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Simultaneous determination of total and extracellular concentrations of the amino acid neurotransmitters in cat visual cortex by microbore liquid chromatography and electrochemical detection

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Abstract

To investigate the influence of a partial sensory deprivation on the total and extracellular concentration of the amino acid neurotransmitters in cat visual cortex, two microbore HPLC methods were developed for the simultaneous determination of aspartate, glutamate, glycine, taurine and γ-aminobutyric acid in cat brain extracts or microdialysis samples. For the determination of the total neurotransmitter concentrations in the visual cortex, the brains were quickly frozen and 200-μm cryostat sections were made. From these sections tissue samples of 2×2 mm^2 containing the six cortical layers were dissected out of the central and peripheral parts of area 17. After homogenisation and centrifugation, the supernatants were used for quantitative amino acid analysis using an *o*-phthalaldehyde–*tert*.-butylthiol pre-column derivatisation HPLC gradient elution method on a microbore column (100×1 mm I.D.; C_8) and single electrochemical detection. Microdialysis samples from area 17 were obtained every 15 min using 2-mm probes perfused with synthetic cerebrospinal fluid at a flow-rate of 1 μl/min. After *o*-phthalaldehyde–*tert*.-butylthiol derivatisation they were analysed on a microbore column by isocratic elution and dual electrochemical detection. The instrumentation and the different separation parameters were optimised and standard curve, recovery, analytical precision and detection limits for each neurotransmitter were determined.

Keywords: Amino acids; Neurotransmitters

1. Introduction

Aspartic acid (Asp), glutamic acid (Glu) and γ-aminobutyric acid (GABA) are well known neurotransmitters in the central nervous system [1,2]. Glycine (Gly), has been suggested to have the same inhibitory function as GABA [3]. Even taurine (Tau) [2] and alanine (Ala) [4] have already been considered to play possible neurotransmitter roles in the visual system. Some of them have been suggested to be involved in the plastic changes underlying the electrophysiologically observed reorganisation of cortical topography in response to sensory deprivation. In our previous work, immunocytochemical methods were used to observe the influence of partial sensory deprivation on the intracellular concentration of GABA and Glu in cat visual cortex [5,6]. In order to investigate this influence on the total (intra- and

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extracellular) and extracellular concentration of the amino acid neurotransmitters, a sensitive high-performance liquid chromatography (HPLC) method for the simultaneous quantification of the different neurotransmitter amino acids at concentrations as low as 10 n*M* was needed.

Various derivatisation methods exist for the conversion of amino acids to detectable forms [7]. Joseph and Davies [8] were the first to demonstrate that the reaction of primary alkylamines, in the presence of an alkyl thiol yielded derivatives which have electroactive properties allowing the use of electrochemical detection (ED). Pre-column derivatisation was found to be more favourable than post-column reaction due to increased resolution and sensitivity [9]. Several HPLC–ED methods have been reported for the simultaneous quantification of amino acid neurotransmitters in brain tissue and microdialysis samples [10–12]. These methods provide the possibility to analyse these neurotransmitters at the sub-pmol level.

Donzanti and Yamamoto [10] described a method for the determination of amino acid neurotransmitters from brain tissue and microdialysis samples. However, the relative instability of *o*-phthalaldehyde (OPA)–β-mercaptoethanol (ME) derivatives and the use of conventional columns makes the sensitivity (1 pmol) of this method insufficient for our purpose. To improve the stability of the derivatives and the sensitivity of the system, Smolders et al. [11] used OPA–*tert*.-butylthiol (tBT) for the derivatisation in combination with a microbore column. The sensitivity of this method is sufficient for measurement of several amino acids, but the baseline drift caused by gradient elution influences the quantification of GABA. Rowley et al. [12] used ED of amino acid derivatives formed by a reaction with OPA in the presence of sulphite ions. The N-alky-1-isoindole sulphonate derivatives formed are electroactive, odourless and stable. This method is sensitive enough for the simultaneous determination of Glu, GABA, Tau, Gly and Ala but the important neurotransmitter Asp cannot be measured by this method as it is eluted with the solvent front.

In the present paper, a modification of the method of Smolders et al. allowing the simultaneous determination of 13 amino acids, including Asp, Glu, GABA, Tau, Gly and Ala, in tissue samples of cat visual cortex is described. A method using isocratic elution and dual ED allowing the simultaneous determination of the same amino acids in microdialysis samples of cat visual cortex is also described.

