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Development of a protocol for the automated analysis of amino acids in brain tissue samples and microdialysates

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Abstract

An automated precolumn derivatisation method has been developed for the measurement of fourteen amino acids in brain tissue and microdialysate samples. The method involves labelling amino acids with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide (CN^-). The resulting highly stable N-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives were separated using a binary gradient elution profile and detected fluorometrically. The order of elution of the derivatised amino acids was confirmed by using liquid chromatography with fluorescence and mass spectrometric detection in tandem. Linear calibration plots were obtained for all amino acids in the range studied (0.2–12.5 μM). The limit of detection for CBI derivatives of amino acids was in the range 5–20 fmol ($S/N=2$) using a 5 μl injection volume. The method has been used for the measurement of amino acids in microdialysates from rat brain and tissue homogenates from different regions of mouse brain.

Keywords: Amino acids

1. Introduction

Amino acids in the central nervous system (CNS) can be divided into two categories depending on whether they have a neurotransmitter or nonneurotransmitter role. Those that are classed in the former group include aspartate, glutamate, glycine, taurine and λ-aminobutyric acid (GABA). The remaining amino acids are thought to have a metabolic function [1]. The differential localisation in various brain regions of the former amino acids is thought to provide evidence for their role as neurotransmitters [2,3]. Changes in the level of certain neurotransmitter amino acids are known to be associated with neurodegenerative diseases, such as Alzheimer's disease [4] and Huntington's chorea [5]. Hence, the measurement of the levels of neurotransmitter amino acids in animal models may provide an additional tool for drug design. Furthermore, identifying changes in the concentration of selected amino acids in different regions of the brain after drug treatment may also play a role in monitoring drug efficiency.

The concentration of amino acids in the brain is in the micromolar to submicromolar range. To quantitate these levels accurately, highly selective assays are required. In comparison, the level of amino acids in microdialysates [6] is approximately two orders of magnitude lower than in tissue samples. This emphasises the need for an assay with high sensitivity. None of the neurotransmitter amino acids possess a

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suitable chromophore or fluorophore that would allow these neurochemicals to be detected directly at trace levels. An approach that is commonly used to measure sub-micromolar levels of amino acids in biological samples is precolumn derivatisation. Two precolumn reagents that have been used extensively for the measurement of neurotransmitter amino acids are *o*-phthalaldehyde (OPA) [7] and naphthalene-2,3-dicarboxaldehyde (NDA) [8]. Both OPA and NDA react with compounds that possess a free primary amine group to yield N-substituted derivatives that can be measured at trace levels using either chemiluminescence [9], electrochemical [10] or fluorescence [11,12] detection. These reagents have also been used for the analysis of the monoamines, dopamine and noradrenaline [13]. Furthermore, both HPLC and CE based methods have been developed for the analysis of N-substituted derivatives of amino acids.

The aim of the present study was to develop a reproducible, robust, rugged, and sensitive precolumn derivatisation method for the measurement of amino acids in brain tissue and microdialysis samples using NDA.

2. Experimental

2.1. Reagents

Analytical reagent grade, ammonium acetate and unstabilised HPLC grade tetrahydrofuran were purchased from Fisher Scientific (Loughborough, UK). Boric acid and sodium hydroxide of ARISTAR grade were obtained from BDH (Poole, UK). Potassium cyanide, γ-aminobutyric acid and taurine were purchased from Sigma (Poole, UK). Amino acid standard mixture containing hydrochloride of L isomer of alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine, each at a concentration of 2.5 m*M*, was purchased from Pierce (Cheshire, UK). Naphthalene-2,3-dicarboxaldehyde was purchased from Molecular Probes (Eugene, OR, USA). HPLC grade methanol 210 and acetonitrile far UV190 were bought from Romil (Cambridge, UK).

2.2. Preparation of buffers

Ammonium acetate (3.854 g) was dissolved in 1 l of deionised water and the pH was adjusted to 6.8 with 0.1 *M* aqueous sodium hydroxide to give a 50 m*M* solution. Borate buffer (0.1 *M*, pH 9.5) was prepared by titrating boric acid solution with 0.1 *M* aqueous sodium hydroxide. This buffer was stored at ambient temperature and discarded after one week.

