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Short Communication

Measurement of glutamate, aspartate and glycine and its potential precursors in human brain using high-performance liquid chromatography by pre-column derivatization with diethylaminoazobenzene sulphonyl chloride

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ABSTRACT

This paper describes a high-performance liquid chromatographic technique, with dimethylaminoazobenzene sulphonyl chloride derivatization, for the measurement of glutamate, aspartate and glycine and its potential precursors in human brain tissue. The derivatization procedure is simple, sensitive and highly reproducible. The derivatized amino acids are stable and can be analysed by reversed-phase chromatography with visible detection at an absorption wavelength of 436 nm. A preliminary application to the determination of the concentrations of several amino acids in several regions of the human brain is described.

INTRODUCTION

Brain amino acids are involved not only in general metabolic processes but also in synaptic transmission as neurotransmitters. Recently, excitatory amino acids (aspartate and glutamate) have been implicated in various neuropsychiatric diseases [1]. Glycine, previously known as an inhibitory amino acid neurotransmitter, has been shown to enhance excitatory amino acid neurotransmission by modulating the N-methyl-D-aspartate receptor complex [2]. Therefore, measurements of glutamate, aspartate and glycine and its potential precursors (serine and threonine) in human brain can provide useful information about the biochemical bases of neuropsychiatric diseases.

Although ion-exchange chromatography with post-column derivatization with ninhydrin has been used routinely for amino acid analysis, it involves long analysis times and the use of rather complex instrumentation. More recently, the use of high-performance liquid chromatography

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(HPLC) with reversed-phase column separation has become popular owing to its relative simplicity and easy maintenance [3].

Numerous HPLC derivatization procedures have been developed for the measurement of amino acids [4–6], with pre-column *o*-phthalaldehyde (OPA) derivatization being the most popular procedure. However, although good resolution and sensitivity are readily obtained, the chemical instability of the derivatives necessitates an automated on-line derivatization system that allows rapid and exact reaction time. This derivatization procedure involves the use of dimethylaminoazobenzene sulphonyl chloride (DABS-Cl) [7]. DABS derivatives are chemically stable and can be separated by reversed-phase liquid chromatography with a high sensitivity. However, this approach has been limited to the determination of protein hydrolysates [8,9], and there is only one report on the determination of free amino acids in brain specimens [10].

This paper describes the measurement of aspartate, glutamate, glycine, serine and threonine in *post-mortem* human brain tissue after DABS-Cl derivatization, using reversed-phase HPLC with visible detection.

EXPERIMENTAL

Materials and reagents

The amino acid standard mixture was obtained from Amino Chrome Kit (Ciba-Corning, UK). DABS-Cl was purchased from Nacalai Tesque (Japan) and recrystallized twice from acetone. Acetonitrile of HPLC grade and N,N-dimethylformamide were purchased from Nacalai Tesque and Kanto Kagaku (Japan), respectively.

Brain dissection

Brains were collected from control subjects, and the cause of death and the *post-mortem* interval were established as described previously [11]. The brains were from seven males and three females (mean age ± S.D. 66.7 ± 8.5) without evidence of neurological or psychiatric disorders. Brains were sectioned coronally into 10-mm-thick sections, and each specific region was dissected out in a cold box (−15°C). Brain tissue was homogenized with a glass–PTFE homogenizer in 0.32 *M* sucrose and stored as aliquots at −80°C until the assay.

Sample preparation

On the day of analysis the aliquotted homogenates (60 μl) were thawed at room temperature, deproteinized by mixing with 100 μl of 0.4 *M* perchloric acid and centrifuged (10 000 *g*, 10 min). Then, 100 μl of supernatant were neutralized with 65 μl of 2 *M* $KHCO_3$, and the precipitates were removed by centrifugation (10 000 *g*, 10 min). After this, 10 μl of clear supernatant or amino acid standard solution, 10 μl of internal standard (1 m*M* α-aminoadipic acid), 20 μl of 25 m*M* $NaHCO_3$ and 80 μl of 4 m*M* DABS-Cl solution in acetonitrile were placed into a small Pyrex tube. The tightly closed tube was vortex-mixed thoroughly and incubated at 70°C for 12 min. The reaction was stopped by adding 380 μl of 20% acetic acid, and a 20-μl aliquot was injected into the HPLC apparatus.