2. Experimental

2.1. Chemicals

Asp, GABA, Ala, arginine (Arg), asparagine (Asn), Glu, Gly, histamine (His), serine (Ser), Tau, threonine (Thr) and tyrosine (Tyr) were obtained from Sigma (St. Louis, MO, USA). The 20 amino acid standard mixture (2.5 m*M*) was from BAS (Bioanalytical Systems, West Lafayette, IN, USA). Standard stock solutions were prepared at a concentrations of 2.5 m*M* in Milli-Q water and stored at −70°C. Working solution was freshly diluted from stock solution. Analytical-grade disodium ethylenediaminetetraacetate (EDTA) was obtained from Merck (Belgolabo, Overyse, Belgium). Sodium acetate from Aldrich (Sigma–Aldrich, Bornem, Belgium); tBT, iodoacetamide and N,N-dimethylacetamide (99+% spectrophotometric grade) from Acros (Geel, Belgium). Methanol was of gradient grade for HPLC (Hiper Solv, BDH, Poole, UK).

2.2. Tissue preparation and extraction

One cat was used for the determination of total neurotransmitter concentration in the visual cortex. It was sacrificed with an overdose of nembutal (60 mg/kg, i.v.). The brain was rapidly removed and immediately frozen by immersion in liquid-nitrogen cooled isopentane and stored at −70°C. Two-hundred μm brain sections were cut on a cryostat. From these sections 2×2 mm^2 cortical tissue samples from the peripheral and central portion of area 17, and containing all six cortical layers, were dissected under the microscope using a surgical blade and expelled into 1.5-ml conical tubes, containing 200 μl 0.1 *M* chilled sodium acetate buffer (pH=6.8). The tissue was homogenised by grinding for 1 min at 4°C and the homogenate centrifuged at 12 000 *g* for 15 min at 4°C. Twenty μl of the supernatant was used for HPLC analysis. The tissue pellet was dissolved in

0.1 *M* NaOH, and the protein content of each sample was determined using a modification of the dye-binding Bradford method [13]. The total concentration of neurotransmitter was expressed as pg/μg protein.

2.3. Microdialysis

Two normal cats were used for microdialysis. Each cat was anaesthetised with 1.0 ml ketamine (i.m.) before a catheter was implanted in a leg vein. A 50% nembutal in 0.9% NaCl solution was administered through the catheter to keep the animal anaesthetised throughout the experiment. The cat was placed on a heating pad to maintain body temperature and fixed in a stereotaxic apparatus (Kopf, Germany). After surgery, the dialysis probe (Carnegie Medicin, CMA 12) with an external diameter of 0.5 mm and a 2-mm dialysing membrane at the tip was implanted into area 17. The probe was perfused with an artificial cerebrospinal fluid (aCSF) [14] with the following composition (NaCl, 124 m*M*; KCl, 5 m*M*; KH_2PO_4, 1.2 m*M*; $MgSO_4$, 1.3 m*M*; $CaCl_2$, 2.4 m*M*; glucose, 5 m*M*; $NaHCO_3$, 26 m*M*; pH=7.4) at a rate of 1 μl/min by a CMA 100 micro-injection pump (Carnegie Medicin, Stockholm, Sweden). Dialysis samples were collected at 15-min intervals using a CMA 170 fraction collector (Carnegie Medicin).

At the end of the experiments, the animal was killed with an overdose of nembutal (60 mg/kg) and perfused with 0.9% saline to remove the blood followed by a fixative containing 4% paraformaldehyde in 0.15 *M* phosphate buffer (pH 7.4, in 0.9% NaCl). After post-fixation in the same fixative for 48 h, the tissue was dehydrated, embedded in paraffin and serial sections (50 μm) were cut and stained with cresyl violet to allow the histological verification of the position of the dialysis probe. The in vitro recovery of the probe was tested before the dialysis experiment.