2.3. Preparation of amino acid solutions

A stock solution of amino acids each at a concentration of 12.5 μ*M* in 0.3 *M* borate buffer (pH 9.5) was prepared using the mixture of 17 amino acids and glutamine, GABA and taurine. Aliquots (50 μl) of this stock solution were stored at −20°C for calibration studies and inter-assay analysis. These samples were discarded after a month. The required concentrations of mixture of amino acids were prepared from the stock solution by serial dilution with borate buffer.

2.4. Preparation of derivatising reagents

Potassium cyanide (6.5 mg) was dissolved in 10 ml of deionised water to give a 10 m*M* solution. The solution was stored at 4°C and discarded after one month. A 3 m*M* solution of NDA was prepared by dissolving 0.552 mg in methanol. This solution was stored in an amber vial at 4°C and discarded after one week.

2.5. Preparation of microdialysates

Male Sprague-Dawley rats (260–300 g) were used. General anaesthesia was induced with a mixture of Sublimaze (0.9 ml/1100^{-1} g i.p.) and Domitor (0.4 ml/100^{-1} g i.p.). Upon reaching deep anaesthesia the rat was transferred to a stereotaxic frame and guide probe inserted and secured [14] so that the tip placement was directly above the striatum. Anaesthesia was reversed with Anisedan (0.2 ml/100^{-1} g i.p.) and Nubain (0.2 ml/100^{-1} g

i.p.). Rats were allowed to recover for 2 weeks. On the experimental day the rat was lightly anaesthetised with 3.5% isoflurane with 2 l O_2 min^{-1} and the guide replaced with a dialysis probe so that the dialysis probe protruded into the striatum. The probe was perfused with artificial cerebrospinal fluid (aCSF) consisting of 125 m*M* NaCl, 2.5 m*M* KCl, 1.18 m*M* $MgCl_2$, 1.26 m*M* $CaCl_2$, (pH 7.4). Probes were perfused for 2 h before samples were collected every 4 min into borate buffer (0.1 *M*, pH 9.5). Dialysis samples are relatively clean and can therefore be analysed directly without further sample clean up.

2.6. Preparation of brain tissue samples

Male Sprague Dawley rats (300–400 g) were used. The rats were decapitated and samples of the required brain area were taken. The samples were frozen immediately and stored at −70°C until required. Samples for analysis were homogenised in 0.4 *M* perchloric acid containing 0.1% w/v sodium metabisulphite, 0.01% w/v EDTA and 0.1% w/v cysteine (1 mg of tissue 10 μl^{-1} of homogenising solution). The homogenate was centrifuged at 10 000 rpm for 10 min at 4°C and an aliquot (10 μl) of supernatant was diluted 20-fold with borate buffer.

2.7. Derivatisation procedure

The derivatisation was carried out using a Gilson 231XL autosampler. For the analysis of standards, the autosampler was programmed to dispense sequentially to an empty vial, 10 μl of amino acid mixture, 90 μl of borate buffer, 4 μl of potassium cyanide and 16 μl of NDA. The derivatisation programme included steps to rinse the injection needle between transfer of KCN and NDA, to mix the final sample by aspirating it twice with 200 μl of air and a 10 min wait time. An alternative derivatisation programme was used for the analysis of brain samples. In this programme, the step for the addition of standard was omitted and buffer and reagents were added to a vial containing 10 μl of sample. Also, for microdialysis studies the volume of borate buffer used for derivatisation of standards and samples was reduced to 16 μl.

2.8. Instrumentation

A Waters HPLC system model 2690 separation module composed of a quaternary gradient solvent management system, a thermostatted autosampler fitted with a 100 μl loop and a 25 μl sample syringe and an in-line vacuum degasser was used. Samples were separated using Waters Symmetry 250×4.6 mm I.D. C_{18}, 5 μm column. Eluates were detected using a Waters model 474 scanning fluorescence detector fitted with a 5 μl flow-cell. The column was connected to the detector using minimal length of 0.13 mm I.D. PEEK tubing.