Chromatography

A Tri Rotar VI HPLC system (Jasco, Japan) was used. It comprised two pumps, a system controller, a Model DG-3510 degasser and a Model AS-L350 autosampler. Peak areas were integrated by a Model 7000B integrator (SIC, Japan). An Amino Chrome CCD-ODS column (packed with LiChrospher C_{18}, 5 μm particle size, 125 mm × 4 mm I.D., Ciba-Corning, UK) was used. The column temperature was kept at 40°C, and the flow-rate was 1.0 ml/min.

Gradient elution

Two mobile phases were used. Mobile phase A was 25 m*M* sodium acetate buffer (pH 6.4) containing 4% N,N-dimethylformamide, and mobile phase B was 100% acetonitrile. Mobile phase A was filtered through a 0.45-μm nitrocellulose filter. The gradient programme is shown in Table I. The total running time, including reequilibration, was 55 min.

TABLE I

GRADIENT PROGRAMME FOR HPLC

Time (min)	A (%)	B (%)
0.0	85	15
2.0	85	15
30.0	70	30
35.0	59	41
37.0	46	54
39.0	43	57
41.0	33	67
42.0	10	90
44.0	85	15

Detection and calculation

A spectrophotometric detector (Model S/3250, Soma, Japan) with the absorption wavelength set at 436 nm was used. Amino acid concentrations were determined by the ratio of the peak area of each amino acid to the peak area of internal standard.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms of the amino acid standard and an extract from hippocampus (CA 1-3). N,N-Dimethylformamide in mobile phase A was used for the separation of serine and threonine.

The relationship between the peak area and the amount of amino acids was linear from 0.5 to 500 pmol. Proper dilution of the samples may be needed before derivatization. Using detection in the visible range, the sensitivity can be increased because of the low signal-to-noise ratio. Thus, the detection limit of DABS-amino acids in our system was 0.5 pmol.

The stability of DABS-amino acids was tested using pooled brain homogenates. The DABS-amino acids from brain homogenates and standard amino acid mixtures were kept at room temperature for up to twenty days, at 4°C for up to three months or at −20°C for up to a year. There

