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Short communication

Simultaneous determination of amino acids in discrete brain areas in *Suncus murinus* by high performance liquid chromatography with electrochemical detection

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ABSTRACT

An improved and simple reversed-phase high performance liquid chromatography method with electrochemical detection for the simultaneous determination of amino acids in brain tissue of *Suncus murinus* was developed. Homogenates from 5 different brain areas were derivatized with *o*-phthalaldehyde in the presence of sodium sulphite. Subsequent separation was achieved using linear gradient elution over 30 min. The derivatives were stable for up to 20 h at 4 °C. The method was accurate, reproducible, and showed good linearity. The recoveries were >88% for aspartate, glutamine, glutamate, glycine and γ-aminobutyric acid, with the limit of quantification varying from 5 to 30 pmol. The method was successfully applied for the measurement of amino acids under fed and fasted conditions.

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1. Introduction

There is a differential distribution of amino acids in the central nervous system (CNS). For example, glutamate (GLU) is a major transmitter in the hippocampus [1]; aspartate (ASP) is involved in hippocampal pathways [2]; and γ-aminobutyric acid (GABA) predominates in the cerebellum and cerebral cortex [3,4].

Recent studies using selective antagonists suggest that amino acids are implicated in CNS pathways involved in emesis control. Thus, N-methyl-D-aspartic acid (NMDA) receptor antagonists prevent motion-induced emesis in the cat [5] and NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor antagonists prevent cisplatin-induced emesis in ferrets [6,7]. However, no studies have examined if amino acid levels increase during emesis. Another species used in emesis research is *Suncus murinus* [8], but there is an absence of data on amino acid function in its CNS. The development of an analytical method for the determination of amino acid neurotransmitters in this latter species may provide new insights for an improved understanding and management of nausea and vomiting in man.

Determination of the levels of amino acids in brain tissue has conventionally been performed using high performance liquid chromatography (HPLC) with electrochemical detection (ECD) [9,10]. Pre-column derivatization of amino acids using *o*-phthaladehydes (OPA) has been the most widely used method for their quantification [11,12]. However, the derivatives are only typically stable for 5 h at 0 °C [11].

In the present studies, we aimed to develop an improved HPLC-ECD method using OPA derivatization for the simultaneous determination of ASP, glutamine (GLN), GLU, glycine (GLY) and GABA in discrete brain areas of *S. murinus*. The brain areas that we selected included the amygdala, frontal cortex, hippocampus, hypothalamus, and brainstem (pons and medulla). Experiments were done under both fed and fasted conditions, to mimic previous investigations by others using rodents [13].

2. Materials and methods

2.1. Animals

Female *S. murinus* (30–40 g) obtained from the Chinese University of Hong Kong were housed at 24 ± 1 °C and relative humidity maintained at 50 ± 5%, under artificial lighting (12 h light/dark cycle). Water and dry pelleted cat chow (Feline Diet 5003, PMI® Feeds, St. Louis, USA) were given *ad libitum*. All experiments were conducted under the licence from the Government of Hong Kong,

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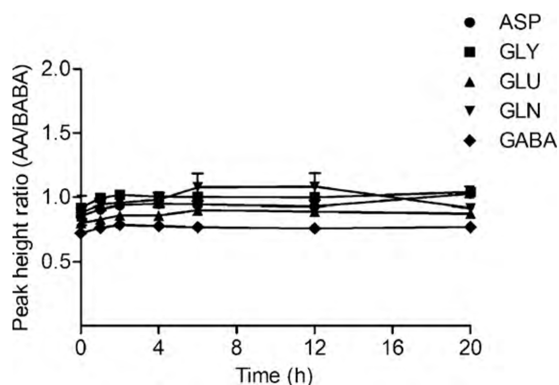

Fig. 1. Stability of the 5 amino acid (150 μM) OPA derivatives at 4 °C. AA: amino acid and BABA: β-aminobutyric acid.

and the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong.

2.2. Chemicals and reagents

All amino acid standards, β-aminobutyric acid (BABA), ethylenediaminetetraacetic acid (EDTA), OPA, sodium sulphite, and sodium tetraborate were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Sodium dihydrogen phosphate monohydrate was obtained from British Drug Houses (Poole, Dorset, UK). HPLC-grade methanol (MeOH) and absolute ethanol were obtained from Merck (Darmstadt, Germany).