2.4. Chromatographic system

The LC system was a BAS 200A Chromatograph equipped with an amperometric detector (BAS). Separation was performed on a SepStik microbore column: 100×1 mm I.D., 3 μm C_8 (BAS). A flow splitter (BAS) was used to provide the low volumetric flow-rates required for the microbore column. The split ratio was 1/16. Operating the pump at 1.2 ml/min, yielded a microbore column flow-rate of approximately 75 μl/min. Automated pre-column derivatisation and sample injection was performed by a BAS Sample-Sentinel (BAS). The injection volume was 10 μl. The microbore column was coupled directly to the amperometric detector cell to minimise the dead volume. The ED system was equipped with a dual glassy carbon working electrode. The operating potentials were set at 750 mV versus an Ag/AgCl reference electrode. The cell volume was reduced by a 16-μm gasket. The chromatographic system was controlled by BAS CONTROL software and the chromatograms were integrated with CHROMGRAPH software (BAS).

For the quantification of neurotransmitters in visual cortex tissue extracts the method described by Smolders et al., but using a modified gradient as shown in Table 1, was used. Mobile phase A consisted of 66% (v/v) 0.1 *M* sodium acetate buffer (pH=6.8), containing 40 mg/l EDTA, 24% methanol and 10% N,N-dimethylacetamide; mobile phase B contained 15% 0.1 *M* sodium acetate buffer (pH=6.8), containing 40 mg/l EDTA, 45% methanol and 40% N,N-dimethylacetamide. The dual glassy carbon electrode was positioned in parallel.

An isocratic mobile phase, consisting of 57% 0.1 *M* sodium acetate buffer (pH=6.8, containing 40 mg/l EDTA), 27% methanol and 16% N,N-dimethylacetamide, was used for separation of the microdialysis samples. After 20 min, the pump was switched to mobile phase B for the elution of the amino acids having a retention time longer than that of GABA and in which we were not interested. The sodium acetate buffer was filtered through a 0.22-μm filter and the temperature of the whole system was

Table 1
Gradient programme

Time (min)	A (%)	B (%)
0.0	100	0
0.1	90	10
18.0	70	30
20.0	5	95
30.0	5	95
30.1	100	0

maintained at 35°C. The dual glassy carbon electrode was in the serial position.

2.5. Derivatisation procedure

The derivatisation was the same as described by Smolders et al. [11] but now adapted to the use of the Sample-Sentinel (BAS). Three μl of A was added to 15 μl of sample (brain extract or microdialysate) and automatically vortex-mixed by the Sample-Sentinel for 0.1 min. Immediately after mixing 3 μl of B was added, vortex-mixed for 2.0 min and injected on the microbore column. The whole derivatisation procedure, including flushing of the injection system, took 10 min.

3. Results and discussion

3.1. Optimisation of separation and detection

Using the method developed by Smolders et al. for the simultaneous determination of Asp, Glu and GABA in tissue extracts resulted in a low reproducibility of the GABA quantification. This was mainly due to the baseline drift even at a sensitivity setting of 20 nAFS. Changing the composition of mobile phase A and using the gradient shown in Table 1 resulted in an important decrease of the baseline drift as shown in Figs. 1 and 2 and a more reproducible quantification of GABA (R.S.D. 4.87%). As illustrated by the chromatograms (Figs. 1 and 2) 13 amino acids, including Asp, Glu, Gly, Ala, Tau and GABA, could be completely separated. The other seven amino acids, isoleucine, lysine, leucine, tryptophan, methionine, phenylalanine and valine, also present in brain tissue and microdialysis samples, were eluted after GABA by a washout step (10 min, 95% mobile phase B).

As the total amino acid concentrations in different brain regions are sometimes very different, ED was performed using two parallel electrodes set at a different sensitivity. In that way ''off scale'' problems could be avoided.