Two eluents were used in a linear elution profile and for convenience these will be referred to as eluent A and B. Eluent A was composed of a mixture of ammonium acetate buffer (50 m*M*, pH 6.8) and tetrahydrofuran in a ratio of 95:5, v/v. Eluent B was prepared by mixing acetonitrile, ammonium acetate buffer (50 m*M*, pH 6.8) and methanol in a ratio of 55:35:10, v/v. Each eluent was filtered using GS 0.22 μm filter that contains a mixture of cellulose acetate and nitrate (Millipore, Watford, UK). A linear gradient from 10 to 67.5% B over 45 min and then to 100% B over 2 min was used for the separation of CBI-amino acid derivatives. A flow rate of 0.7 ml min^{-1} was used for the separation of amino acid derivatives.

The LC-mass spectrometry (MS) study was performed using an HP1100 binary pump (Hewlett Packard Ltd., Cheshire, UK), a FAMOS autosampler (L.C. Packings, Amsterdam, The Netherlands) and a Jasco FP-920 spectrofluorometric detector (Jasco Great Dunmow, UK) coupled to a Micromass Q-Tof mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray atmospheric pressure ionisation (API) source. Negative ion electrospray ionisation (ESI) was used with the standard Micromass probe. The outlet from the fluorescence detector was connected directly to the ESI probe and to aid desolvation at the high flow rates employed, the desolvation nozzle within the API source was heated to 375°C. Data was acquired over the scan range 180–400 m/z in a 1.1 s cycle.

3. Results and discussions

Naphthalene-2,3-dicarboxaldehyde reacts with primary amines in the presence of a nucleophile, such as cyanide ion, to give the corresponding 1-cyano-2-substituted-benz[*f*] isoindole (CBI) derivative [14].

The addition of a nucleophile, such as cyanide, to the reaction mixture is essential for the formation of stable CBI derivatives. The use of nucleophiles other than cyanide, such as hydrogen sulphite, isothiocyanate, and 2-mercaptoethanol have been reported [11]. Montigny et al. found that CBI-derivatives having a substituent other than a cyano group on the indole ring not only reduced stability but also showed reduced fluorescence quantum efficiency.

Excitation and emission wavelengths were optimised for the detection of CBI derivatives of glutamate and γ-aminobutyric acid (GABA). Stop-flow scans of 100 μ*M* glutamate and GABA treated with NDA were carried out. The excitation spectrum of CBI derivatives of these amino acids was found to have bands with maxima at 420 and 442 nm. The optimum emission wavelengths were found to be 480 nm with excitation set at 442 nm.

3.1. Stability of CBI derivatives

The stability of CBI-amino acid derivatives was measured using a mixture of amino acids each at a concentration of 0.5 μ*M*. The sample was derivatised with NDA and placed in an autosampler thermostatted to 10°C. Repeated analysis of this sample was carried out over 13 h and it was found that the HPLC-peak area for the CBI-amino acid derivatives changed by 4–6%. This change is within the coefficient of variation for repeated injections for pre-column methods, which can be as high as 10%. Hence, the observed change in HPLC-peak area of CBI amino acid derivatives may not be entirely attributable to decomposition of the derivative. A similar level of stability has been reported for CBI derivative of glycine formed using NDA [11]. The stability of CBI amino acid derivatives formed using NDA is markedly better than that reported for corresponding derivatives obtained using OPA/mercaptoethanol [15]. However, it has been reported [16] that stability of OPA amino acid derivatives can be improved using sulphite as a nucleophile in place of mercaptoethanol. The authors reported that OPA/sulphite derivatised amino acids are stable for approximately 5 h at 0°C. However, it is impractical to maintain and run samples at this temperature unattended.

3.2. Optimisation of HPLC conditions

In our study we developed methodologies to resolve both neurotransmitter and putative amino acids. This was deemed necessary because studies carried out to evaluate the effects of novel ligands may involve the use of drugs which affect the levels of both these types of amino acids. Hence, a profile of changes in the concentration of the widest range of amino acids would be of pharmacological value.

