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Rapid and sensitive step gradient assays of glutamate, glycine, taurine and γ-aminobutyric acid by high-performance liquid chromatography–fluorescence detection with *o*-phthalaldehyde–mercaptoethanol derivatization with an emphasis on microdialysis samples

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Abstract

We developed a rapid step-gradient HPLC method for determination of glutamate, glycine and taurine, and a separate method for determination of γ-aminobutyric acid (GABA) in striatal microdialysates. The amino acids were pre-column derivatized with *o*-phthalaldehyde–2-mercaptoethanol by using an automated refrigerated autoinjector. Separation of the amino acids was established with a non-porous ODS-II HPLC column, late-eluting substances were washed out with a one-step low-pressure gradient. Concentrations of the amino acids were determined with a fixed-wavelength fluorescence detector. The detection limit for GABA was 80 fmol in a 15 μl sample, detection limits for glutamate, glycine and taurine were not determined because their concentrations in striatal perfusates were far above their detection limits. Total analysis time was less than 12 min, including the wash-out step. The methods described are relatively simple, sensitive, inexpensive, and fast enough to keep up with the microdialysis sampling.

Keywords: Derivatization, LC; Glutamate; Glycine; Taurine; γ-Aminobutyric acid

1. Introduction

It is well known that various amino acids [e.g., glutamate (Glu), aspartate (Asp), glycine (Gly), taurine (Tau) and γ-aminobutyric acid (GABA)] play important roles in cerebral neurotransmission as well as in different neurological disorders [1–4]. Thus, measurement of their cerebral extracellular concentrations is of interest in the field of neuroscience. Microdialysis is a powerful technique which permits determination of the extracellular concentration of amino acid neurotransmitters during various pharmacological or physiological manipulations. The presence of relatively low concentrations of amino acids in microdialysis samples requires a sensitive analytical method for their determination. This par-

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ticularly concerns GABA since its basal concentrations in cerebral microdialysates are in a range of about 5–100 n*M*. Reversed-phase high-performance liquid chromatography (HPLC) with pre-column derivatization and fluorescence or electrochemical detection are methods of choice for analysis of amino acids in microdialysates [5–14]. So far the best sensitivity has been achieved by using microbore chromatography [8,14]. Although the introduction of this advanced chromatographic technology is very tempting, it requires some additional reconstruction of a conventional HPLC system [14].

In this paper we describe a HPLC method for the simultaneous assay of glutamate, glycine and taurine, and a separate method for the assay of GABA in brain striatal microdialysis perfusates. Both methods described are rapid and sensitive. This was achieved by automated *o*-phthalaldehyde (OPA) derivatization, with isocratic chromatography on a non-porous silica (NPS) column with 3 μm particle size followed by a wash-out step of late-eluting amino acids.

2. Experimental

2.1. Equipment

The HPLC system consisted of a solvent delivery pump (Pharmacia LKB gradient pump 2249, connected to a Pharmacia Biotech low-pressure mixer; Pharmacia Biotech, Sweden), a refrigerated microsampler (Model CMA/200; CMA/Microdialysis, Stockholm, Sweden), an analytical column (Micra NPS ODS-II, 100×4.6 mm I.D., 3 μm particle size; Micra Scientific, IL, USA) protected by a 0.5-μm inlet filter (included with the column) and thermostated by a column heater (Model CROCO CIL; Cluzeau Info-Labo, France), and a fluorescence detector (Model CMA/280; CMA/Microdialysis). The chromatograms were recorded on an integrator (Model C-R 4A Chromatopac; Shimadzu, Kyoto, Japan).

2.2. Reagents

2.2.1. Chemicals

Calcium chloride, disodium hydrogenphosphate, magnesium chloride, potassium chloride, sodium chloride and L-(+)-ascorbic acid (all pro analysi grade) were obtained from Merck (Darmstadt, Germany). All amino acids (β-alanine, L-alanine, L-arginine hydrochloride, L-aspartate, L-asparagine, L-glutamic acid, L-glutamine, glycine, L-histidine hydrochloride, L-threonine, L-serine) except taurine (Fluka, Buchs, Switzerland) were obtained from Sigma (St. Louis, MO, USA). Methanol and tetrahydrofuran (HPLC grade) were obtained from Rathburn (Walkerburn, UK), and acetonitrile (HPLC grade) from J.T. Baker (Deventer, The Netherlands). Orthophosphoric acid (OPA) (pro analysi grade) was obtained from Riedel-de Haën (Seelze, Germany).

