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A novel liquid chromatography/mass spectrometry method for determination of neurotransmitters in brain tissue: Application to human tauopathies

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ABSTRACT

Neurotransmitters, small molecules widely distributed in the central nervous system are essential in transmitting electrical signals across neurons via chemical communication. Dysregulation of these chemical signaling molecules is linked to numerous neurological diseases including tauopathies. In this study, a precise and reliable liquid chromatography method was established with tandem mass spectrometry detection for the simultaneous determination of aspartic acid, asparagine, glutamic acid, glutamine, γ-aminobutyric acid, N-acetyl-L-aspartic acid, pyroglutamic acid, acetylcholine and choline in human brain tissue. The method was successfully applied to the analysis of human brain tissues from three different tauopathies; corticobasal degeneration, progressive supranuclear palsy and parkinsonism-dementia complex of Guam. Neurotransmitters were analyzed on ultra-high performance chromatography (UHPLC) using an ethylene bridged hybrid amide column coupled with tandem mass spectrometry (MS/MS). Identification and quantification of neurotransmitters was carried out by ESI + mass spectrometry detection. We optimized sample preparation to achieve simple and fast extraction of all nine analytes. Our method exhibited an excellent linearity for all analytes (all coefficients of determination > 0.99), with inter-day and intra-day precision yielding relative standard deviations 3.2%–11.2% and an accuracy was in range of 92.6%–104.3%. The present study, using the above method, is the first to demonstrate significant alterations of brain neurotransmitters caused by pathological processes in the brain tissues of patient with three different tauopathies.

1. Introduction

Neurotransmitters (NT) are critical chemical messengers which allow the transmission of signals between neurons across synapses or between neurons and other cells within the central nervous system (CNS). Apart from acetylcholine and choline, all small-molecule transmitters belong to the family of biogenic amines or amino acids [1–3]. NTs are widely distributed in CNS, brain tissue and body fluids of mammals. The determination of concentration changes of NTs in the brain tissue is of great importance for studying normal neuronal processes such as behavioral effects and aging, but also in various neurodegenerative diseases including tauopathies, Huntington's disease, schizophrenia, epilepsy, depression, drug addiction, and attention deficit hyperactivity disorder [1–4]. Today, the exact pathological mechanisms behind most of these disorders remain unclear and without a deep understanding of them, the development of therapeutics represents a significant challenge. From an analytical point of view, one of the problems in advancing brain therapeutic treatments is the combination of different analytical approaches and strategies of data analysis in the process of discovering the biochemical basis of mental disorders and neurodegenerative diseases [5].

In tauopathies, abnormal deposition of microtubule associated protein tau occurs, in addition to other pathological hallmarks such as amyloid plaques, synuclein or TDP43 deposits. Alzheimer's disease

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(AD) is the most common tauopathy, characterized by cognitive deficits and memory impairment [6,7]. The major component of AD hallmark—neurofibrillary tangles (NFTs)—are depositions of abnormally hyperphosphorylated microtubule-associated protein tau, which is essential for assembly and stabilization of microtubules [8]. Several biochemical pathways involving neurotransmission and receptors are influenced in AD [9]. An imbalance of different neurotransmitters—glutamic acid, acetylcholine, dopamine and serotonin, in specific brain regions responsible for emotional activities, has been proposed as a neurobiological determinant of AD dementia. Previous studies for example, indicated that amino acids such as aspartic acid, γ-aminobutyric acid and glutamic acid are significantly reduced in AD brains. Glutamatergic and cholinergic abnormalities are strongly associated with cognitive deterioration in AD. The glutamatergic and cholinergic systems are involved in memory and cognition and are implicated in the progression of AD. Acetylcholine (ACh) synthesis, choline acetyltransferase activity, and nicotinic receptor binding, which can facilitate the release of ACh, have been found to be reduced in the AD brain. There is no cure for AD, although reversible acetylcholinesterase inhibitors employed in the therapy, such as donepezil, rivastigmine and galantamine treat the symptoms related to memory, thinking, language, judgment and other thought processes [10,11]. A disturbed serotonergic system is commonly associated with depression and aggression. Antidepressants and antipsychotics, frequently employed in treating AD patients, often interact with neurotransmitter reuptake transporters. In recent years, abundant evidence has emerged to support the notion that the GABAergic signaling system undergoes pathological alterations and contribute to AD pathogenesis. Accordingly, targeting GABAergic neurotransmission is being explored as a potential therapy for AD treatment [8,9,11,12]. The changes of different neurotransmitters have been found also in other tauopathies such as frontotemporal dementia and Pick's disease [13].

Different approaches often requiring chromatographic separation have been used for the determination of neurotransmitters. HPLC is the most suitable technology for the simultaneous determination of NTs in human diseases [12]. The most common detection methods used previously include electrochemical detection [14], UV detection [15] or fluorescence detection [16–18]. These methods have limitations as they are restricted the number of compounds that can be analyzed simultaneously [19]. Electrochemical detection tends to lack reproducibility, mainly because of hysteretic degradation of the electrode, and has low detection limits [20]. UV detection is not sensitive and selective for monoamines. It is difficult to use for amino acids due to their challenging separation, long running times and the use of potentially toxic derivatization reagents such as o-phthalaldehyde, 9-fluorenylmethyl chloroformate, benzoyl chloride, dansyl chloride, or 2,4-dinitrofluorobenzene, ninhydrin and fluorescamine [21,22]. Liquid chromatography with mass spectrometry (MS) detection has been used to quantify NTs in various biological matrices. However, derivatization or addition of ion pair reagents was used to achieve good chromatographic separation [1,4,19,23,24].

