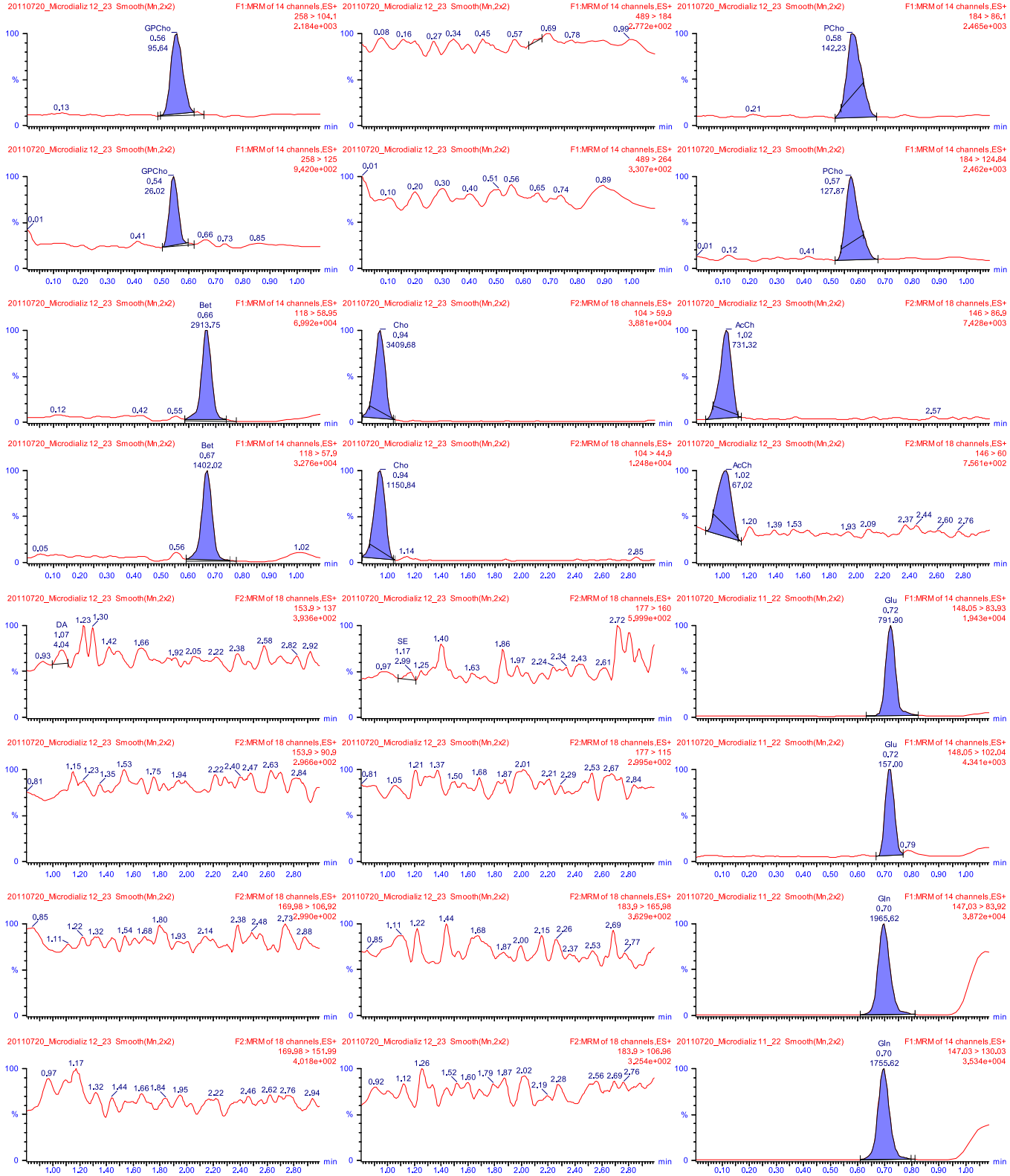

Fig. 3. Chromatograms of each analyte in a rat microdialysate sample (two transitions). The first transition of each analyte was used for quantification and the second for confirmation.