The HPLC conditions detailed in the Experimental Section are optimal for the baseline resolution of 14 of the 19 primary amino acids in the standard mixture (Fig. 1). As expected, the order of elution is related to a large extent to the hydrophobicity of the derivatised amino acids. Thus, the more hydrophobic CBI derivatives elute towards the end of the chromatographic run.

Under the HPLC conditions used in this study, CBI derivatives of isoleucine and phenylalanine co-eluted. Both these derivatives can be resolved by increasing the flow rate from 0.7 to 1.4 ml min^{-1}.

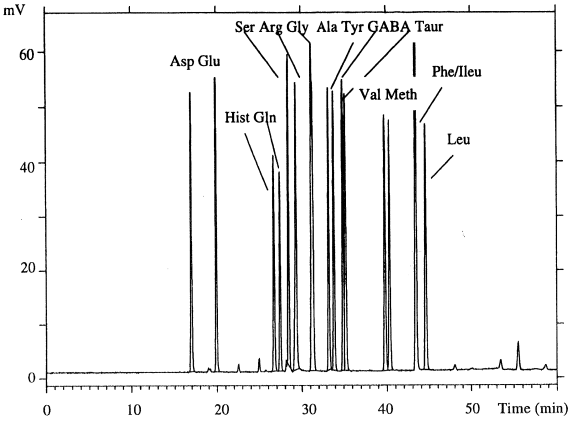

Fig. 1. Chromatogram of a mixture of 14 amino acids at level of 2 pmol injected onto the column following derivatisation with NDA. Sensitivity range was set to 0.1 mV. Derivatisation and HPLC conditions as described in the Experimental Section.

However, in view of these being putative amino acids and not of key value to the study of neurotransmitter function, the lower flow rate was used. Multiderivatised lysine and cysteine could not be detected at low levels because of fluorescence quenching [17].

3.3. Linearity, limits of detection and reproducibility

Calibration studies of amino acid concentration as function of HPLC-peak area of CBI derivatives were carried out for seven concentrations of a mixture of amino acids in the range 0.2–12.5 μ*M*. Linear plots with correlation coefficients between 0.997 to 0.999 were obtained in all cases. The limit of quantification of amino acids was found to be in the range 100–200 fmol injected with a signal-to-noise ratio of 10:1. The background level of amino acids present in reagents and contaminated glassware govern these detection limits. The limit of detection obtained in our studies is comparable to those reported for amino acids using OPA, 9-fluroenylmethylchloroformate (FMOC-Cl) [18] and 6-aminoquinoly-*N*-hydroxy-succiminimidyl carbamate (AQC) [19]. The reproducibility of the assay was measured by ten repeat injections of solutions containing a mixture of amino acids each at a concentration of 0.2, 6 and 12.5 μ*M* treated with NDA. The coefficients of variation for 0.2, 6.0 and 12.5 μ*M* solutions were in the range 5–10.9%, 3.7–4.4% and 1.1–3.5% respectively. The recovery was determined by adding a mixture of standard amino acids to microdialysates from the nucleus accumbens at final concentrations of 2.5, 5.0 and 10.0 μ*M*. Table 1 shows recovery obtained for six amino acids at these concentrations was between 98 and 108%.

3.4. HPLC-Mass spectrometric analysis

The identity of the CBI amino acid derivatives was confirmed by using fluorescence and mass spectrometric detection in tandem. The total ion chromatogram (TIC), representing the change in intensity of all ions within the scan range against time, obtained using mass spectrometric detection was in very good agreement with that generated by fluorescence detection (Fig. 2). The CBI amino acid derivatives typically showed fragment ions corresponding to decarboxylation of the amino acid moiety of the CBI group, as shown for the fragmentation of CBI-glycine in Fig. 3. Several other peaks were observed in the TIC but examination of their mass spectra indicated that they were not amino acids, and were assigned as impurities arising from the reagents and solvents used. The TIC shown in Fig. 2B represents an injection of 400 pmol of each amino acid on-column, however it was found that 40 pmol was sufficient to generate unambiguous mass spectra for each CBI amino acid derivative. Hence, dual detection can be used for the unequivocal identification of derivatised amino acids in biological samples.