Here we developed and validated an ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the simultaneous determination of nine NTs in human brain tissues: aspartic acid (Asp), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), γ-aminobutyric acid (GABA), N-acetyl-L-aspartic acid (NAA), pyroglutamic acid (PyrogGlu), acetylcholine (ACh) and its metabolite choline (Chol). We performed an extensive optimization of sample preparation and chromatographic conditions. This simple method, without derivatization or addition of ion-pair reagents, has been validated according to international guidelines. With its use we analyzed levels of NTs in three different tauopathies—corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and parkinsonism-dementia complex of Guam (PDC). To the best of our knowledge, our study is the first to provide brain tissue levels of these neurotransmitters in three different human tauopathies.

2. Material and methods

2.1. Reagents and materials

Glutamic acid, γ-aminobutyric acid, aspartic acid, ascorbic acid, asparagine, N-Acetyl-L-aspartic acid, acetylcholine, choline, pyroglutamic acid, D9-choline, ammonium formate and LC/MS grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was purchased from Merck, glutamine was purchased from Calbiochem. D6-4-aminobutyric acid, D3-aspartic acid, D5-N-Acetyl-L-aspartic acid, D3-glutamic acid, D5-glutamine, D9-acetylcholine and D5-pyroglutamic acid were from C/D/N isotopes (Quebec, Canada), D5-asparagine was from Cambridge isotopes laboratories. Water was purified using a Millipore system (Bedford, MA, USA). All reagents were of analytical grade.

2.2. Preparation of standards

Individual stock solutions of aspartic acid, glutamic acid, glutamine, choline, N-acetyl-L-aspartic acid, pyroglutamic acid, D5-asparagine, D3-aspartic acid, D3-N-acetyl-L-aspartic acid, D3-glutamic acid, D5-glutamine, D9-choline and D5-pyroglutamic acid were prepared in 0.5 M HCl solution. Acetylcholine, GABA, D6-GABA and D9-acetylcholine stocks were prepared in water. All stocks were stored at −80 °C until use. The analyte stock solutions were serially diluted with 80% acetonitrile to provide working standard solutions of the desired concentrations. Working solutions were prepared from the freshly prepared stock solutions every day for each sample set. Calibration curves of the analytes were prepared by diluting the stock solutions with 80% acetonitrile/water (v/v) and spiked into matrix. Concentration ranges varied from 0.025–250 μg/ml.

2.3. Ultra-performance liquid chromatography coupled to tandem mass spectrometry

A Waters (Waters, Prague, CZ) triple-quadrupole (XEVO TQD) mass spectrometer with electrospray ionization source in positive mode (ESI +) was used. The mass spectrometer was operated with the following parameters: capillary voltage 3 kV, source temperature 150 °C, desolvation temperature 350 °C, cone gas 50 L/h, desolvation gas 600 L h^{-1}. The source cone voltages and collision energies were manually optimized for each SRM transition. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis. Chromatographic separation was performed on an Acquity UPLC BEH Amide column (2.1 mm × 100 mm × 1.7 μm practicle size, HILIC separation) with VanGuard pre-column. Column temperature was maintained at 50 °C. Mobile phase A consisted of 50 mM ammonium formate/0.25% formic acid in water. Mobile phase B consisted of 100% acetonitrile. The gradient of mobile phase was as follows: 90% B (0–0.5 min), decreased to 85% B (0.5–2 min), to 80% B (2–4 min), to 60% B (5–7 min), returning to 90% B and re-equilibrating (7–9 min) before the next injection. The total run time was nine minutes. The flow rate was set at 0.7 mL/min, the column temperature was maintained at 50 °C and the injection volume was 5 μL.

2.4. Human brain tissue samples

We used brain tissues (frontal cortex) from 14 patients with three different tauopathies (5 PDC, 4 PSP and 5 CBD). As controls, 7 subjects were used (Table 3S; Supplementary materials). Brain tissues were obtained from Netherlands Brain Bank (Amsterdam, Netherlands), Queen Square Brain Bank for Neurological Disorders (London, UK) and Binghamton University (Binghamton NY, USA). All tissues were stored at −80 °C until analysis.

Table 1
SRM conditions of neurotransmitters and their internal standards.

