HIGH-SENSITIVITY AMINO ACID ANALYSIS: MEASUREMENT OF AMINO ACID NEUROTRANSMITTER IN MOUSE BRAIN

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1. Introduction

In the conventional amino-acid analyser, free amino acids are separated by ion-exchange chromatography and detected by post-column derivatization with ninhydrin or fluorescent reagents [1–5]. The sensitivity of post-column derivatization sytems is essentially limited due to impurities which react with ninhydrin or themselves have UV-absorption [3,4]. These artefacts raise the baseline, especially during the change of buffer, and make it difficult to analyse basic amino acids at low picomole levels. To reduce such contaminants, a highly purified buffer or a two-column system must be used.

Pre-column derivatization of amino acids, followed by analysis of the derivatives, completely avoids the problem of buffer contamination and should thus be the method of choice for high-sensitivity amino acid analysis. The reproducibility of derivatization and the stability of the derivatized amino acids, however, have been questioned [3,4].

We have developed a new method of amino acid analysis using pre-column derivatization with a colour-producing reagent, dimethylamino-azobenzene sulphonyl chloride (DABS-Cl) [6–8], followed by analysis of DABS-amino acid by reverse-phase HPLC [9]. DABS-amino acids can be detected in the visible region at a sensitivity of <1 pmol. Kinetic studies have shown that 'dabsylation' is reproducible and the derivatized amino acids are both photostable and chemically stable [6,9]. The sensitivity and accuracy of this new method have been demonstrated by analyses of only 10–30 ng protein hydrolysates [9].

Abbreviations: DABS, dimethylamino-azobenzene sulphonyl; HPLC, high-performance liquid chromatography; GABA, γ-aminobutyric acid

This communication describes the use of DABS-Cl in the analysis of the amino acid neurotransmitters taurine, GABA, glutamate, glycine and β-alanine in mouse brain. We have developed a gradient system which separates these neurotransmitters from all other common amino acids within 30 min. This sensitive method is especially useful in the study of the release of amino acid neurotransmitters.

2. Materials and methods

2.1. *Chemicals*

DABS-Cl was purchased from Fluka (Switzerland) and recrystallized from boiling acetone. Taurine, GABA, β-alanine and leucine amide were obtained from Sigma (USA). All organic solvents used in both dabsylation and HPLC analysis were of analytical grade and supplied by Merck (Darmstadt).

2.2. *High performance liquid chromatography*

The HPLC system (Waters Associates) comprises two 6000A pumps, a 720 system controller, a U6K sample injector and a model 440 detector (fixed wavelength at 436 nm). The sensitivity of the detector was set at 0.01 AUFS. DABS-amino acids were separated on a Zorbax-ODS column (4.6 mm × 25 cm, Dupont). Peak areas were integrated by a SP 4000 central processor (Spectraphysics).

2.3. *Extraction of amino acids from mouse brain*

Mice (Tierfarm Sisseln, Switzerland) were killed by microwave radiation (2.8 kW operating power, 2450 MHz, Medical Engineering Consultants, Lexington MA). The heads of the animals were positioned perpendicularly to the microwave *E*-field. Exposure was for 4 s. The brains were removed from the skulls and

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dissected immediately into discrete regions. The brain tissue samples were weighed and homogenized by ultrasonication in 1 ml 0.6 N perchloric acid containing 200 nmol leucine amide as internal standard. The suspensions were left for 30 min at 4°C and centrifuged (20 min at 12 000 × *g*, 4°C). The clear supernatants were neutralized with 250 μl 2 M $NaHCO_3$ and shaken for 10 min at 300 rev./min. Finally, the suspensions were kept at 0°C for 15 min and centrifuged at 3000 × *g*. The clear supernatants were diluted to 5 ml with 0.2 M $NaHCO_3$–NaOH (pH 9) and used for dabsylation.

2.4. *'Dabsylation'*

Of the 0.2 M $NaHCO_3$–NaOH (pH 9) diluted extract, 10 μl was mixed with 20 μl DABS-Cl solution (4 nmol/μl in acetone) in a 1 ml Eppendorf tube. Dabsylation was performed at 70°C for 10 min. The dabsylated mixture was diluted to 200 μl with 50% acetonitrile, and 10–25 μl was applied for HPLC analysis.

