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Development and application of a liquid chromatography/tandem mass spectrometric assay for measurement of *N*-acetylaspartate, *N*-acetylaspartylglutamate and glutamate in brain slice superfusates and tissue extracts

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ABSTRACT

A liquid chromatography–tandem mass spectrometric method has been developed for measurement of *N*-acetylaspartate, *N*-acetylaspartylglutamate and glutamate. The analytes were separated within 5 min using an anion exchange/reverse phase column. The lower limit of quantification for Glu, NAA and NAAG was found to be 5, 50 and 6 nM, respectively, with a signal-to-noise ratio of 5:1. Using this methodology the basal levels of Glu, NAA and NAAG could be measured consistently in *in vitro* superfusion samples from rat hippocampus. The assay was also used for measurement of the distribution of Glu, NAA and NAAG in different regions of the rat brain.

1. Introduction

N-Acetylaspartate (NAA) and *N*-acetylaspartylglutamate (NAAG) are two of the most abundant biochemicals in the mammalian brain. NAA is synthesized within the brain, primarily in neurons and represents around 7% of neuron osmolarity [1]. Its presence in neurons is used as a marker of neuronal integrity and the concentration of NAA is altered in all neurological disorders involving neuronal loss or degeneration [2,3]. In addition, NAA is the precursor of NAAG and its availability may limit the rate of synthesis of NAAG by glia under some conditions [4]. NAAG has been shown to have neurotransmitter or neuromodulator activity in the CNS and is often co-localised with other neurotransmitters including glutamate (Glu) and γ-aminobutyric acid (GABA) [3,5]. It has been shown using receptor binding studies that NAAG is a low-potency agonist at the NMDA receptor and a highly selective agonist at the type II metabotropic glutamate receptors (i.e. mGluR2 and mGluR3) [6]. It is proposed to act via these receptor subtypes to reduce cAMP levels, decrease voltage-dependent neurotransmission, suppress excitotoxicity, influence long-term potentiation and depression, regulate $GABA_A$ receptor subunit expression, and inhibit release of GABA from cortical neurons [7–10]. In addition, NAAG is rendered inactive in the extracellular space by hydrolysis to Glu and NAA, a reaction catalysed by the astrocyte membrane bound enzyme, glutamate carboxypeptidase II/III [11]. The Glu derived from NAAG may serve as a releasable pool of the excitatory neurotransmitter [12]. Hence, any changes in the synthesis or release of NAAG either under normal circumstances or under pathological conditions will alter glutamatergic neurotransmission.

Using magnetic resonance spectroscopy (MRS) it has been shown that there is regional-specific alterations of NAA/NAAG levels in the brain of patients with bipolar disorders and schizophrenia [13,14]. Additionally, post mortem studies have found evidence of regional NAA and NAAG alterations in schizophrenia and bipolar disorder patients [15,16]. Furthermore, regional deficiencies in NAA and NAAG were also found in rats reared in isolation and in rats administered chronically with the NMDA antagonist phencyclidine (PCP), which are considered to be putative animal models of schizophrenia [17,18].

One of the first reported methods for the measurement of NAA and NAAG in brain extracts was based on anion-exchange chromatography with UV detection at 210 nm [19]. The possible co-elution of the analytes in brain extracts with other negatively

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charged low molecular weight UV absorbing metabolites of the brain was not shown by these authors. Tavazzi et al. [20] reported an ion pair chromatography assay based on UV detection at 210 nm for measurement of NAA and NAAG in brain extracts and showed separation of the analytes from other brain metabolites. The limits of detection achieved using these UV based methods are adequate for measurement of NAAG in brain tissue extracts but these are insufficient for measurement of the analytes in superfusates. Other techniques that have been reported for measurement of NAAG are gas chromatography with mass spectrometry [21,22] and pre-column derivatisation with fluorescence detection [23]. Although the limits of detection obtained using these methods are adequate for measurement of NAAG in superfusates they involve complicated sample preparation steps and the separation time is relatively long. Over last few years assays for measurement of NAA based on mass spectrometry have been reported by several authors. The limits of detection reported for NAA are acceptable for measurement of the analyte in brain extract [24] and urine [25]. However, these limits of detection are inadequate for determination of NAA and NAAG in superfusates.