Compound name	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)	
Asn	133.11	87.02	0.030	16	12	Used for quantification
	133.11	116.01	0.020	16	12	
D5-Asn	137.96	97.03	0.030	16	12	
Asp	134.08	74.00	0.030	16	12	Used for quantification
	134.08	87.90	0.020	16	12	
D3-Asp	136.98	91.03	0.030	16	12	
NAA	176.20	134.10	0.063	16	10	Used for quantification
	176.20	88.00	0.063	16	10	
D3-NAA	179.07	137.10	0.063	16	10	
Glu	148.20	84.00	0.030	20	18	Used for quantification
	148.20	101.80	0.030	20	18	
D3-Glu	151.10	86.10	0.030	20	18	
Gln	147.10	84.00	0.030	20	18	Used for quantification
	147.10	130.10	0.030	20	18	
D5-Gln	152.10	89.10	0.030	20	18	
GABA	104.00	86.90	0.063	20	11	Used for quantification
	104.00	69.00	0.063	20	11	
D6-GABA	110.10	92.10	0.063	20	11	
ACh	146.11	60.00	0.106	16	14	Used for quantification
	146.11	67.00	0.106	16	14	
D9-ACh	155.09	87.02	0.106	16	14	
Chol	104.10	44.90	0.106	41	30	Used for quantification
	104.10	60.00	0.106	41	30	
D9-Chol	113.10	69.00	0.106	41	30	
PyroGlu	130.10	84.00	0.100	20	13	Used for quantification
	130.10	57.30	0.100	20	13	
D5-PyroGlu	135.10	89.00	0.080	20	13	

2.5. Preparation of brain tissue samples and quality controls (QCs)

The samples were prepared by adding 600 µL of cold extraction solution (80% acetonitrile + 0.1% formic acid) into brain tissue (20–40 mg). Then 10 µl of internal standard (10ug/ml) was added and samples were homogenized using a FastPrep homogenizer on dry ice 3 times for 20 s. The homogenized mixture was centrifuged (30,000*g*/10 min), diluted with 80% acetonitrile (1:1, v/v) and centrifuged again (30,000 × g/4 min). Supernatant was transferred into vials and analyzed with UHPLC/MS. Quality control samples (QCs) were prepared from pooled human brain tissues according to the same extraction method.

2.6. Method validation

The method was validated in accordance with current acceptance criteria for bioanalytical method validation (Food and Drug Administration). Calibrations were performed using six standards. The linear calibration equation (y = ax + b) was generated using the ratio of analyte peak area over IS peak area after quantitative integration by TargetLynx XS software. Calibration linearity was determined by the coefficient of determination (R^2). The limit of detection (LOD, S/N = 3:1) and the limit of quantification (LOQ, S/N = 10:1) were calculated from calibration curves (based on standard deviation of response and slope). The inter-day precision and accuracy of the method was determined by analyzing the three different concentrations over six days. Intra-day accuracy and precision was calculated from six repeat injections. Recovery was evaluated in six replicates at three different spiked concentration levels of analytes. The percentage recovery was determined by comparing of nominal concentration of standard with the concentration difference of analytes between spiked QC sample (homogenized brain tissue) and the non-spiked sample. Recovery was calculated as: Recovery (%) = (concentration after standard spiking of standard—concentration without spiking)/nominal standard concentration × 100. The matrix effect was calculated by comparing area of stable isotope labelled standards in mobile phase and stable isotope labelled standards in brain extracts. Analyte matrix effects were calculated from peak area ratios as follows: Matrix effect (%) = peak area in mobile phase/peak area in brain tissue × 100. The stability of analytes, ISs in the working solution and spiked brain extract was carried out using the autosampler at 4 °C for up to 24 h.

2.7. Statistical analyses

The statistical analyses were performed in R [25]. For all nine variables (Ach, Asn, Asp, Chol, GABA, Gln, Glu, NAA, and PyroGlu) and all six multiple pairs of different tauopathies and controls (CBD vs PDC, CBD vs PSP, CBD vs controls, PDC vs PSP, PDC vs controls, and PSP vs controls), the null hypothesis—population mean difference is equal to zero—was tested against two-sided alternative hypothesis—population mean difference is not equal to zero—by bootstrap two-sample Student *t*-test (number of bootstrap samples 10000) taking to account different variances in each group at a significance level of 0.05 [26]. No global test and multiplicity correction was implemented. The summary tables includes—mean difference (first minus second group), bootstrap empirical 95% confidence interval (CI) for population mean difference (LB: lower bound, UB: upper bound), and bootstrap p-value (in case of bootstrap test, the test statistic is not available). The bootstrap CIs and p-values are visualized as arrows, where statistically significant results (CIs not including zero) are highlighted as black bold. Statistically non-significant results (CIs including zero) are highlighted as grey normal. Longer intervals reflect higher variability. Zero, as a reference, is highlighted as vertical line in grey color.

3. Results and discussion

3.1. Development and optimization of UPLC/MS method

UHPLC conditions were optimized to achieve optimal chromatographic separation and a maximum sensitivity of the mass spectrometry detection. The ionization and fragmentation behaviors of all analytes were also studied. We used positive electrospray ionization because it

easily ionizes molecules with ionizable functional groups such as neurotransmitters. All analytes were detected by MS/MS under collision activated dissociation (CAD) conditions. Using the selected reaction monitoring (SRM) mode, fragment spectra were generated. For each analyte two transitions were measured. One for quantification, the second as confirmatory. For the internal standards one transition was measured. The mass spectrometry parameters are summarized in Table 1.