2.3. Food deprivation studies

Animals were randomly divided into 2 groups of 4 animals each, i.e. fasted and fed groups. In the fed group, animals were fasted overnight (12 h) and then given free access to food for 4 h. In the fasted group, animals were fasted for 12 h.

2.4. Tissue preparation

Brains were dissected over ice into various regions, including the amygdala, frontal cortex, hippocampus, hypothalamus, and brainstem (pons and medulla). The tissue was then homogenized in ice-cold 80% MeOH at 10–30 μl/mg wet weight. The homogenates were centrifuged at 18,000 × *g* for 15 min at 4 °C, and the resulting supernatant was removed and stored at −80 °C until analysis.

2.5. Derivatization procedure

The OPA reagent was prepared daily by dissolving 2.2 mg OPA in 50 μl absolute ethanol, and then mixing with 50 μl sodium sulphite solution (0.03 M) and 90 μl borate buffer (0.1 M, pH 10.4). This reagent was protected from light and prepared 90 min before use. Prior to HPLC-ECD analysis, 20 μl of amino acid mixtures, or brain tissue homogenates, were mixed with 11 μl OPA reagent. 2 μl BABA (2.2 mM) was also added as an internal standard. The mixtures gently mixed for 60 min at room temperature to allow derivatization.

2.6. Chromatographic conditions

The HPLC system consisted of an Agilent 1100 Liquid Chromatography, equipped with a Quat pump delivery system, robotic autosampler (G1329A) and column thermostat (G1316A). Detection was performed using a Hewlett Packard model 1049A programmable electrochemical detector with a glassy carbon working electrode. Samples were separated using a C18 reverse-phase Adsorbosphere OPA-HR column (150 mm × 4.6 mm, 5 μm) with a 5 μm, 7.5 mm × 4.6 mm, C18 Adsorbosphere guard column (Alltech Associates Inc., Deerfield, IL, USA). Mobile phase A consisted of 0.1 M monosodium dihydrogen phosphate and 0.05 mM EDTA (pH adjusted to 5.0 with 5 M sodium hydroxide) with 2% MeOH. Mobile phase B was pure MeOH. The separation was obtained at a flow rate of 0.8 ml/min with a linear gradient from 0 to 15% B over 15–18 min. The column temperature was set at 30 °C. The glassy carbon working electrode combined with an Ag/AgCl reference electrode was set at +0.7 V.

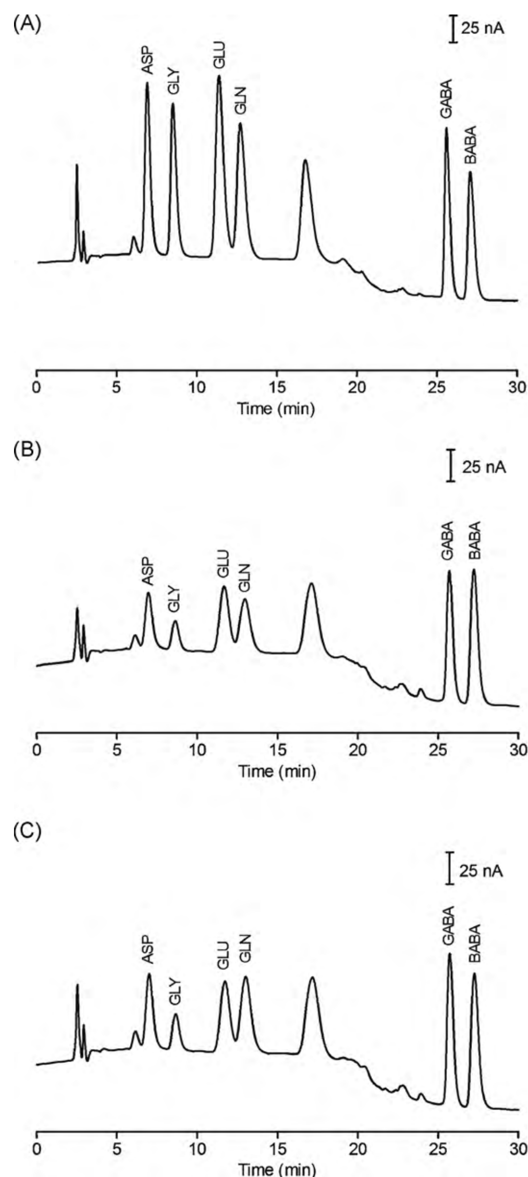

Fig. 2. Representative HPLC chromatograms of amino acids in *S. murinus* brain tissue. (A) The hippocampal homogenate was spiked with 150 μM amino acid standards. The hypothalamic homogenates were obtained from (C) fed and (D) fasted animals.