The present work describes a new rapid liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the simultaneous measurement of Glu, NAA and NAAG in superfusion samples and brain extracts. Using an *in vitro* superfused brain tissue slice preparation we measured basal and stimulated efflux of Glu, NAA and NAAG. We also demonstrated that the efflux of these analytes from rat hippocampal slice is a tetrodotoxin (TTX)-dependent process. Furthermore, we showed that the assay can be applied to the simultaneous measurement of all three analytes (Glu, NAA and NAAG) in tissue samples taken from distinct structures of the rat brain.

2. Materials and methods

2.1. Reagents

Glutamate, NAA and NAAG were obtained from Sigma–Aldrich (Poole, UK). Formic acid of HiperSolv grade was purchased from BDH (Poole, UK). Acetonitrile of far UV grade was obtained from Fischer Scientific (Loughborough, UK). All other chemicals were of analytical reagent grade and purchased from Sigma–Aldrich. All aqueous solutions were prepared using deionised water from an Elga maxima system (Elga, High Wycomb, UK).

2.2. Instrumentation and conditions

A HPLC system consisting of two Jasco model PU-1585 HPLC pumps (Great Dunmow, UK) a Prolab model 2006 degasser (Presearch, Hitchin, UK) a Jasco 1580-32 dynamic mixer and a HTS Pal autosampler from Presearch fitted with a Valco six port injection valve and a 20 μl loop was used. Separations were performed using a 50 mm × 2.1 mm i.d. Primesep D column (Hichrom, Reading, UK). Analytes were separated using a binary gradient elution profile composed of eluent 'A'—0.2% formic acid in a mixture of water and acetonitrile (50:50, v/v) and eluent 'B'—0.5% formic acid in a mixture of water and acetonitrile (25:75, v/v) (Table 1). A flow rate of 0.4 ml/min was used. The column temperature was maintained at 35 °C using a Jones Chromatography (Carnforth, UK) model 7990 column oven. Eluates were detected using an Applied Biosystems Sciex API-4000 triple-quadrupole mass spectrometer (Warrington, UK) equipped with a TurboIonSpray ion-source. The operating parameters of the ion-source, including analyte dependent and source dependent were optimised to obtain the optimum performance from the mass spectrometer for the analysis of Glu, NAA and NAAG. The sensitivity of detection for the three analytes in the positive ion mode was found to be higher than in negative mode. Hence, the mass spectrometric parameters were optimised to generate maximum level of the protonated molecule. The source-dependent parameters for the three analytes consisted of collision gas, curtain gas, ion spray gas 1 and 2, ionspray voltage and the temperature of the heater gas, with optimum values of 4, 10, 60, 10, 4.5 kV and 450 °C, respectively. The analyte-dependent parameters were also tuned to obtain the maximum detector response (Table 2). The ion spray voltage was set at 4.5 kV and the source temperature at 450 °C. The mass spectrometer was operated at unit mass resolution for both Q1 and Q3 in molecular reaction monitoring (MRM) mode. The precursor-to-product transitions of m/z 148.1 → 84.2 for glutamate, m/z 176.1 → 133.9 for NAA and m/z 305.2 → 148.1 for NAAG were monitored. Data were acquired and processed using Analyst version 1.4.1 software.

Table 1
Elution profile used for separation of Glu, NAA and NAAG.

Time (min)	Eluent A (%)	Eluent B (%)	Gradient
0	100	0	
2	100	0	
2.1	0	100	Linear
10	0	100	Isocratic
11	100	0	Linear

2.3. Ion-exchange matrices

Isolute PE-AX (Biotage, Hertford, UK), Oasis MAX (Waters, Elstree, UK), Strata SAX (Phenomenex, Macclesfield, UK) and Vivapure Mini Q high capacity spin column (Vivascience, Epsom, UK) were tested for extraction of Glu, NAA and NAAG from superfusion samples.