3. Results and discussion

3.1. *Dabsylation and HPLC analysis of taurine, β-alanine and GABA*

Dabsylation of taurine, β-alanine and GABA, as of other common amino acids [9], is both quantitative and reproducible. The peak-area responses of synthetic standards (prepared with an excess of amino acids) were used to confirm the quantitative reaction of taurine, β-alanine and GABA with excess DABS-Cl under the conditions described (fig.1, legend). The linear relationship between the peak-area response

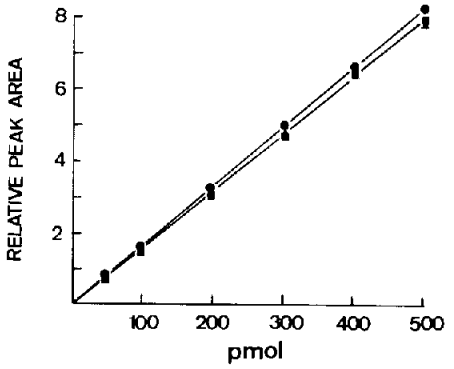

Fig.1. The linear relationship between the peak area and quantity of amino acids taurine (–▲–), β-alanine (–●–) and GABA (–■–) subjected to 'dabsylation'. Of each amino acid 50–500 pmol was dabsylated with 80 nmol DABS-Cl (see section 2) and 10 pmol dabsylated samples (20–2%) analysed by HPLC. The yields were then normalized back to 100% injection.

and the amount of amino acid subjected to dabsylation is shown in fig.1.

The detection limit of DABS-amino acid is dependent upon the baseline noise, which, in turn, is dependent upon the quality of the detector. We have obtained an even more stable baseline (as compared to those shown in fig.2) with the new filter (436 nm) provided by Water Associates. With this new system, the detector can be routinely set at 0.005 AUFS and as little as 0.3 pmol DABS-amino acid can be analysed.

3.2. *Analysis of amino acid neurotransmitters in mouse brain*

An analysis of the amino acid concentrations in different sections of the mouse brain is listed in table 1.

Table 1

Amino-acid concentration (μmol/g wet tissue) in different regions of the mouse brain as determined by the DABS-Cl method[a]

	Cortex		C. striatum		Hippocampus		Hypothalamus		Cerbellum	
	Mouse A	Mouse B	Mouse A	Mouse B	Mouse A	Mouse B	Mouse A	Mouse B	Mouse A	Mouse B
Taurine	15.51	15.08	15.97	14.54	13.58	12.05	12.27	11.61	11.18	14.94
Gln	5.23	4.37	4.59	4.89	4.80	4.60	4.74	4.72	4.71	6.73
Glu	13.62	12.82	13.03	12.48	12.72	11.16	11.08	10.13	10.53	13.67
Gly	0.95	1.12	1.04	1.02	1.08	1.22	1.22	1.04	2.09	2.21
Ala	0.69	0.91	0.77	0.78	0.59	0.82	0.54	0.55	0.47	0.92
β-Ala	0.19	0.20	0.33	0.35	–	0.31	0.46	0.30	0.27	0.28
GABA	1.76	1.64	2.26	2.00	1.96	1.50	2.49	2.04	1.48	1.66

[a] Amino acids with <0.5 μmol/g wet tissue are not listed, except for β-alanine; aspartic acid and serine were not determined