2.7. Precision, accuracy and recovery

Intra-day precision (% relative standard derivation, RSD) and accuracy were evaluated on the same day, whereas inter-day precision and accuracy was evaluated on three separated days. Recovery (%) was calculated by comparing the peak heights of spiked brain homogenates with those of the controls.

Table 1
Intra- and inter-day precision and accuracy of the assay.

Amino acid	Amount added (pmol)	Intra-day ($n = 3$) Amount detected (pmol)	Intra-day Accuracy (%)[a]	Intra-day RSD (%)[b]	Inter-day ($n = 3$) Amount detected (pmol)	Inter-day Accuracy (%)[a]	Inter-day RSD (%)[b]
ASP	50	46.60 ± 1.31	93.3	2.81	46.40 ± 1.60	92.9	3.45
	500	463.70 ± 9.10	92.7	1.96	463.30 ± 8.22	92.7	1.77
	800	733.82 ± 9.18	96.7	1.19	730.10 ± 50.06	91.3	6.86
GLY	50	47.00 ± 3.31	94.1	7.03	46.07 ± 1.70	92.9	3.69
	500	511.40 ± 6.97	97.7	1.36	506.50 ± 5.14	98.7	1.02
	800	777.50 ± 5.14	97.2	0.66	743.90 ± 43.20	93.0	5.81
GLU	50	51.73 ± 3.06	96.5	5.92	52.13 ± 1.36	95.7	2.61
	500	503.40 ± 3.61	99.3	0.72	453.40 ± 49.62	90.7	10.94
	800	783.10 ± 6.09	97.9	0.78	715.20 ± 56.99	89.4	7.97
GLN	50	44.73 ± 1.33	89.5	2.98	46.93 ± 3.16	93.9	6.74
	500	520.40 ± 6.35	95.0	1.22	471.90 ± 46.70	94.4	9.89
	800	783.00 ± 11.85	95.0	1.51	720.00 ± 53.38	90.0	7.41
GABA	50	43.53 ± 0.90	87.1	2.07	51.40 ± 2.00	97.3	3.89
	500	475.20 ± 0.87	97.9	0.18	476.30 ± 9.19	95.3	1.93
	800	784.50 ± 2.19	98.1	0.28	755.50 ± 26.27	94.4	3.48

[a] Accuracy (%) = (1 − (mean concentration measured − concentration spiked)/concentration spiked)] × 100%.
[b] RSD, relative standard deviation (%) = (SD/mean) × 100%.

2.8. Limitation of the assay

The limit of quantification (LOQ) was defined as the lowest concentration of amino acid standards that could be determined with sufficient precision and accuracy, i.e. an RSD less than 11% and an accuracy >85%.

2.9. Statistic analysis

The significance of differences between fed and fasted groups was assessed by an unpaired Student's-*t* test using GraphPad Prism version 5.0 (GraphPad Software Inc. Version, California, USA). Results are expressed as the mean ± SD for the analytical method validation and the mean ± SEM for amino acid concentrations. In all cases, the differences between treatment groups were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Optimization of the derivatization procedure

The present derivatization procedure is a modification of a previously developed method by Rowley et al. [11]. In the present studies, amino acids were derivatized with OPA in the presence of sodium sulphite to form *N-alkyl*-isoindole-sulphonate derivatives that were electroactive. However, sulphite is itself electroactive, and a high content of sodium sulphite in the resultant mixture may produce high background currents that interfere with the baseline separation of amino acids [12]. We found that an OPA reagent containing 7.83 mM of sodium sulphite yielded a baseline separation of all amino acids. Further, a reaction time of 60 min or longer produced a maximal detector response (data not shown).

The stability of the amino acid derivatives kept in an autosampler at 4 °C was examined. As shown in Fig. 1, no significant degradation was observed within 20 h. Comparing with the previously reported stability of OPA derivatives for 30 min at room temperature, or 5 h at 0 °C [11], and stability of naphthalene-2,3-dicarboxaldehyde derivatives for 16 h at 4 °C [10], our derivatization procedure improved the stability of the amino acid derivatives.