2.4. Animals

Animals were housed in a temperature and humidity controlled environment with free access to food and water. Rats were kept on a 12 h light–dark cycle with lights on at 06:00 h. Studies were conducted in compliance with the Home Office Guidance on the operation of the UK Animals (Scientific Procedures) Act 1986, and were approved by the GlaxoSmithKline Animal Procedures Review Panel.

2.5. Steady-state measurement of glutamate, NAA and NAAG in brain tissue extracts

Male Lister Hooded rats (Charles River, UK) were killed and brains were removed, snap frozen and then stored at −80 °C until dissection. Brain microdissections were performed using the Maps and Guide to Microdissection of the Rat Brain [26]. Tissue samples of frontopolar cortex (FPC; A5400–A3000 μm), cingulate cortex (CCi; A3000–A900 μm), caudate nucleus (c; A3000–A900 μm), nucleus accumbens shell (a (shell); A3000–A900 μm), nucleus accumbens core (a (core); A3000–A900 μm), dentate gyrus (DG; P3000–P4800 μm including dentate gyrus and CA1) dorsal hippocampus (dHI; P3000–P4800 μm including CA2, CA3 and CA4), temporal cortex (CTe; P3000–P4800 μm), entorhinal cortex (CE; P3000–P4800 μm) and ventral hippocampus (vHI; P4800–P6600 μm including CA2, CA3 and CA4) were dissected bilaterally from 2 mm coronal slices of each brain. Frontopolar cortex, temporal cortex and entorhinal cortex were dissected using a scalpel. Caudate nucleus was dissected using a 4 mm

Table 2
Analyte-dependent parameters of the MS detector.

Compound	Q1 m/z (Th)	Q3 m/z (Th)	Time (ms)	Parameter[a]			
				DP (V)	EP (V)	CE (V)	CXP (V)
Glu	148.1	84.2	150	31	10	19	7
NAA	176.1	133.9	150	61	10	13	8
NAAG	305.2	148.1	150	45	10	15	9

[a] DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

microdissection needle. A 2 mm microdissection needle was used to obtain tissue from the remaining regions. Each tissue sample was homogenised using a Gallenkamp Soniprep 150 (Sanyo, Loughborough, UK) in 0.1% formic acid in a mixture of methanol and water 95:5, v/v (40 μl/mg wet weight tissue). The resultant slurry was centrifuged using a Labofuge 400 R (Heraeus Instruments, Langenselbold, Germany) at 5590 × *g* for 10 min at 4 °C. A small volume (10 μl) of the supernatant was diluted with 90 μl of 0.1% formic acid in a mixture of methanol and water (95:5, v/v). An aliquot (5 μl) of the diluted sample was loaded onto the HPLC column.

2.6. Recovery and assessment of matrix effects

Standards, pre- and post-extraction samples used for recovery and assessment of matrix effects were prepared using two mixtures of Glu, NAA and NAAG at different concentrations. Two sets of four samples of mixture of Glu, NAA and NAAG were prepared in eluent 'A'. Frontopolar cortex tissue samples from Lister Hooded rats were dissected as described in Section 2.5. Two sets of four tissue samples were homogenised (40 μl per mg wet weight tissue) in mixture of methanol, water and formic acid (95:4.9:0.1, v/v/v) containing a blend of Glu, NAA and NAAG. The resultant slurry was treated as described in Section 2.5. Another three sets of four tissue samples were extracted using a mixture of methanol, water and formic acid alone (95:4.9:0.1, v/v/v). The supernatant from two of these sets were diluted in a mixture of methanol and water (95:5, v/v) containing a combination of Glu, NAA and NAAG. The third set of supernatant was diluted in a mixture of methanol and water alone (95:5, v/v).