The optimization of HPLC separation was started by selecting a proper stationary phase. Reversed phase column are common in biomarker studies however they do not offer sufficient retention of small highly polar compounds [18,27]. We tested three types of columns: #1 ethylene-bridged hybrid inorganic–organic particle (BEH) containing columns with reverse stationary phases (Acquity BEH C18, Acquity BEH C8), #2 silica based columns with improved retention of polar compounds (Acquity HSS T3, Acquity HSS Cyano) and #3 Kinetex pentafluorophenyl column that combines several different interaction mechanisms. The initial mobile phase consisted of acetonitrile, as an organic solvent and ammonium formate buffer (10:90, v/v). The concentration of the ammonium formate buffer in the mobile phase was tested at 10 mmol/l, 20 mmol/l and 50 mmol/l. The column temperature was tested at 30 °C, 40 °C and 50 °C. There was no retention of studied analytes using hybrid reverse phase columns and specialized silica based columns. A slightly higher retention (column efficiency range 72–416) was obtained using the pentafluorophenyl column and methanol as an organic phase. Low retention may lead to the co-elution with other endogenous compounds present in the biological matrix and to ion suppression in mass spectrometer. Derivatization or ion pair reagents have been used to overcome low retention problems of small polar compounds [28–30]. Hydrophilic interaction liquid chromatography (HILIC) is a good approach to improve the retention of hydrophilic analytes. HILIC allows high-resolution separation of highly polar substances with mass spectrometry compatible mobile phases [12,31–33]. In our study, Acquity BEH HILIC column and Acquity BEH amide column were tested. Using the Acquity BEH HILIC column retention with column efficiency ranging from 366 to 2428 was achieved. Using the Acquity BEH Amide column we achieved higher separation efficiency, however we also observed peak splitting. This was removed by increasing the ionic strength to 50 mM and temperature to 50 °C. The column optimization results are summarized in Table 1S (Supplementary materials).

In conclusion, the BEH Amide column and the gradient of 50 mM ammonium formate were selected for further validation. The retention time of analytes and associated internal standards was 0.87 min for ACh, 1.50 min for Ch, 3.16 min for PyroGlu, 3.44 min for NAA, 3.8 min for GABA, 4.8 min for Gln, 4.9 min for Asn, 5.14 min for Glu and 5.33 min for Asp (Fig. 1).

3.2. Optimization of sample preparation

Preparation of biological samples is a critical step in an accurate and reliable LC– MS/MS assay. Homogenization and metabolite extraction are the most common sample pre-treatments in metabolomics of brain tissue. The extraction is usually done at low temperature in order to avoid sample degradation and metabolites conversion. Brain tissue analysis also depends on the size of the region which to be analyzed, therefore the small sample (20–30 mg) is recommended for increasing the protocol applicability [5]. To avoid sample degradation, we homogenized brain tissue on dry ice using the FastPrep-24 instrument.

Extraction with organic solvents is preferable method in animal studies [5,22,23,33,34]. We tested several different types of extraction solvents (methanol, acetonitrile, water), at various concentrations, and concentrations of mobile phase additive. Formic acid was used as a suitable additive because it does not cause ion suppression like TFA [35]. The results of sample extraction of nine analytes at different conditions are shown in Fig. 1S (Supplementary materials). The extraction with organic solvent only (acetonitrile or combination of acetonitrile and methanol), regardless of the concentration of FA used was generally not effective (Fig. 1S, conditions 1–18). In some cases (Asp, Glu) the efficiency of extraction was reduced to only 10%. The extraction efficiency of 50–90% was obtained with acidified water only without using any organic solvent (Fig. 1S, conditions 37–39). Pyroglutamate was the only exception, where the extraction efficiency with water was strongly dependent on the concentration of formic acid (5–70%), 0.2% FA being the most effective. For aspartic and glutamic acid, the acidified water was the best studied extraction solvent. These results are in a good agreement with physico-chemical properties of studied analytes. Hydrophilic, water soluble molecules such as NTs are usually less soluble in solvents that are less polar as water. However, using only the water for extraction has a disadvantage that protein precipitation step that must be performed before the injection of samples into the HPLC system. Subsequently, the lower (20%), middle (50%) and higher (8%) concentration of the organic solvent were tested (Fig. 1S, conditions 19–36). Increasing the concentration of organic solvent to 20% or to 50% resulted in 70–100% extraction process efficiency. Protein precipitation is an additional step necessary after extraction, since it has been shown that more than 75% of organic solvent is required to effectively remove proteins from samples before LC/MS analysis [36]. The 80% concentration acetonitrile extracts more than 90% of analytes with the exception of Asp, Glu or Chol where 80% concentration of methanol was more effective. Significant differences were observed for some analytes, when 0.1%, 0.2% or 0.5% FA was added to the extraction solvent as an additive (Fig. 1S, conditions 25–27). Overall, the 0.1% FA helped the extraction process therefore it was subsequently used also for validation studies. In conclusion, our data showed that 80% acetonitrile/0.1% FA is the most effective combination for sample preparation when analyzing neurotransmitters in human brain tissue. Further, this does not require any additional sample preparation steps before HILIC separation. Accordingly. this sample preparation method was used for the method validation and human brain tissue analysis.