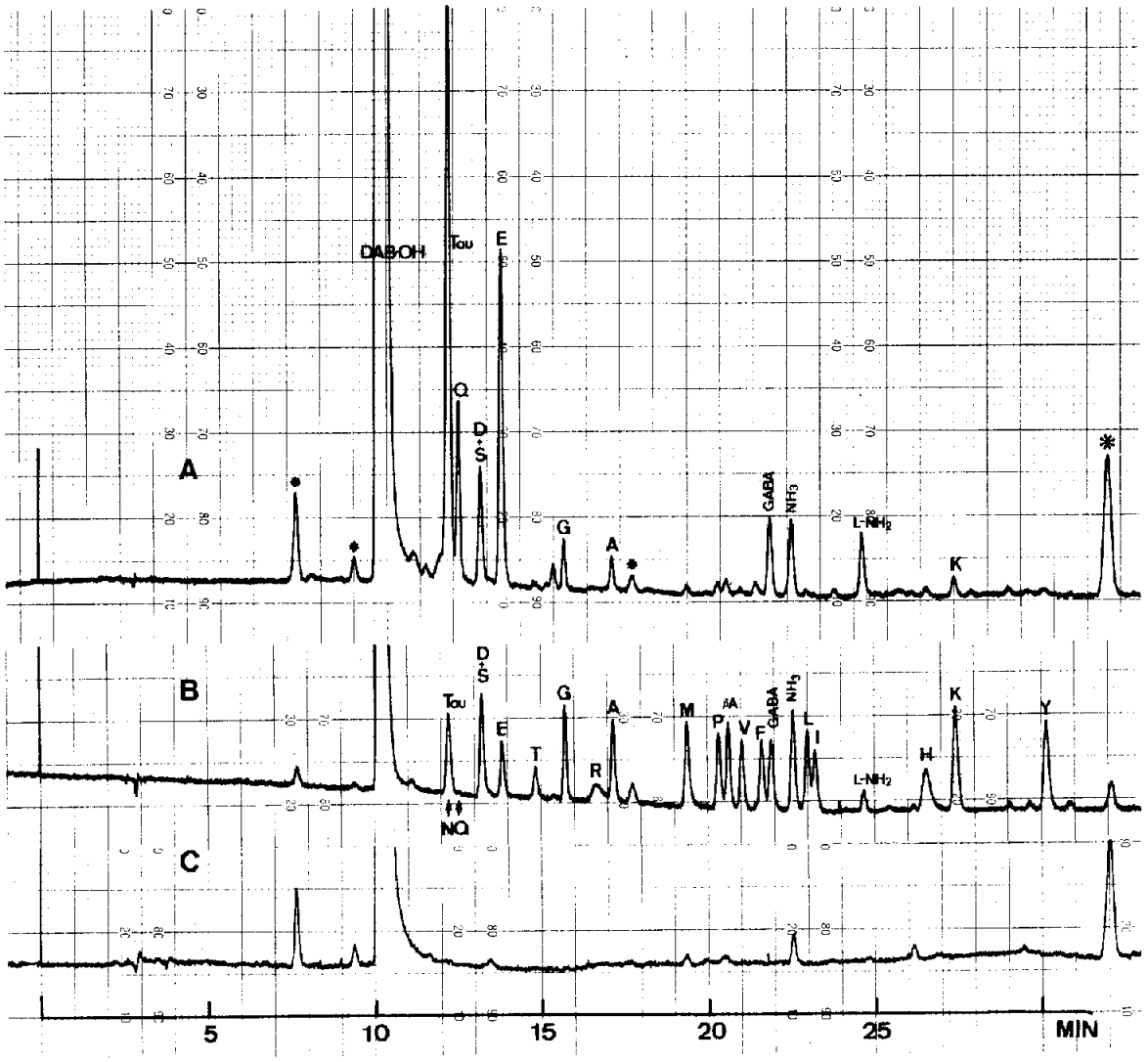

Fig.2. Chromatograms from amino-acid analyses by the DABS-Cl method:

(A) Analysis of amino acid extract from C. striatum. Only 1/500 of the striatal tissue extract from one mouse was dabsylated and 1/20 of the dabsylated sample was injected for HPLC. By-products originating from the reagent are marked with asterisks.

(B) Analysis of 5 pmol common amino acid standard (Hamilton) plus taurine, β-alanine, GABA and leucine amide. The low response of leucine amide may be due to the impurity of the sample. However, a linearity between the peak response and quantity of leucine amide has also been established (unpublished).

(C) Blank run. Chromatographic conditions: solvent (A) is 0.045 M acetate (pH 4.1); solvent (B) is acetonitrile; gradient, 20–70%B in 25 min (linear); flow rate, 1.1 ml/min; column temp. 22°C; chart speed, 1 cm/min. DABS-amino acids are symbolized by the one-letter abbreviation of their corresponding amino acids: Tau, βA, GABA and L-NH_2 stand for taurine, β-alanine, γ-aminobutyric acid and leucine amide, respectively.

These results are comparable to those obtained from a standard amino acid analyser (Biotronik LC 6000E). However, only 0.01% of the total regional brain extract from one mouse was required for each analysis.

In the chromatographic system described (fig.2), GABA and β-alanine have baseline separation from other common amino acids, but asparagine overlaps with taurine and serine overlaps with aspartic acid. Asparagine in mouse brain is ~0.1 μmol/g wet tissue, which is <1% of the taurine concentration and thus should not interfere with the quantitative analysis of taurine.

3.3. *Comparison of the DABS-Cl method with the conventional amino acid analyzer*

In performing amino acid analysis at low picomole levels, there are distinct advantages to be gained from using the pre-column derivatization method instead of the post-column detection system:

(1) The ninhydrin-sensitive impurities or impurities with UV-absorbent material contained in the chromatographic buffer do not interfere with DABS-amino acid identification.

(2) The pre-column derivatization method requires simpler instrumentation and the HPLC system can be easily converted for the analysis of peptide as well as amino acid thiohydantoins during sequence analysis, simply by changing the column.

(3) Complete amino acid analysis of a physiological sample takes only 30 min as compared to 1–3 h in the post-column detection system.

3.4. *Comparison of the DABS-Cl method with other pre-column derivatization methods of amino acid analysis*

Several pre-column derivatization methods affording sub-nanomole sensitivity are available [10–17]. Each of them, however, has some intrinsic defect. Phenylisothiocyanate does not form phenylthiohydantoin derivatives with taurine, β-alanine and GABA. The fluorescent quenching of dansyl amino acids in aqueous solutions, such as those used in reverse-phase HPLC has forced most researchers to resort to the less sensitive UV-detection system [12,13]. The *o*-phthalaldehyde method also suffers from the instability of the derivatized amino acid [16,17] and the incapability of reacting with amino acids (proline and hydroxyproline).

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