2.7. In vitro hippocampal slice superfusion

Six male Sprague–Dawley rats (Charles River, UK) were killed, the brains removed and dissected out immediately and placed in ice-cold Krebs solution that contained 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 10 mM glucose, 0.06 mM L-ascorbic acid and 0.03 mM Na_2EDTA (pH 7.4) which was saturated with 95% O_2/5%CO_2. The Krebs solution was sterilised by passing it through a 0.2 μm autoclaved Nylon filter. The hippocampi were isolated and 300 μm slices were prepared on a McIlwain tissue chopper (Mickle Laboratory Engineering, Guilford, UK). The tissue samples were rinsed with oxygenated Krebs Ringer solution that had been maintained at 37 °C. Release experiments were performed using a Brandel Suprafusion 2500 series system (Gaithersburg, USA). Immediately after rinsing the samples (120 μl) were transferred to superfusion chambers. The tissue slices were retained in the chambers using nylon mesh filter discs (Semat International, St. Albans, UK). Following a 30 min equilibration, the slices were superfused with Krebs solution at a flow rate of 250 μl/min. Individual samples (1.0 ml) were collected every 4 min for 12 min. These superfusates were used to determine the efflux under resting conditions. One-half of the tissue samples were then superfused with normal Krebs solution and the remainder with Krebs solution containing 10 μM TTX. Samples were collected every 4 min for 32 min. The tissue samples were then given a 2 ms biphasic pulses of 15 Hz at 20 mA for 3 min and fractions were collected every 4 min for further 28 min.

2.8. Preconcentration of superfusates

A small volume (400 μl) of superfusion sample was diluted with an equal volume of deionised water and 100 μl of 0.5 M ammonium hydroxide. Samples were desalted and concentrated using Vivapure Mini Q high capacity spin column. This involved equilibration, loading, washing and elution steps. The column was centrifuged at 5590 × *g* for 10 min at 4 °C between each step and filtrate resulting from the equilibration, loading and washing steps was discarded. The column was equilibrated with two aliquots (450 μl) of 1 M phosphate buffer (pH 6.5) and the sample was then loaded. The column was washed sequentially with 1 ml of 2 mM phosphate buffer (pH 6.0) and 500 μl of deionised water. The bound analytes were eluted from the column using 50 μl of a mixture of 0.3 M HCl and methanol (80:20, v/v). A small portion (5 μl) of the extract was loaded onto the HPLC column. Calibration standards were extracted using an identical procedure to that described for samples.

3. Results and discussion

3.1. HPLC–MS/MS optimisation

Several different anion-exchange columns were tested for separation of Glu, NAA and NAAG. Optimal separation of all three analytes was achieved using a Primesep D column. The column is comprised of anion exchange and long alkyl sites chemically bonded to a silica support. The ion-exchange group is positively charged throughout the recommended working pH range of 1.5–7.0. This allows small negatively charged molecules to be retained and separated by anion exchange and reverse phase mechanisms. For Glu, NAA and NAAG it was found that increasing the ionic strength and organic content of mobile phase reduced their retention on the column. This demonstrates that both reverse phase and ion-exchange mechanisms contribute to the retention mechanism of these analytes. Isocratic and linear gradients were tested for separation of Glu, NAA and NAAG. A steep linear gradient elution profile gave optimal separation and good HPLC-peak shape for all three analytes. Since the Glu that arises from hydrolysis of NAAG may serve as pool of the excitatory amino acid, this additional measurement provides valuable information on the potential impact of these analyte changes on the overall glutamatergic neurotransmission in the system under evaluation.

Glutamate, NAA and NAAG were found to predominantly form protonated molecules ($[M+H]^+$) in the TurboionSpray ion-source. The collision associated fragmentation of Glu, NAA and NAAG precursor ions at m/z 148, 176 and 305 produced a number of discrete product ions. Of these precursor-to-product ion transitions, the reactions 148 → 84, 176 → 134 and 305 → 148 produced the highest ion currents with the best signal-to-noise ratio, which are consistent with those reported by other authors [21,27]. These product ions were used for the simultaneous measurement of Glu, NAA and

NAAG. Fig. 1 shows a MRM chromatogram of mixture of the three analytes in a superfusion sample.