3.3. Method validation

For each neurotransmitter, the calibration was performed using internal standard procedure with stable isotope labelled standards [37]. The linear calibration curves (coefficients of determination > 0.99) were obtained for all analytes within the studied concentration ranges (Fig. 2S; Supplementary materials). The linear regression equations, correlation coefficients, the limit of detection (LOD) and the limit of quantification (LOQ) are summarized in Table 2. The intra-day and inter-day precision and accuracy results at three concentrations levels are shown in Table 3. The intra-day precision (% RSD) for all analytes ranged from 0.7% to 7.1%, and the accuracy was within 93.6% to 102.6%. Similarly, for inter-day experiments (for six consecutive validation days), the precision varied from 3.2% to 8.9% and the accuracy was within 92.6% to 104.3%. The matrix effects of the NTs ranged from 89 % to 122 % in the matrix of the brain extract. The recovery values during extraction process ranged from 83% to 113% (Table 4). Two different brain tissues were used as QC samples. The RSDs (%) for all analytes ranged from 0.1 to 3.86% (n = 6) (Table 3S, Supplementary materials). All analytes were stable with values within the acceptance limits of accuracy (± 15% RE) and precision (± 15% CV) (Table 2S; Supplementary materials). The results of stability studies also indicate that stock solutions of standards and internal standards were stable at least for two months when stored at −20 °C or six months when stored at −80 °C (data not shown).

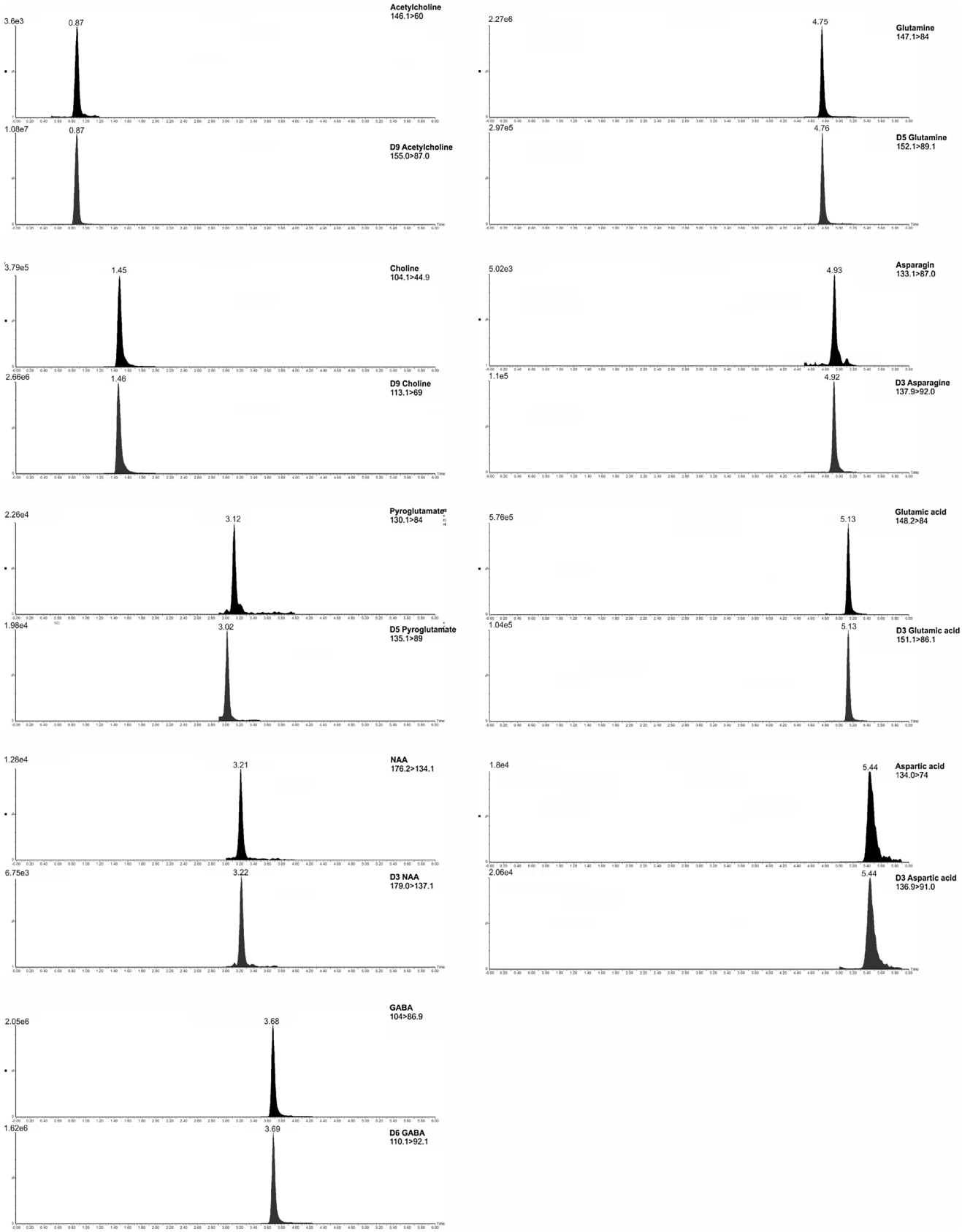

Fig. 1. Extracted ion chromatograms of 9 targeted analytes in human brain tissue. Selected reaction monitoring (SRM) was used to detect targeted analytes. Chromatographic conditions: column, Acquity BEH Amide column (2.1 mm × 100 mm, 1.7 µm particle size) with VanGuard pre-column; flow rate = 0.7 mL min^{-1}; column temperature 50 °C.