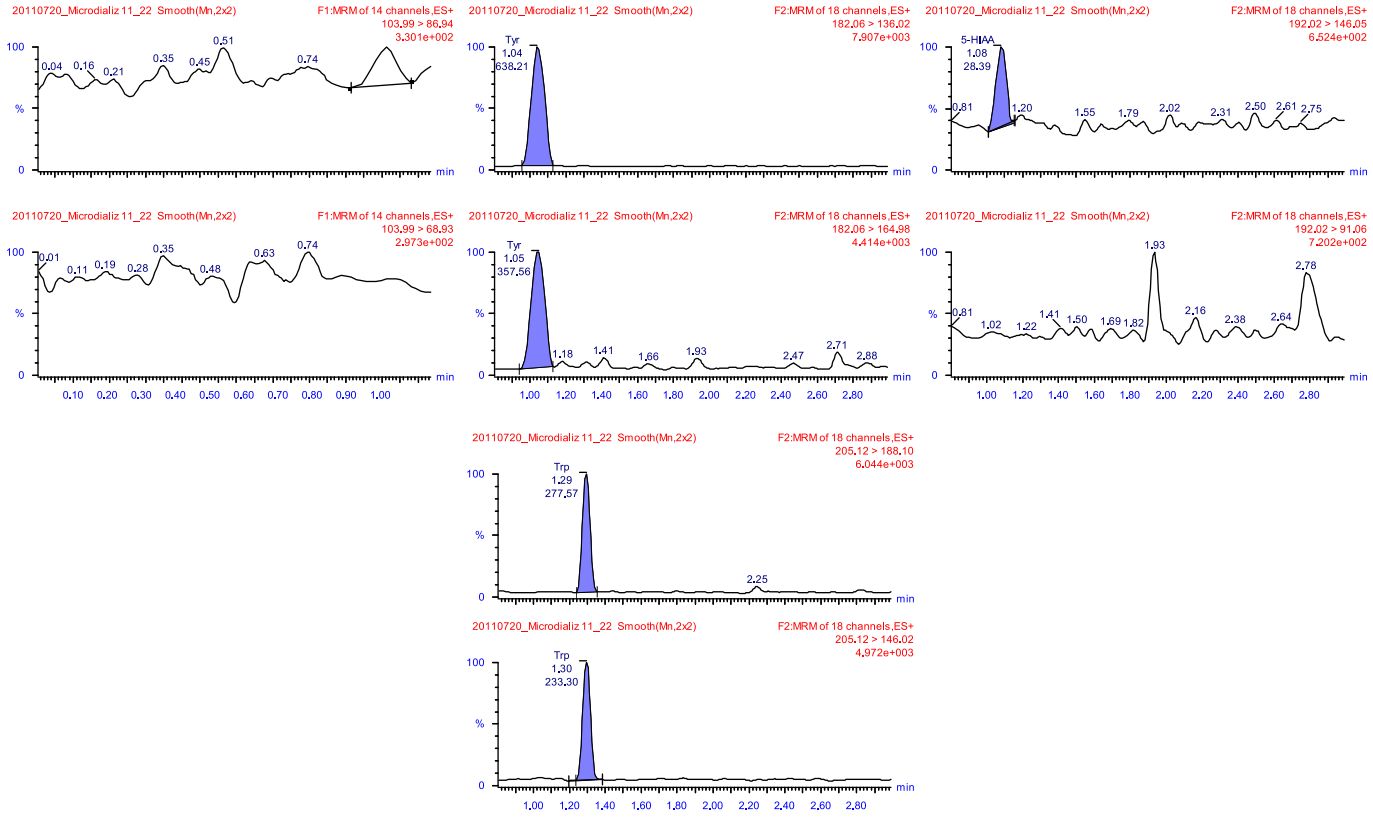

Fig. 3. Continued.

CDPCho, PCho, Bet, Gln, Glu, GABA, Cho, NE, E, AcCh, Tyr, DA, 5-HIAA, SE, and Trp respectively. This finding suggested that the LC–MS/MS conditions provided sufficient selectivity.

Accuracy: *precision and trueness.* Recoveries and repeatability and within-laboratory reproducibility are summarized in Table 3.

The recoveries were between 85% and 115% in all cases. Relative standard deviation values (RSD) were between 0.6% and 18.2%. The highest RSDs were for NE and 5-HIAA at 18.2% and 18%, respectively. This was due to the fact that their concentrations were close to or below the limit of quantification (LOQ). Therefore, all compounds were within the acceptable limits for the bioanalytical method validation, which are considered ≤15% of the actual value, except at the LOQ, where it should not deviate by more than 20%. Precision and trueness data indicated that the methodology to determine the target compounds in microdialysates from rat brain is highly accurate and precise, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Sensitivity: The calculated LODs (signal-to-noise ratio = 3) are in the range from 0.1 ng mL^{-1} for AcCh to 50 ng mL^{-1} for CDPCho, and the corresponding LOQs (signal-to-noise ratio = 10) ranged from 0.3 ng mL^{-1} to 200 ng mL^{-1}. The values obtained are shown in Table 2.