3.2. Linearity, repeatability and detection limits

Studies of HPLC-peak area as a function of concentration were carried out using standard mixtures of Glu, NAA and NAAG prepared in either Krebs solution or 0.1% formic acid in a mixture of methanol and water (95:5, v/v). Standards used for superfusion studies were processed using Vivaspin columns prior to analysis. For seven concentrations in Krebs solution in the range 0–10 μM of Glu and 0–1.0 μM for NAA and NAAG linear correlations were obtained with coefficients of 0.99. Similarly, for seven concentrations of Glu and NAA in 0.1% formic acid in a mixture of methanol and water (95:5, v/v) in the range 0–50 μM and NAAG in the range 0–2.5 μM linear coefficients were obtained in the range 0.98–0.99. The repeatability of the assay was determined by analysing three samples of mixture of Glu and NAA at concentration of 0.1 and 1 μM NAAG in a mixture of methanol and water (95:5, v/v). The intra-day precision was found to be between 0.8 and 7.3% ($n = 3$); the inter-day precision was 6–16% ($n = 4$).

Lower limit of quantification of 5, 50 and 6 nM was obtained for Glu, NAA and NAAG, respectively with a signal-to-noise ratio of 5:1. The method was used for the analysis of brain tissue extracts and samples arising from *in vitro* efflux experiments.

3.3. Preconcentration of glutamate, NAA and NAAG from superfusates

Analysis of hippocampus superfusion samples using LC–MS/MS demonstrated that basal levels of NAAG were below the limit of detection. Consequently, superfusates were desalted and concentrated using anion-exchange separation. A variety of anion-exchange solid phase extraction (SPE) resins together with an anion-exchange spin column were tested. The recovery of Glu, NAA and NAAG was found to be similar to all anion-exchange SPE resins tested. However, the final elution volume that was used to recover the analytes from the SPE resins was too high to allow sufficient concentration of the analytes without an additional evaporation and re-constitution step. In comparison to SPE, the analytes could be eluted from the spin column using a fraction of the load volume. This allowed Glu, NAA and NAAG to be concentrated by a factor of three to four. The recovery of Glu, NAA and NAAG was found to be 75, 80 and 85%, respectively. This was sufficient to allow quantification of NAAG in superfusion samples using tandem mass spectrometry.

3.4. Superfusion study

The effects of the voltage-dependent Na^+ channel blocker, TTX on basal efflux and stimulated efflux of Glu, NAA and NAAG from rat