Table 2
Linearity parameters, limit of quantification and limit of detection.

Analyte	Retention time	r^2	Regression equation[a]	Linear range[b]	LOQ[c]	LOD[d]
GABA	3.80	0.9996	0.0419x + 0.005	2.5–250	6.06	1.820
Chol	1.50	0.9994	0.0647x + 0.050	2.5–250	2.07	0.620
PyroGlu	3.16	0.9984	0.0070x + 0.006	0.25–25	0.154	0.046
Asn	4.90	0.9986	0.0059x + 0.001	0.25–25	0.29	0.088
Asp	5.33	0.9984	0.1222x + 0.078	2.5–250	6.42	1.930
Ach	0.87	0.9997	0.0005x + 0.0002	0.025–2.5	0.037	0.012
Gln	4.80	0.9996	0.1771x + 0.022	2.5–250	3.51	1.050
Glu	5.14	0.9995	0.2260x + 0.023	2.5–250	5.05	1.520
NAA	3.44	0.9989	0.0084x + 0.006	0.25–25	0.84	0.250

[a] The calibration curves were constructed by plotting the IS/analyte ratio versus the concentration of each analyte. Each calibration curve was derived from seven data points (n = 7).
[b] Linear range in $\mu g\,ml^{-1}$.
[c] LOQ referred to the limits of quantification $\mu g\,ml^{-1}$.
[d] LOD referred to the limits of detection in $\mu g\,ml^{-1}$.

3.4. Analysis of brain samples from different tauopathies

Transition of tau protein from its highly soluble form into the insoluble aggregates is a common process in several neurodegenerative disorders known as tauopathies [38]. Our aim was to investigate how tau induced neurodegeneration affects other biological pathways in CNS. Here we analyzed the concentrations of nine neurotransmitters in brain tissue in the areas affected by tauopathy process. The clinical data and pathological findings of the control subjects and the patients with different tauopathies are summarized in Table 3S (Supplementary materials). We found a significant decrease of brain tissue concentrations of GABA, asparagine, glutamine and N-acetyl-L-aspartic acid in all three tauopathies. The level of pyroglutamic acid levels was reduced only in PSP and CBD, there was no change observed in PDC. Glutamic acid levels were slightly reduced in PSP, there was no change in PDC and CBD. There was a significant increase of aspartic acid in all tauopathies when compared to the controls. Acetylcholine and choline levels were not changed in the patients (Table 5 and Fig. 2). The results of bootstrap two-sample Student *t*-test are summarized in Table 4S (Supplementary materials).

We found significant decrease of GABA and glutamine levels in the brain tissues of the patients with tauopathies. In brain glutamine synthesis occurs exclusively in astrocytes. Glutamine serves as an important precursor for the synthesis of two important neurotransmitters, glutamic acid and GABA. Glutamate-glutamine-GABA pathway is important in neuron-glia communication [39]. Glutamic acid is

Table 3
Intra-day and inter-day accuracy and precision statistics.

		Intra-day (n = 6)			Inter-day (n = 18)		
Compound	Spiked concentration[a]	Mean concentration[a]	RSD (%)	Accuracy (%)	Mean concentration[a]	RSD (%)	Accuracy (%)
GABA	5.00	4.84	0.72	96.81	4.78	3.46	95.64
	25.00	24.40	2.07	97.60	25.08	6.04	100.30
	250.00	252.84	1.40	101.13	250.32	4.12	100.13
Chol	2.50	2.47	7.11	98.61	2.48	5.91	99.03
	10.00	10.03	0.98	100.30	9.75	5.90	97.48
	250.00	250.64	1.44	100.26	249.99	4.54	99.99
PyroGlu	0.25	0.25	5.32	100.27	0.25	8.93	100.72
	2.50	2.49	3.71	99.41	2.41	6.56	96.47
	25.00	25.28	2.90	101.11	25.74	4.84	102.97
Asn	0.50	0.51	3.11	102.56	0.50	7.48	100.33
	2.50	2.55	6.40	101.99	2.46	5.63	98.34
	25.00	25.22	1.13	100.87	25.67	4.25	102.67
Asp	5.00	4.68	2.23	93.55	4.63	4.99	92.59
	25.00	24.64	3.07	98.54	24.35	7.27	97.41
	250.00	251.26	2.18	100.50	253.15	3.96	101.26
ACh	0.050	0.048	1.60	96.68	0.047	3.69	93.90
	0.25	0.25	6.77	101.77	0.25	5.52	98.69
	2.50	2.53	1.39	101.02	2.56	3.74	102.27
Gln	5.00	4.85	2.64	96.95	4.81	3.57	96.29
	50.00	48.66	1.35	97.32	48.93	4.23	97.85
	250.00	251.10	0.88	100.44	254.33	3.22	101.73
Glu	5.00	4.85	1.91	97.07	4.75	4.73	94.92
	50.00	48.29	5.21	96.57	49.05	4.85	98.11
	250.00	250.68	2.83	100.27	255.45	3.56	102.18
NAA	0.50	0.47	3.29	94.08	0.48	7.32	96.47
	5.00	4.91	3.06	98.17	4.92	8.17	98.31
	25.00	25.27	4.46	100.91	26.07	6.29	104.27

[a] in $\mu g\,ml^{-1}$.