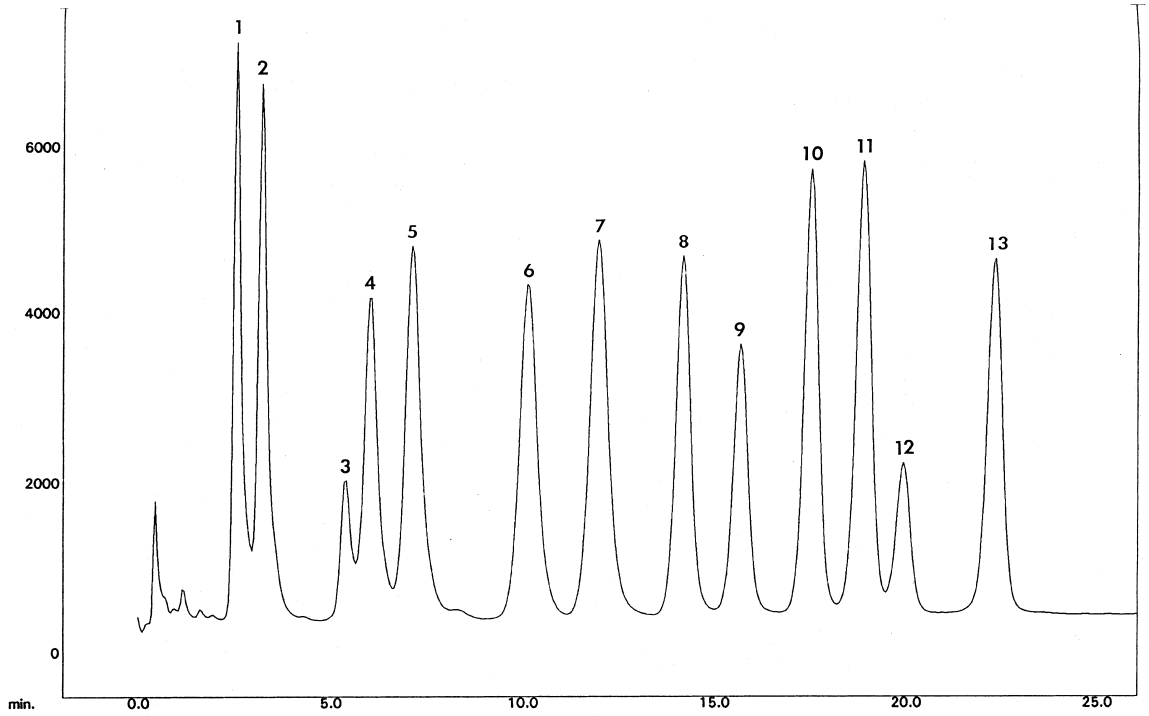

Fig. 1. Chromatogram of a 5.0 μ*M* amino acid standard mixture: range 20 nAFS; the numbered peaks represent: (1) Asp; (2) Glu; (3) Asn; (4) His; (5) Gln; (6) Ser; (7) Arg; (8) Gly; (9) Thr; (10) Tyr; (11) Ala; (12) Tau and (13) GABA.