3.5. Measurement of amino acids in brain tissue samples and micodialysates

Fig. 4 illustrates the levels of 14 amino acids found in tissue from the frontal cortex, nucleus accumbens, hippocampus and hypothalamus regions of mouse brain tissue. With the exception of glycine, amino acids that are present at relatively high levels are the neurotransmitters aspartic acid, glutamic acid, taurine and GABA. The first two are excitatory neurotransmitters, whereas taurine and GABA show inhibitory or neuromodulatory action, and act as a

Table 1
Recovery of amino acids at final concentrations of 2.5, 5.0 and 10 μ*M* in microdialysates from nucleus accumbens

Concentration of amino acid added (μ*M*)						Concentration of amino acid found (μ*M*)						% Recovery					
Asp	Glu	Gln	Gly	Tau	GABA	Asp	Glu	Gln	Gly	Tau	GABA	Asp	Glu	Gln	Gly	Tau	GABA
2.5	2.5	2.5	2.5	2.5	2.5	3.1	3.0	15.0	4.3	3.9	2.71	108	107	101	106	104	106
5.0	5.0	5.0	5.0	5.0	5.0	5.5	5.5	17.1	6.4	6.3	5.2	103	102	99	98	101	102
10	10	10	10	10	10	10.6	10.6	22.5	11.6	11.2	10.2	103	102	101	100	99	101

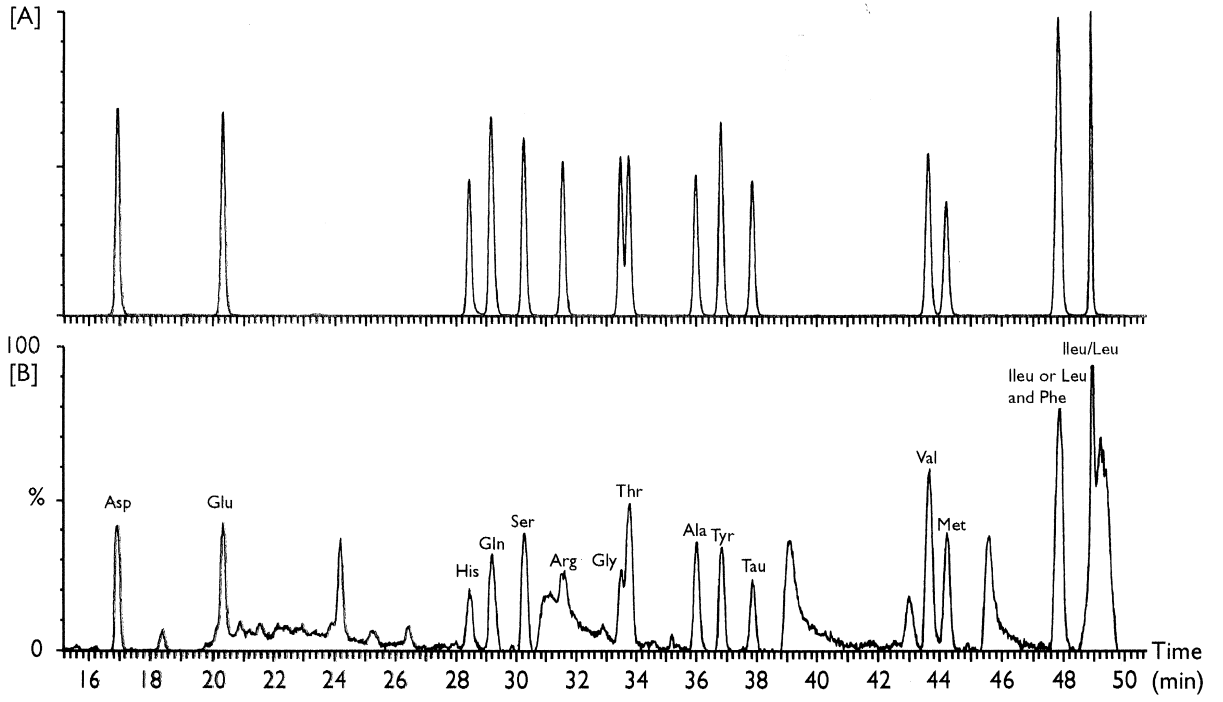

Fig. 2. (A) Chromatogram of amino acids each at 400 pmol treated with NDA; (B) TIC of sample used to record chromatogram in (A); Derivatisation, HPLC and mass spectrometric conditions as described in the Experimental Section.