Table 4
Extraction recovery and matrix effects.

Compound	Spiked concentration[a]	Mean concentration[a]	Recovery%	Matrix effect%
GABA	5	4.65	93.00	95.7
	10	9.55	95.50	
	25	23.00	92.00	
Chol	0.5	0.49	98.00	98.7
	2.5	2.66	106.40	
	5	4.95	99.01	
PyroGlu	1	0.56	111.40	89.0
	5	5.31	106.20	
	10	11.16	111.60	
Asn	0.50	0.51	102.31	116.8
	1	1.03	103.06	
	5	5.18	103.70	
Asp	5	5.20	104.00	111.9
	10	11.00	110.00	
	25	28.30	113.20	
Ach	0.025	0.021	82.30	108.1
	0.05	0.043	85.10	
	0.10	0.086	85.90	
Gln	25	22.78	91.10	112.5
	50	51.67	103.30	
	100	102.78	102.80	
Glu	25	24.60	98.40	115.5
	50	54.41	108.80	
	100	89.01	89.01	
NAA	5	5.46	109.20	122.5
	10	9.77	97.70	
	50	47.76	95.50	

[a] in $\mu g\,ml^{-1}$.

transported to astrocytes via glutamate transporters where it is converted into glutamine by glutamine synthetase (GS). Therefore, the changes in glutamine could be related to reduced activity of GS due to astrogliosis present in PSP and CBD cases. Reduced GS expression was described previously in AD patients [40,41].

GABA is the major inhibitory neurotransmitter in CNS that is synthetized by glutamic acid decarboxylase (GAD). In neurodegeneration, the decrease of GABA is often associated with reduced activity of GAD. Although in all three tauopathies, subcortical structures are primarily affected by pathology, the neuropathological studies demonstrated changes also in cerebral cortex [42,43]. The PET and histopathological studies in PSP in demonstrated loss of GABAA receptors in the cerebral cortex [44]. Here we found significant decrease of GABA in frontal cortex of PSP patients. Interestingly there was no change of GAD in PSP and as previous reports demonstrated, no change/increase of GABA in the subcortical structures [45].

Similarly, to the previous studies of FTD and Pick patients we did not find any significant changes in cholinergic system in PDC, PSP and CBD. This is in contrast to AD where abnormalities in cholinergic pathways are common and several cholinergic drugs are successfully used in humans to treat the symptoms of neurodegeneration [46].

We found a significant decrease of asparagine in all three tauopathies. Asparagine is the common N-glycosylation site on proteins. Moreover, asparagines have alpha-helix inducing and stabilizing role [47]. Depletion of asparagine from the CNS is associated with altered mental status [48]. The transport of asparagine across the blood-brain barrier is limited, which suggests that brain depends on local de novo synthesis. Asparagine synthetase catalyzes the conversion of aspartic acid to asparagine. Reduced activity of asparagine synthetase is responsible for congenital microcephaly and a progressive form of encephalopathy [49].

The levels of NAA were reduced in studied tauopathies. NAA is a suitable marker for neuronal damage in human brain during neurodegeneration. In our study the tissue concentrations of NAA did no vary significantly with the age [50]. A decrease of NAA in PDC, PSP and CBD suggest massive neurodegeneration and neuronal loss. Reduced levels of NAA in frontal and temporal cortices was already described for other tauopathies such as AD [51].

4. Conclusions

In conclusion, we developed and validated a simple and effective method for the quantification of nine neurotransmitters in human brain tissue. The presented method has several advantages compared to the previously reported methods as it provides higher selectivity, no sample pre-treatment and chromatographic separation without the use of ion-pair reagents. The concentrations of neurotransmitters in frontal cortices of patients with three different tauopathies were quantified, demonstrating the applicability of our method in human clinical studies. We were the first to show the significant differences of cortical concentrations of NTs that correlate with the tauopathy process in the brain. In the future, we would like to apply this method to study different tauopathies, which may aid the development of novel therapeutic approaches.