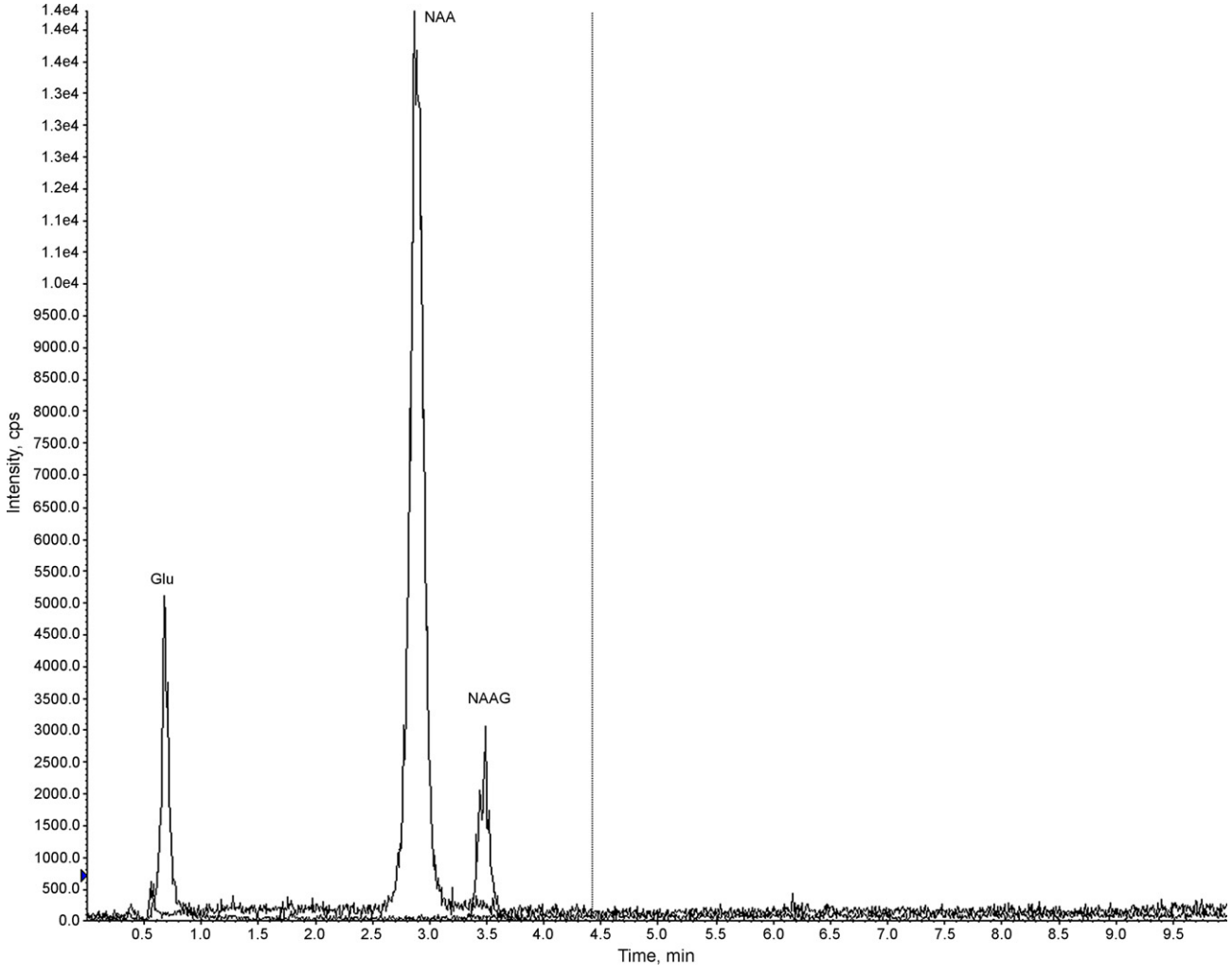

Fig. 1. MRM chromatogram of hippocampal superfusate analysed for Glu, NAA and NAAG. LC–MS/MS conditions as described in Section 2.

superfused hippocampal slices are shown in Fig. 2. Pre-treatment baseline levels were in nM range, Glu = 140 ± 10, NAA = 280 ± 30 and NAAG = 5.5 ± 0.5. In keeping with literature data, spontaneous/basal efflux of Glu is TTX-insensitive [28]. In absence of TTX in the superfusion medium, the efflux of Glu increased upon electrical stimulation reaching a maximum of 290 ± 50% of basal pre-treatment baseline levels. In contrast with previous reports [29,30] this was achieved in absence of uptake inhibitors and using relatively mild electrical parameters. This increase in Glu efflux was completely abolished by TTX, suggesting that the increase in efflux is mediated via membrane depolarization and subsequent neuronal release [29]. Interestingly, NAAG and NAA efflux followed a pattern that mirrors the efflux of Glu; basal efflux was TTX-insensitive but electrical stimulation elicits a TTX-sensitive increase in the efflux of both NAAG (maximum increase 330 ± 50%) and NAA (300 ± 20%). These data are in agreement with previous studies where NAAG efflux was elevated in an impulse-dependent manner following K^+-evoked depolarization [21]. Since NAAG is mainly concentrated in axonal terminals of neurons [12,31] it can be argued that increases in NAAG efflux upon membrane depolarization are mainly produced by neurons. Under physiological conditions, NAAG is enzymatically cleaved in the extracellular space to NAA and Glu by glutamate carboxypeptidase II or III [10] which suggest that some of the concurrent increases in NAA levels may be, at least in part, a result of NAAG breakdown. However, the high concentration of phosphate buffer used in the perfusion media may be inhibiting the carboxypeptidases activity [32] which would indicate the existence of a releasable pool of NAA.