3.2. Method development and validation

Fig. 2 shows typical chromatograms of amino acid derivatives in the hypothalamus of fed and fasted *S. murinus*. Single peaks (retention time in min) were demonstrated for ASP (7.0), GLY (8.6), GLU (11.7), GLN (12.9), and GABA (25.7). The method showed a good overall intra-day (7.03%) and inter-day (10.94%) variation, with an accuracy >87% (Table 1). The method had adequate sensitivity with LOQ of 5–30 pmol (Table 2). The calibration curves for all 5 amino acids yielded good linearity with correlation coefficients of >0.99 in the concentration range of 30–900 pmol/2 μl (15–450 μM; Table 2). Furthermore, 87.9–115.9% recovery (Table 3) was obtained by spiking known amounts of a mixture of amino acid standards into the brain homogenates.

3.3. Measurement of amino acids in S. murinus brain tissue samples

The levels of the 5 major amino acid neurotransmitters were determined in the range of 100–1500 μg/g (Fig. 3). These results are in good agreement with findings in rat brain tissues [10,14]. Previously, using microdialysis, we reported that the basal extracellular levels of GLU in the ventromedial hypothalamus of *S. murinus* was 0.22 ± 0.07 μM [15]. In the present studies, GLU was determined as the most abundant excitatory amino acid neurotransmitter in all the selected brain areas. With the exception of GLY and GABA, the amino acids were expressed at similar levels among the different brain areas that we examined. Furthermore, the ratio of GLU, GLN, and GABA were similar among all the selected areas except for the hypothalamus. In the hypothalamus, similar levels of GLU and GABA were detected whereas in other brain areas, GLU was

Table 2
Calibration data for 5 amino acid neurotransmitters in the range of 15–450 μM (30–900 pmol).

Amino acid	Retention time (min)	Slope	Intercept	R^2	Limit of quantification (pmol)
ASP	6.4	0.007186	−0.01165	0.9919	10
GLY	8.5	0.006838	−0.03028	0.9935	30
GLU	9.7	0.004948	−0.01066	0.9919	10
GLN	12.8	0.004685	−0.00688	0.9921	5
GABA	24.9	0.006551	−0.01878	0.9936	10