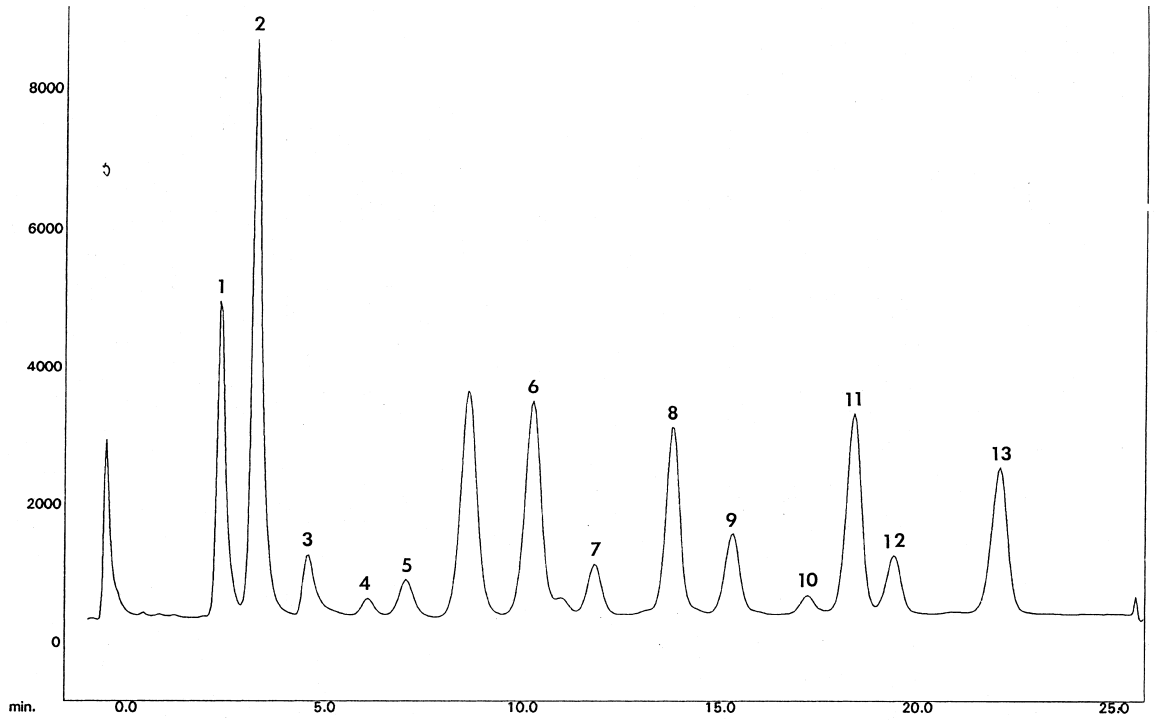

Fig. 2. Chromatogram of amino acids in homogenates of the central portion of normal cat area 17; range 20 nAFS; peaks as in Fig. 1.

When applying this protocol on microdialysis samples of cat visual cortex, we had to increase the sensitivity setting to 0.1 nAFS which again resulted in an important baseline drift interfering with the quantification of GABA. To eliminate this baseline drift we tried an isocratic elution that after optimisation of pH, ion strength and organic modifier concentration resulted in the chromatogram shown in Fig. 3. The N,N-dimethylacetamide concentration seems to be the most critical parameter for this separation. The influence of N,N-dimethylacetamide on the capacity factors of the different amino acids is shown in Fig. 4.

As the concentration of Asp and Glu in visual cortex dialysates is about 5- to 10-times higher than the GABA concentration, the Asp and Glu peaks are off scale at a sensitivity setting of 0.1 nAFS and this interferes with an exact integration of the peak area. We could however overcome this problem by installing a serial dual working electrode set at different sensitivities (0.1 nAFS for the upstream and 2.0 nAFS for the downstream electrode). This set up has at the same time the advantage that the large front peak, otherwise interfering with the detection of Asp and Glu, is now eliminated by the electrochemical reaction at the upstream electrode.

3.2. Optimisation of the injection and microbore system

During our experiments it became clear that small temperature changes in the flow splitter, the injection system, the microbore column and the detector were responsible for bad reproducibility. This was due to changes of diffusion coefficients which influence the retention time and resolution of some analytes, redox kinetics, background noise and flow speed. Therefore, the microbore column, the flow splitter system, the detector cell and the mobile phase were thermally controlled at 35°C by the BAS 200 system.

To obtain a reproducible derivatisation and to avoid the pungent odour of tBT, an autosampler was used for automated pre-column derivatisation and injection. Different mixing methods, needle positions