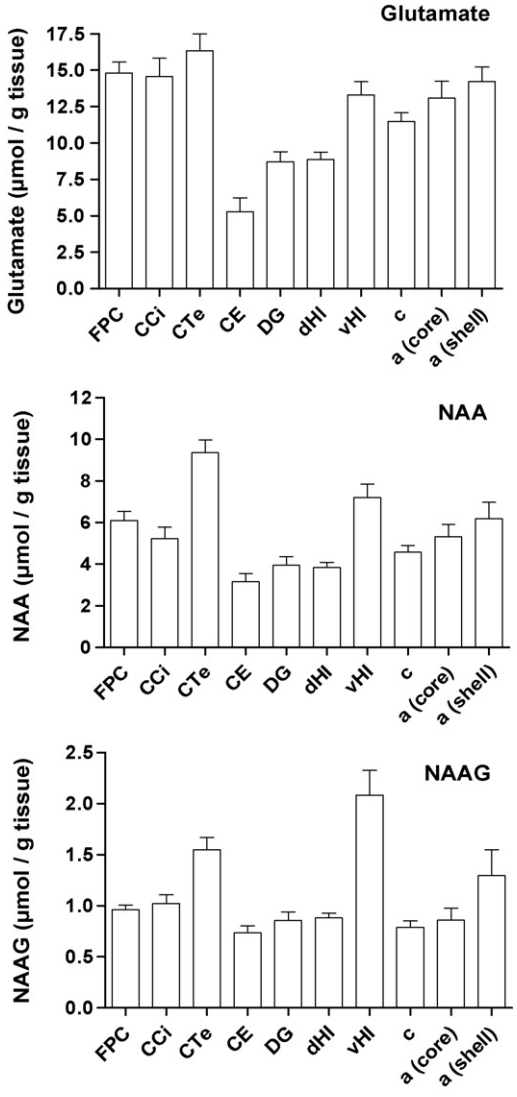

Fig. 2. Regional differences in steady-state concentrations of Glu, NAA and NAAG in the rat frontopolar cortex (FPC), cingulate cortex (CCi), temporal cortex (CTe), entorhinal cortex (CE), dentate gyrus (DG) dorsal hippocampus (dHI), ventral hippocampus, (vHI), caudate putamen (c), nucleus accumbens core (a (shell)) and nucleus accumbens shell (a (shell)). Data are expressed as mean ± S.E.M. (n = 6–9) as µmoles per g of wet tissue (µmol/g tissue).