Table 5
Brain tissue concentrations of neurotransmitters in different tauopathies.

	arithmetic average ± SD[a]			
	CBD	PDC	PSP	controls
Acetylcholine	0.397 ± 0.293	0.111 ± 0.06	0.424 ± 0.35	0.287 ± 0.076
Asparagine	0.044 ± 0.01	0.03 ± 0.012	0.06 ± 0.005	0.828 ± 0.355
Aspartic acid	0.448 ± 0.161	0.434 ± 0.141	0.577 ± 0.127	0.245 ± 0.063
Choline	0.038 ± 0.018	0.052 ± 0.05	0.068 ± 0.011	0.026 ± 0.011
GABA	0.103 ± 0.028	0.077 ± 0.032	0.112 ± 0.005	0.16 ± 0.014
Glutamine	0.797 ± 0.166	1.193 ± 0.172	1.083 ± 0.202	1.788 ± 0.2
Glutamic acid	1.693 ± 0.652	1.431 ± 0.412	2.208 ± 0.317	1.722 ± 0.373
N-acetylaspartic acid	1.524 ± 0.673	1.575 ± 0.238	1.554 ± 0.267	2.946 ± 0.376
Pyroglutamate	0.066 ± 0.043	0.237 ± 0.093	0.032 ± 0.023	0.211 ± 0.156

SD – standard deviation.
PDC – parkinsonism-dementia complex of Guam.
CBD – corticobasal degeneration.
PSP – progressive supranuclear palsy.
[a] in $\mu g\,mg^{-1}$ of tissue.

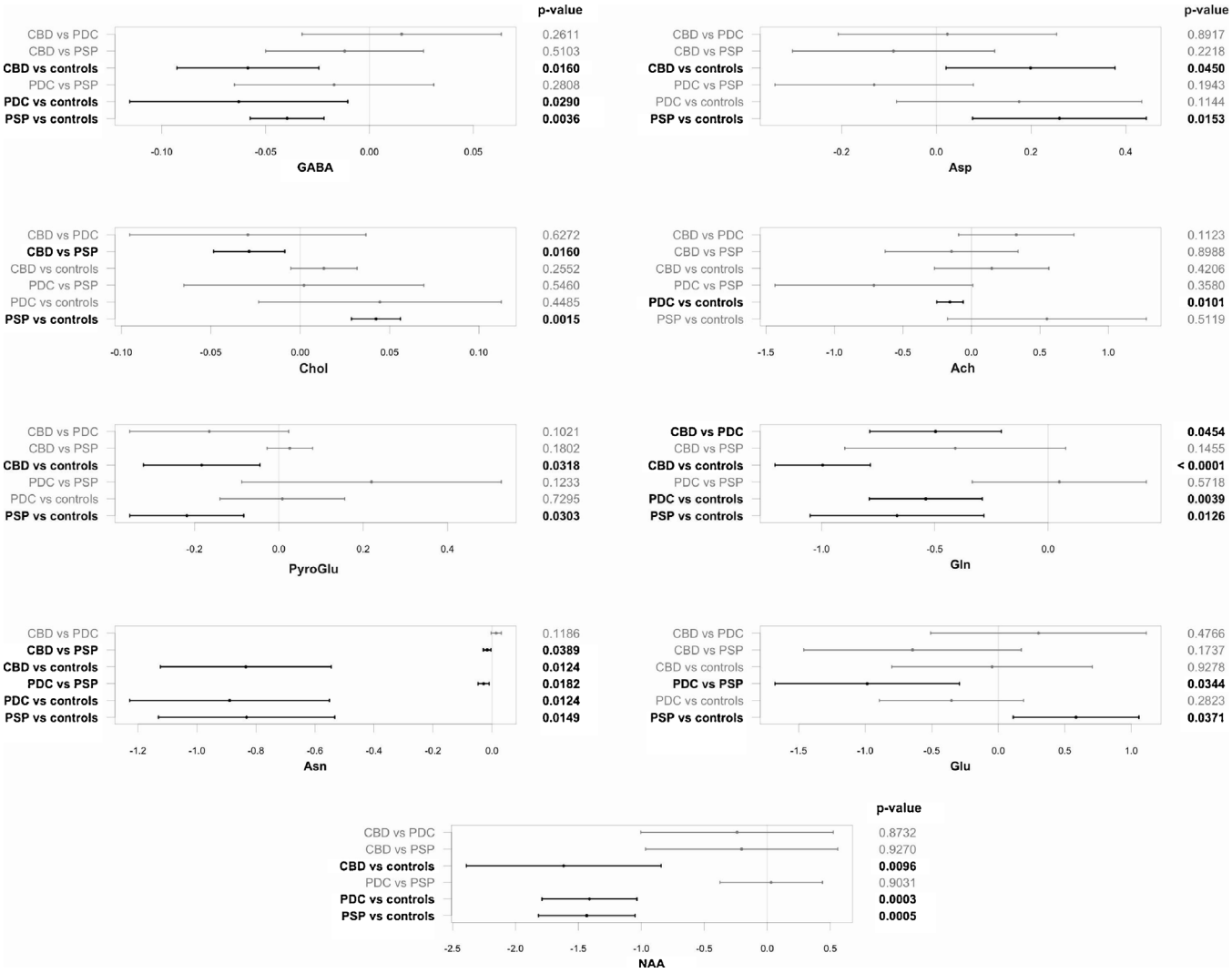

Fig. 2. Changes of neurotransmitters in different tauopathies. The bootstrap confidence intervals were visualized as arrows, statistically significant results (confidence intervals not including zero) are highlighted in black bold, statistically nonsignificant results (confidence intervals including zero) are highlighted in gray normal. Longer interval reflects higher variability. Zero, as a reference, is highlighted as vertical line in gray.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jchromb.2017.12.015.

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