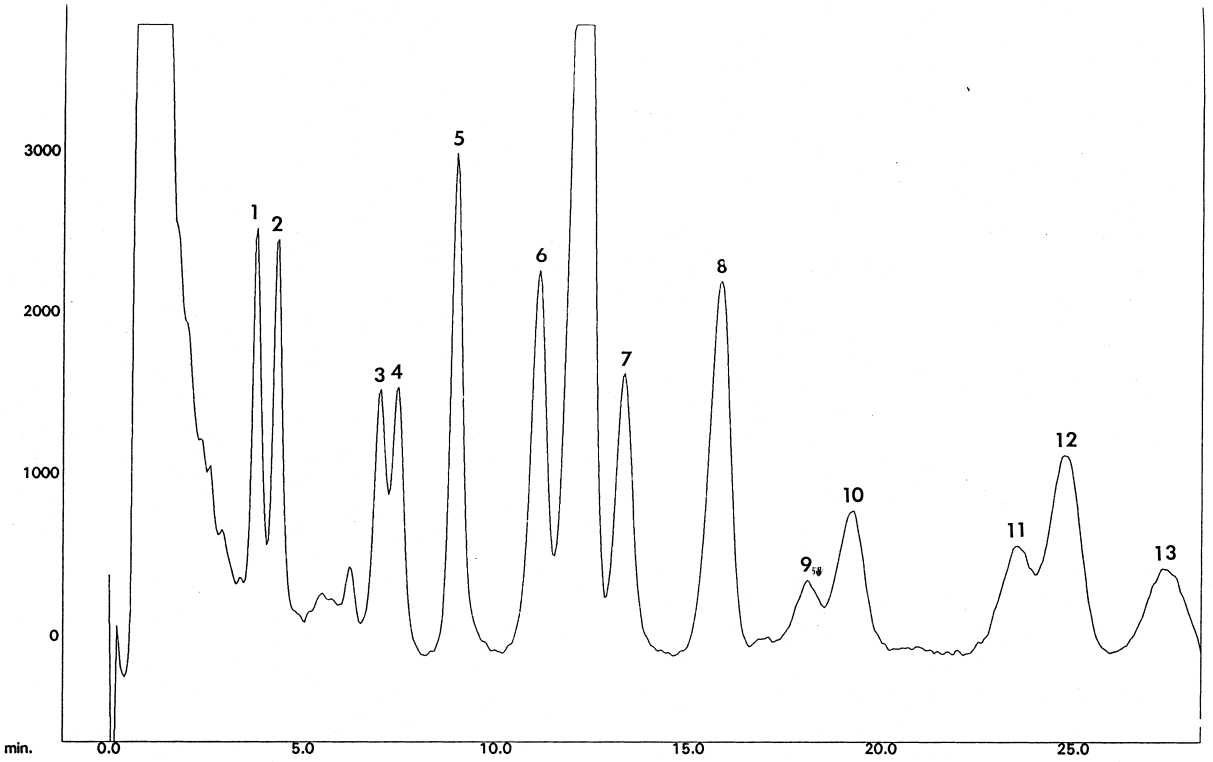

Fig. 3. Chromatogram of a 0.05 μ*M* amino acid standard mixture: range 0.1 nAFS; numbered peaks as in Fig. 1.

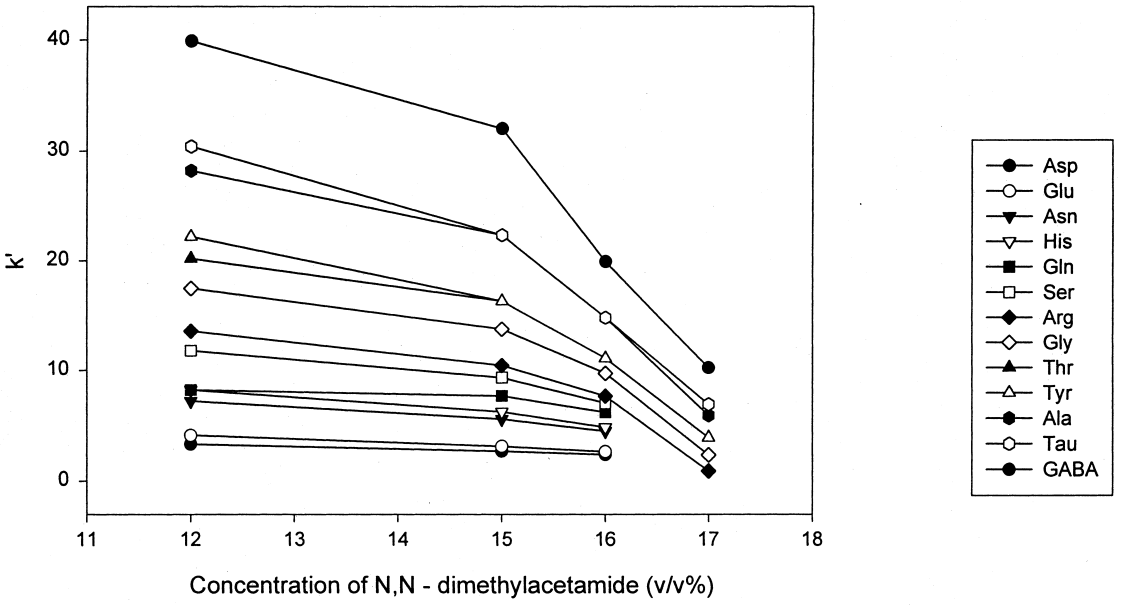

Fig. 4. Effect of N,N-dimethylacetamide concentration on the capacity factors of 13 amino acids.