counterbalance in the control of fast synaptic transmission. Unlike the levels of aspartic and glutamic acid, the concentrations of taurine and GABA differ widely from one brain region to another. It is also interesting that glutamine appears to be present at very high levels in all the brain regions studied. This is a metabolite of glutamic acid, and its formation may be partly due to a mechanism that regulates levels of glutamic acid. The levels of amino acids detected in tissue samples from the different brain regions are comparable to those reported by Tossman et al. [20].

As expected, the levels of amino acids in microdialysates were found to be significantly lower

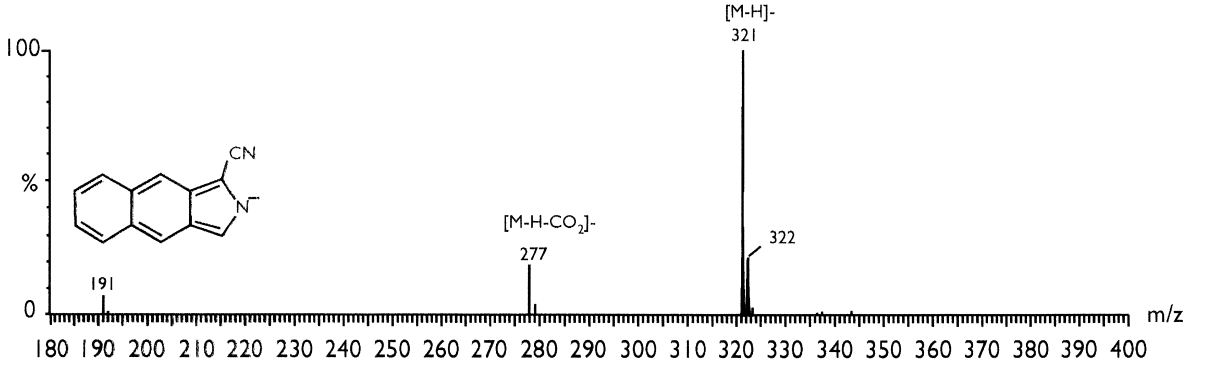

Fig. 3. Mass spectrum of CBI-derivative of glutamic acid, extracted from TIC of mixture of amino acids treated with NDA.