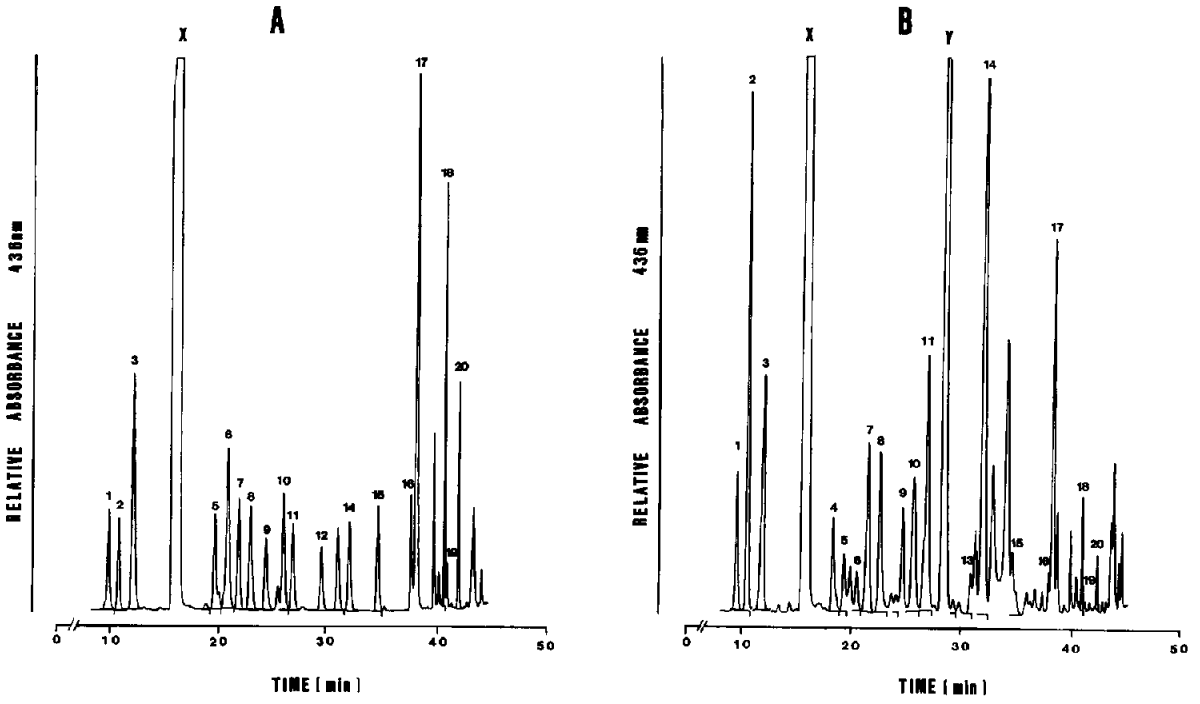

Fig. 1. Representative chromatograms showing amino acid profiles after DABS-Cl derivatization of a standard mixture (A) and an extract of the hippocampus (CA 1–3) (B). Peaks: 1 = aspartate; 2 = glutamate; 3 = α-aminoadipic acid (internal standard); 4 = glutamine; 5 = serine; 6 = threonine; 7 = glycine; 8 = alanine; 9 = arginine plus taurine; 10 = proline plus GABA; 11 = valine; 12 = methionine; 13 = isoleucine; 14 = leucine; 15 = phenylalanine; 16 = cystine; 17 = NH_3; 18 = lysine; 19 = histidine; 20 = tyrosine; X = DABS-ONa; Y = noise peak derived from reagents. Peaks 11 and 14 in tissue samples are contaminated with an unidentified peak.

TABLE II

AMINO ACID CONCENTRATIONS IN DIFFERENT REGIONS OF NORMAL HUMAN BRAIN

Values are expressed as mean ± S.D., with the number of samples in parenthesis.

Brain region	Concentration (μmol/g of protein)				
	Aspartate	Glutamate	Serine	Threonine	Glycine
Parietal cortex (angular gyrus)	36.7 ± 7.8 (9)	85.2 ± 18.9 (9)	9.6 ± 2.4 (9)	3.2 ± 0.9 (9)	22.3 ± 6.6 (9)
Temporal cortex (lateral occipito-temporal gyrus)	13.2 ± 4.2 (8)	44.4 ± 1.7 (8)	6.2 ± 1.7 (8)	2.1 ± 0.6 (8)	12.0 ± 4.5 (8)
Occipital cortex (secondary visual cortex)	24.9 ± 10.5 (9)	89.2 ± 25.5 (9)	4.1 ± 1.2 (9)	2.4 ± 1.2 (9)	12.5 ± 4.2 (9)
Putamen	24.8 ± 10.4 (10)	100.8 ± 46.2 (10)	6.2 ± 3.2 (10)	7.2 ± 2.2 (10)	21.3 ± 10.4 (10)
Caudate	13.2 ± 5.7 (10)	73.7 ± 32.6 (10)	5.4 ± 1.6 (10)	3.4 ± 1.9 (10)	17.3 ± 5.1 (10)
Hippocampus (A 1–3)	14.5 ± 11.4 (10)	38.2 ± 7.9 (10)	4.6 ± 6.0 (10)	2.9 ± 1.9 (10)	8.2 ± 7.3 (10)
Thalamus (N. lateralis posterior)	17.5 ± 7.9 (10)	53.4 ± 14.5 (10)	3.2 ± 1.9 (10)	2.2 ± 0.9 (10)	12.4 ± 3.2 (10)

was no significant change in resumed concentrations during these periods. These data illustrate the excellent stability of this derivatization procedure. Thus, derivatized samples can be stored at room temperature and analysed later without any noticeable degradation.

The reproducibility was tested using five samples from pooled brain homogenates, which yielded coefficients of variation (C.V.) of 2.7% for aspartate, 2.2% for glutamate, 1.6% for serine, 1.2% for threonine, 0.9% for glycine, 1.0% alanine, 1.3% for arginine, 1.0% for methionine, 1.0% for isoleucine and 1.2% for leucine. This confirms the good reproducibility of derivatization for each amino acid.

Using this method, the amino acid concentrations in several brain regions have been measured (Table II). These values are comparable with those obtained with other derivatization procedures: ninhydrin [12,13] or OPA [15,16]. Glutamate concentrations in Table II are also in agreement with those shown in our previous paper [11], where we used enzymic–fluorimetric method.

The disadvantage of our method is the poor resolution of γ-aminobutyric acid and taurine. A good resolution of these amino acids can be achieved with a different analytical column [10].

CONCLUSION

The use of DABS-Cl for the determination of amino acids has several advantages: (1) the derivatives are chemically stable and can be stored at room temperature before analysis, so an expensive automated derivatization apparatus is not necessary; (2) the derivatives can be separated by reversed-phase liquid chromatography and detected in the visible range with a detection limit of 0.5 pmol; (3) amino acids from brain samples can be measured with good reproducibility and sensitivity.

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