Table 2
System suitability test for binary gradient HPLC method

Amino acid	R.S.D. (%) of peak area	Concentration range of standard curve (μ*M*)	Correlation coefficient (*r*)
Asp	1.90	1.0–20.0	0.9954
Glu	1.54	1.0–20.0	0.9937
Gly	6.30	1.0–20.0	0.9985
Ala	4.61	1.0–20.0	0.9990
Tau	3.74	1.0–20.0	0.9974
GABA	4.87	1.0–20.0	0.9979

and reaction times were tested. Vortex-mixing method resulted in a better sensitivity than syringe mixing.

To minimise the dead-volume and obtain the highest sensitivity, the microbore column was directly connected to the ED cell. For the connection with the injection valve a tubing with an I.D. of 50 μm was used to avoid diffusion.

3.3. System suitability

A system suitability test was performed by five consecutive injections of a 1 μ*M* and a 0.1 μ*M* standard mixture for respectively the gradient and isocratic elution (see Tables 2 and 3).

3.4. Extraction of tissue sample

As the pH during a commonly used perchloric acid extraction is incompatible with the derivatisation method used, the extraction of the brain tissue samples was done in 0.1 *M* acetate buffer pH 6.8. The recovery of the neurotransmitters was determined by comparison of the amounts of standard mixture added and measured in brain homogenates. The results are shown in Table 4.

Table 4
Recovery of neurotransmitter amino acids in cat brain tissue ($n=3$)

Amino acid	Recovery (%)
Asp	91.90
Glu	100.37
Gly	93.43
Ala	78.74
Tau	84.69
GABA	89.45

3.5. Neurotransmitter concentrations

The results obtained for brain tissue samples using the gradient elution method are summarised in Table 5. As expected for normal cat visual cortex no significant differences in amino acid concentrations were found between the central and the peripheral portions of area 17. A significant decrease for both Glu and GABA was however found in the deafferented part of the visual cortex of a cat in which a central binocular retinal lesion had been induced two weeks before sacrifice [15] (to be published). This is in agreement with our previous immunocytochemical [5,6] and in situ hybridisation [16] studies on the

Table 5
Neurotransmitter concentration in the peripheral and central part of area 17 in normal cat

Amino acid	Concentration (pg/μg protein)	
	Central area 17	Peripheral area 17
Asp	5.78±0.89	4.72±1.26
Glu	21.15±2.03	21.29±2.08
Gly	5.74±0.81	6.04±0.73
Ala	2.32±0.44	2.27±0.24
Tau	13.43±1.81	14.88±1.97
GABA	8.72±0.92	8.74±0.92

Table 3
System suitability test for isocratic HPLC method

Amino acid	R.S.D. (%) of peak area	Concentration range of standard curve (μ*M*)	Correlation coefficient (*r*)	Detection limit ($S/N=3$, μ*M*)
Asp	5.6	0.1–5.0	0.9998	0.0001
Glu	4.6	0.1–5.0	0.9997	0.0002
Gly	6.2	0.5–5.0	0.9948	0.0001
Ala	7.0	0.1–5.0	0.9977	0.006
Tau	6.3	0.1–5.0	0.9974	0.005
GABA	8.3	0.01–1.0	0.9981	0.005

same paradigm suggesting a decrease for both Glu and GABA after sensory deprivation of the part of area 17 subserving central vision.

Using the isocratic elution method on three different microdialysis samples taken in the central portion of area 17 we obtained the following concentrations (mean±S.E.M., μ*M*): Asp (0.0593±0.017); Glu (0.243±0.066); Gly (0.604±0.17); Ala (1.42±0.37); Tau (1.30±0.35) and GABA (0.0168±0.0040). The in vitro recovery of the probe was: (mean±S.E.M., $n=5$) Asp (12.17±0.64%); Glu (9.93±0.44%); Gly (15.77±0.72%); Ala (10.23±0.53%); Tau (9.72±0.38%) and GABA (9.37±0.35%).

4. Conclusions

A minor modification of the gradient elution method developed by Smolders et al. allowed the simultaneous quantification of Asp, Glu, Gly, Ala, Tau and GABA in brain tissue extracts. We developed an isocratic system which permit the simultaneous measurement of amino acids including GABA in microdialysis samples of cat visual cortex. Both methods are easy, fast and highly reproducible. The isocratic method has a detection limit of 5 n*M* for GABA at a signal-to-noise ratio of 3/1. The main drawback of this method is that an inter-sample washout is necessary. This delays the analysis time with about 15 min.

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