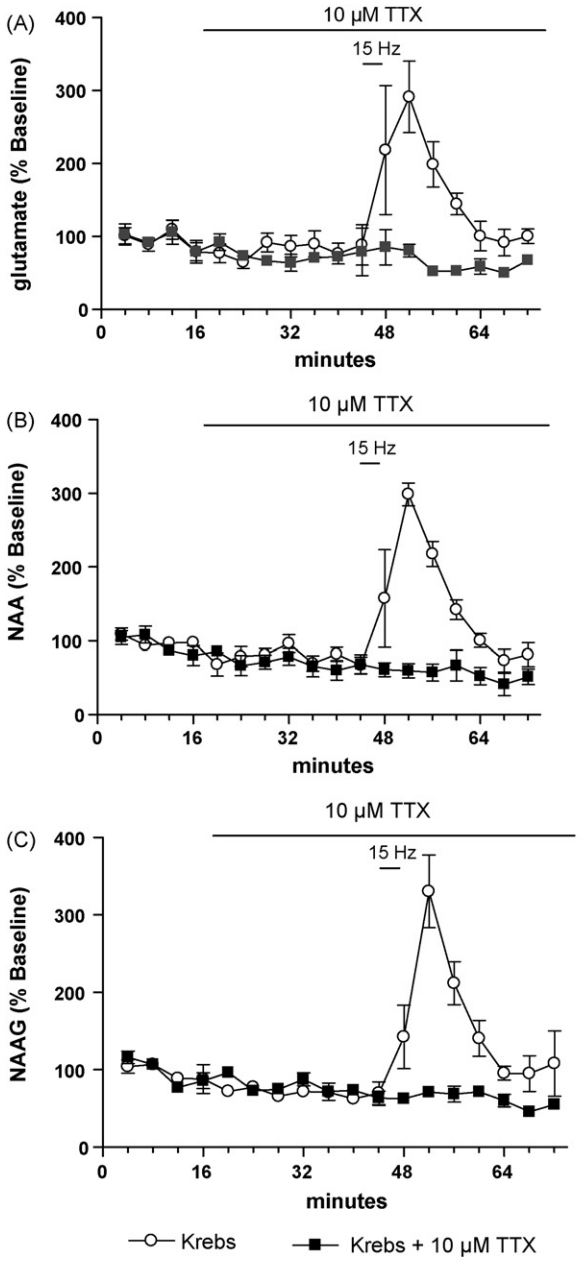

Fig. 3. Effects of TTX (10 µM) on basal and stimulated glutamate (A), NAA (B) and NAAG (C) efflux from rat hippocampal slices. Data are expressed as mean ± S.E.M. (n = 3 per group) and expressed as concentration of analyte as a percentage of pre-treatment baseline. Perfusion with TTX had no effect on basal efflux of Glu, NAAG or NAA but inhibited the increase in the efflux of the three analytes in response to electrical stimulation (2 ms biphasic pulses of 15 Hz at 20 mA during 3 min).

Table 3
Matrix effect and recovery for two different concentrations of glutamate, *N*-acetylaspartate and *N*-acetylaspartylglutamate.

Analyte	Mean peak area[a]							Matrix effect[b] (%)		Recovery[c] (%)	
	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Set 7	A	B	C	D
Glu	1.10E+07	2.10E+07	1.00E+07	1.50E+07	1.80E+07	1.40E+07	1.70E+07	40	40	89	89
NAA	3.40E+06	7.50E+06	2.20E+06	4.70E+06	6.80E+06	4.30E+06	6.00E+06	70	60	85	82
NAAG	3.00E+06	6.80E+06	4.00E+05	2.90E+06	5.10E+06	2.40E+06	4.20E+06	82	69	80	82

Sets 1, 4 and 6–20 μM Glu and NAA and 0.2 μM NAAG. Sets 2, 5 and 7–40 μM Glu and NAA and 0.4 μM NAAG. A and C are matrix and recovery values for Glu, NAA and NAAG spiked at 20, 20 and 0.2 μM. B and D are matrix and recovery values for Glu, NAA and NAAG spiked at 40, 40 and 0.4 μM.

[a] In *arbitrary* units, $n = 4$.

[b] Matrix effect expressed as the ratio of mean peak area of analyte added post-extraction (Set 4 or 5) minus amount present in sample (Set 3) to the mean peak area of analyte standards in eluent 'A' (Sets 1 and 2) multiplied by 100. A value above 100% indicates ionisation enhancement and a value below 100% indicated ionisation suppression.

[c] Recovery calculated as ratio of the mean peak area of analyte spiked before extraction (Set 6 or 7) minus amount of analyte present in sample (Set 3) to mean peak area of analyte spiked post-extraction (respective concentration from Sets 4 and 5) multiplied by 100.

3.5. Applicability of the methodology to brain tissue extracts analysis

Ion suppression and enhancement are significant factors that affect the quantitative performance of a mass spectrometer, particularly when an electrospray interface is used [33]. Ion suppression values obtained for Glu, NAA and NAAG are depicted in Table 3. The recovery of Glu, NAA and NAAG was determined as a ratio of the response of the analyte added to the sample before extraction to the response of the analyte spiked into the sample after extraction. Recovery values determined in this way are not affected by any ion suppression or enhancement effects. The recovery obtained for the three analytes was found to be between 80 and 89%.

Fig. 3 illustrates the steady-state levels of Glu, NAA and NAAG found in tissue extracts of frontopolar cortex, cingulate cortex, temporal cortex, entorhinal cortex, caudate nucleus, nucleus accumbens, and hippocampus of rat brain. Glutamate levels were consistent with previous data but showed a wider regional variation [34]. A similar regional variation was found for NAA and NAAG levels, which are also consistent with previous reports [15,18,24]. However, in contrast with superfusion samples the analyte concentrations found in brain tissue samples may be affected by the extraction method used.

4. Conclusions

A LC–MS/MS assay for measurement of Glu, NAA and NAAG has been developed. This is the first reported protocol which allows all three analytes to be measured in a single run. We have shown that the procedure can be applied to investigate alterations on steady-state levels of Glu, NAA and NAAG and to study changes in their *in vitro* efflux from hippocampal slices. The analytical method thus permit the thorough investigation of all of the analytes of the NAA/NAAG pathway; a pathway which has been suggested to be involved in the pathophysiology of psychiatric disorders such as schizophrenia.

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