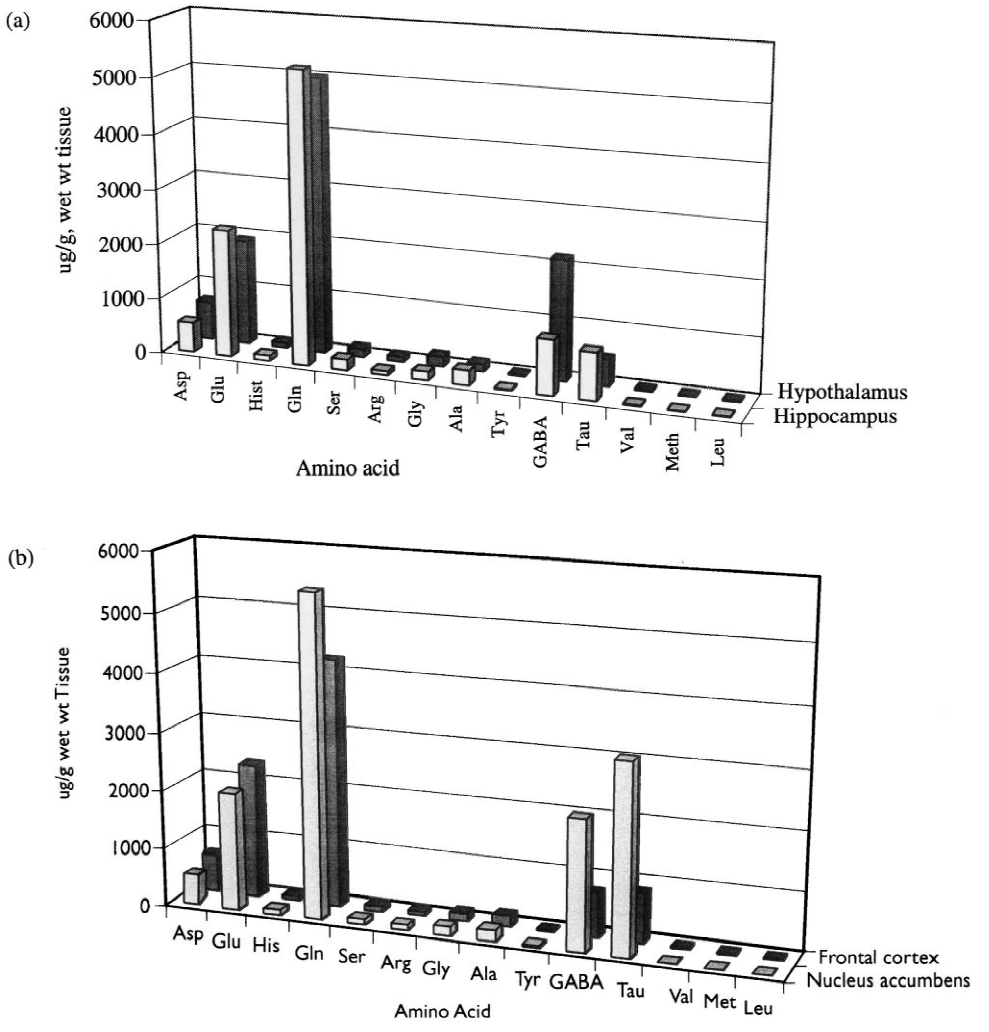

Fig. 4. Comparative histogram illustrating the levels of amino acids found in tissue samples of (a) nucleus accumbens and frontal cortex regions and (b) hippocampus and hypothalamus of mouse brain.

than those detected in brain tissue samples. In addition, the relative levels of amino acids were also markedly different. This can be seen clearly by comparing the relative levels of glutamic acid and glutamine, in tissue samples the latter amino acid is present at much higher levels than glutamic acid whereas no difference was observed in the relative amount of these amino acids in microdialysates (see Fig. 5). The difference in the relative amounts of amino acids observed between tissue samples and dialysates may be attributable to turnover samples equating to level of amino acids from synthesis vs. release vs. metabolism whereas amino acids in dialysates reflect the levels released into the interstitial fluid surrounding the nerve cells. The difference could also be due to differences in binding to proteins abundant in tissues. The results obtained for the frontal cortex in our study are markedly different from those reported by Tossman et al. [20]; the reason for this discrepancy is unclear. The differences observed may be attributable to slight variations in the placement of the microdialysis probe in

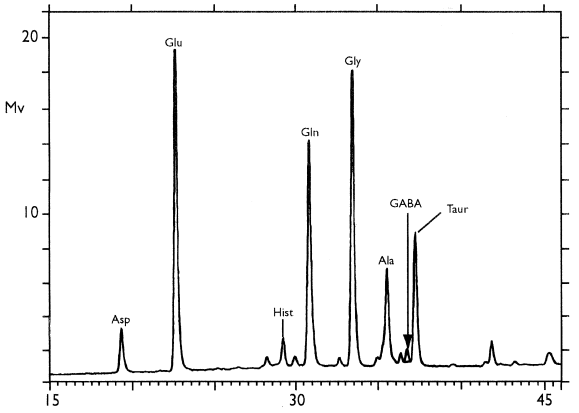

Fig. 5. Chromatogram of microdialysate sample from frontal cortex of rat treated with NDA. The level of glutamate and GABA in this sample were found to be 120 and <10 fmol respectively on column. Derivatisation and HPLC conditions as described in the Experimental Section.

the frontal cortex and or the higher concentration of calcium ions in the artificial CSF used by Tossman et al. [20].

The methodology outlined in this study is currently being used to monitor changes in amino acid levels in various brain regions following drug treatment. It is hoped that such studies will provide information on the localisation of drug action in the brain and will be of value in the design of more effective therapeutic agents.

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