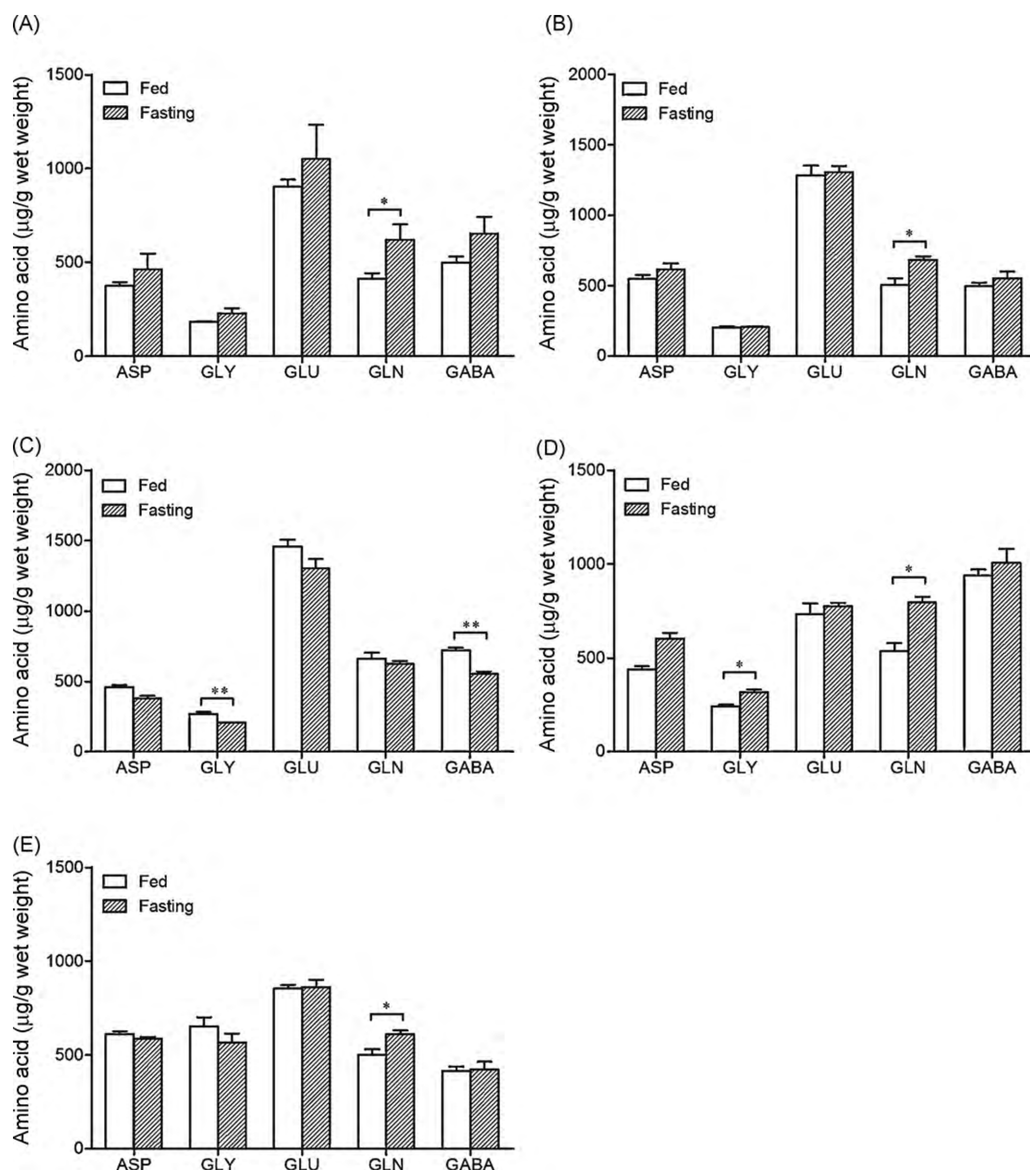

Fig. 3. Levels of amino acids detected in the (A) amygdala, (B) frontal cortex, (C) hippocampus, (D) hypothalamus and (E) brainstem (pons and medulla) of fed and fasted *S. murinus*. Data are expressed as μg/g wet weight tissues and represent the mean ± SEM ($n = 4$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the corresponding fed animal groups.

expressed at a much higher level than GABA (see Fig. 3). Our findings are in good agreement with a previous study in the rat [16]. It is possible that the high levels of GABA detected in the hypothalamus of *S. murinus* may be a consequence of species differences.

Table 3
Recovery of amino acids in the assay.

Amino acid	Spiked (μM)	Detected (μM)	% Recovery
ASP	25	25.29 ± 7.87	115.9
	150	142.00 ± 11.21	94.7
	300	306.30 ± 39.30	102.1
GLY	25	26.12 ± 0.95	104.5
	150	149.14 ± 11.21	99.4
	300	313.84 ± 45.90	104.6
GLU	25	27.20 ± 2.79	108.8
	150	135.78 ± 3.84	90.5
	300	302.30 ± 50.23	100.8
GLN	25	26.39 ± 2.47	105.6
	150	137.19 ± 10.25	91.5
	300	296.91 ± 3.99	99.0
GABA	25	25.90 ± 2.85	103.6
	150	131.84 ± 11.43	87.9
	300	293.04 ± 37.97	97.7

Only GLN showed significant differences in levels between the fed and fasted animals. Thus, in the amygdala, frontal cortex, hypothalamus and brainstem (pons and medulla), the amount of GLN was higher in fasted animals than that in fed animals ($P < 0.05$). Interestingly, Yudkoff et al. [13] demonstrated that reducing dietary carbohydrates failed to modify the levels of ASP, GLU, or GABA, but significantly increased the levels of GLN and leucine in the CNS of rats. Moreover, during feeding, a significantly lower level of GLY was also detected in the hypothalamus ($P < 0.01$). In the hippocampus, GLY and GABA levels were reduced significantly in fasted animals ($P < 0.05$; Fig. 3C). The changes in GLY in both hypothalamus and hippocampus and GABA in the hippocampus might be attributed to the nutrients contained in the food. However, the mechanism of how diet affects the levels of brain GLY and hippocampus requires further investigation.

4. Conclusions

In conclusion, we successfully developed an improved HPLC-ECD method for the simultaneous measurement of amino acids.

The method was successfully applied to measure 5 major amino acid neurotransmitters in *S. murinus* brain tissues. To the best of our knowledge, we are the first to report the levels of GLU, GLN, GABA, ASP, and GLY, and their distribution in the brain of fed and fasted *S. murinus*.

Little is known about the role of amino acid neurotransmitters in emesis control. However, the previous determination of monoamine function in species capable of emesis provided valuable information towards hypotheses that culminated in the discovery of several anti-emetic drugs, such as the $5HT_3$ receptor antagonists [17,18]. Measurement of amino acid neurotransmitters may similarly provide new information towards an improved understanding of emesis control and possibly the discovery of new anti-emetic drugs.

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