3.5. Application of the method

Samples of microdialysates from rat brain were collected following the protocol previously described. The main objective of the work was to determine the time-course of neurotransmitters when different ingredients (individually or together) were supplied to the animals orally by gavage or locally into the brain.

Fig. 3 shows chromatograms obtained for a microdialysate sample. Under the experimental conditions, AcCh, Cho, GPCho, PCho, Bet, Tyr, Gln, Glu and Trp were detected. Figs. 4 and 5 show examples of the time-course of some neurotransmitters when two different stimuli are used. Only those neurotransmitters that were related to the stimuli or that changed during the protocol are shown.

Fig. 4 displays the progress of AcCh, SE, Cho, PCho and GPCho in the brain of an animal for 200 min, during which 100 mM KCl was added to the perfusion media at 140 min after the beginning of the experiment. It is known that KCl stimulates specific areas of the brain to release AcCh and SE [34,35]. As expected, AcCh and SE displayed a rapid increase 20 min after the addition of the KCl, with a peak at 180 min and only 40 min after the addition. Moreover, SE also increased at zero time likely due to the stress of the animal after manipulation and/or to the stress of the tissue after the insertion of the probe. Similarly, Cho, PCho, and GPCho also increased at the beginning of the experiment although they were not affected by the addition of KCl and remained stable over time.

Fig. 5 shows AcCh and Cho overflow from rat hippocampus over 260 min. The data are expressed as % of basal release (mean of samples 60, 80 and 100 min as a basal reference). Rats received a gavage of D-glucose (1 g/kg of body weight) at the end of sample 100 min. A two-fold increase in AcCh overflow was observed 40 min after the gavage, but it progressively returned to baseline values over the ensuing 2 h period. Choline showed a decrease of two-thirds of the baseline concentration at 60 min after the gavage, but gradually returned to the baseline level over the ensuing 40 min.

The observed increase in AcCh levels agrees with the fact that AcCh synthesis is directly dependent on glucose supply (glucose is critical for the production of acetyl-CoA, a precursor of acetylcholine) [36]. In fact, there are a number of hypotheses about the physiological bases of the memory-improving action of glucose. Glucose sources could alleviate localized deficits in extracellular

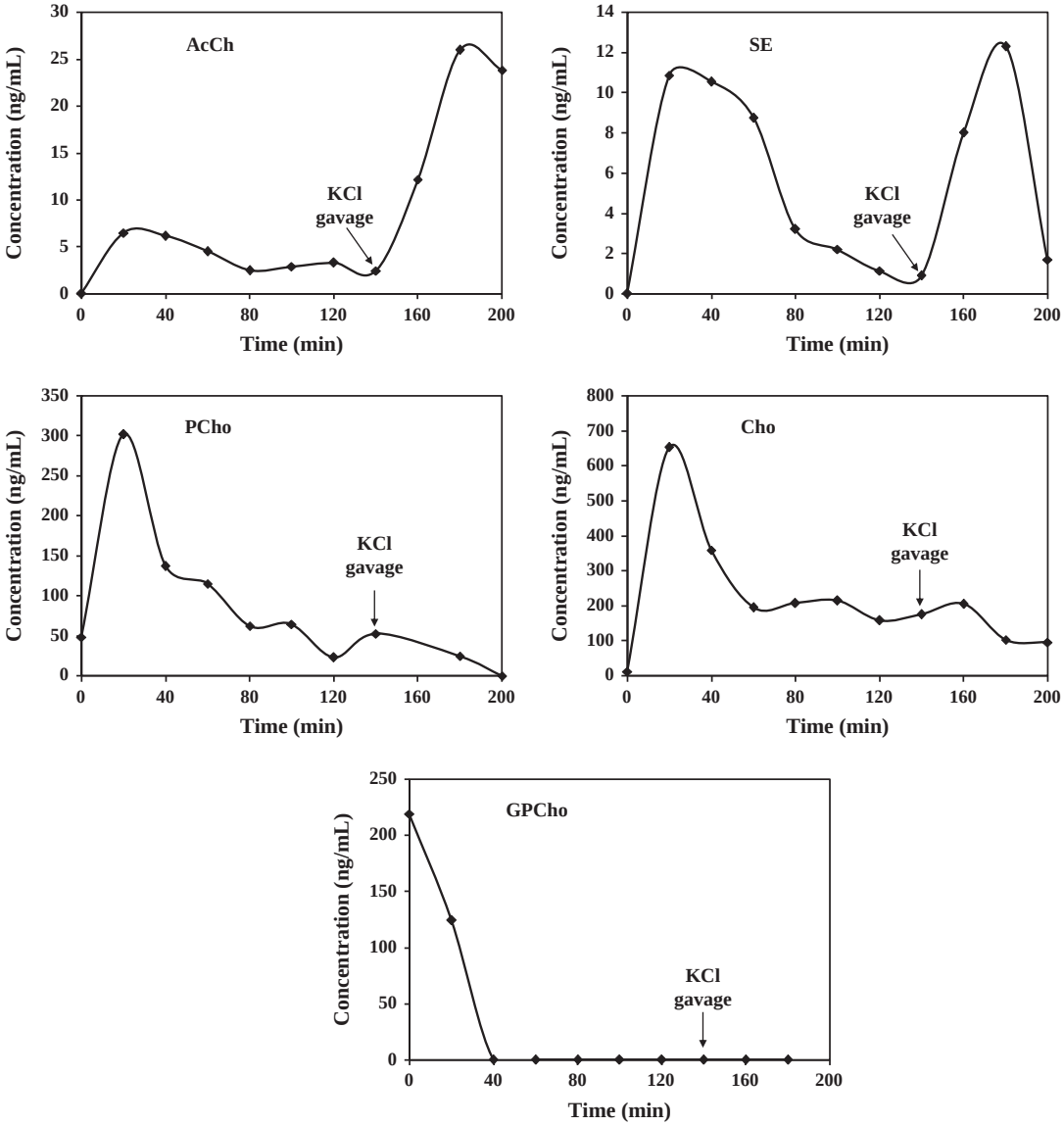

Fig. 4. Time-course of acetylcholine and other metabolites in rat hippocampus after the addition of KCl.

glucose in the hippocampus. Extracellular brain glucose concentrations vary with neuronal activity, indicating that glucose may be critical in modulating memory functioning. Moreover, it has been reported that hippocampal AcCh release increases in rats during a spatial task [37]. AcCh is rapidly hydrolyzed to free Cho and acetate by the enzyme, acetylcholinesterase, into synapses. This process terminates the physiological actions of the neurotransmitter [38]. The observation of a decrease in the concentration of Cho, after an increase of AcCh, could be explained as a high activation of the reuptake process to replenish the intracellular levels of AcCh. Most of the free Cho liberated by the intrasynaptic hydrolysis of AcCh is taken back into its nerve terminal of origin by the high-affinity Cho transporter, and either reacetylated to form AcCh or phosphorylated for ultimate conversion to membrane PCho [39].

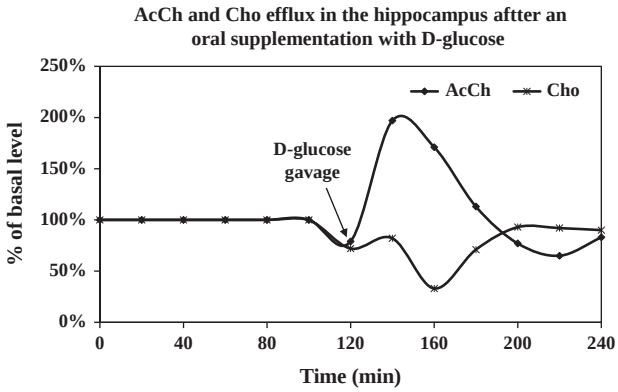

Fig. 5. Time-course of acetylcholine and choline in rat hippocampus after an oral bolus of D-glucose (1.0 g/kg of body weight). Values are expressed as % of basal release (mean of samples 60, 80 and 100 min as a basal reference).

4. Conclusions

The proposed method is a powerful tool for the simultaneous determination of 16 compounds, including neurotransmitters of different chemical families and related metabolites. With this improvement, several previous methods have been combined into a single method, which minimizes the time and total cost of the

analysis. The simplicity and sensitivity of the new method makes it possible to do an in vivo study of changes in chemical signals in the brain of an animal. Therefore, it is helpful in studies of neurotransmitters released in the brain when different ingredients or external agents are administered or when specific situations or activities are monitored, such as learning, memorizing, stress situations, etc. In summary, the proposed method provides opportunities to study all of these components in wide range of situations.

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