2.2.2. Mobile phases

Mobile phase A: 0.05 *M* disodium hydrogenphosphate, pH 6.1 (adjusted with 85% phosphoric acid), 0.5% (v/v) acetonitrile, 1% (v/v) tetrahydrofuran.

Mobile phase B: 0.05 *M* disodium hydrogenphosphate, pH 4.8 (adjusted with 85% phosphoric acid), 7% (v/v) acetonitrile, 1% (v/v) tetrahydrofuran.

Mobile phase C: acetonitrile–tetrahydrofuran–water (70:1:29, v/v).

HPLC-water was first purified with a Milli-RO 12 Plus water purifying system (Millipore, Milford, MA, USA), and then processed with an Elgastat UHQ-II system (Elga, UK). All mobile phases where filtered through 0.2-μm polyamide membrane filters and degassed with helium for 10 min.

2.2.3. Amino acid standards

Standard stock solution of amino acids (1 m*M*) were prepared in a mixture of water–methanol (1:1) and stored at 4°C for 1 month. Working standard solutions were prepared daily by diluting the stock solutions with Ringer solution.

2.2.4. Derivatizing reagent

OPA–mercaptoethanol (2-MCE) reagent was prepared daily by mixing 1 ml OPA borate buffer solution (OPA incomplete, 1 mg/ml; Sigma) with 3 μl of 2-MCE solution (1.114 g/ml; Sigma).

2.3. Derivatization

Automated sample derivatization was carried out using a CMA/200 refrigerated autosampler at 4°C. The autosampler was programmed to add 6 μl of the

derivatizing reagent to 15 μl of a standard or microdialysis sample, to mix two times and to inject 20 μl onto the column after a reaction time of 1 min.

2.4. Chromatographic conditions

2.4.1. Analysis of glutamate, glycine and taurine

A 5-μl volume of a striatal microdialysis sample was diluted with 10 μl 1.5 m*M* β-alanine in Ringer solution (internal standard, I.S.) and derivatized as described above. Mobile phase A was pumped at a flow-rate of 1 ml/min, column temperature was maintained at 37°C. The detector gain was set to 10 times signal amplification. The external low-pressure mixer was programmed to switch to mobile phase C (wash-out step) after 3 min from the beginning of the run and to switch back to mobile phase A after 2 min. Switching from one mobile phase to another takes about 2 min (time needed for solvent replacement in low-pressure mixer and in tubings at a flow-rate of 1 ml/min). The whole step-gradient run (from injection to injection) took less than 12 min in total.

2.4.2. GABA analysis

The GABA analysis conditions are the same as for Glu, Gly and Tau analysis except: undiluted 15 μl of microdialysate sample (without I.S.) was derivatized, mobile phase B was used instead of mobile phase A and detector gain was set to 100 times signal amplification.

2.5. Microdialysis

Male Wistar rats were implanted with guide cannulae (BAS MD-2250) under halothane anaesthesia (3.5% during induction for 5 min and then 2.5–1% during surgery). The guide cannulae were aimed at the point above the caudate–putamen (A/P=+1.0, L/M=+2.7, D/V=−2.0, according to the atlas by Paxinos and Watson [15]. The cannula was fastened to the skull with dental cement (Aqualox, Voco, Germany) and three stainless steel screws. After the surgery the rats were placed into individual test cages (30×30×40 cm) and allowed to recover at least for 4 days before the experiment. In the morning of the experiment days, a microdialysis probe (BAS, MD-2200, 4 mm membrane, 0.32 mm O.D.) was inserted into the guide cannula. Modified Ringer solution (147 m*M* NaCl, 1.2 m*M* $CaCl_2$, 2.7 m*M* KCl, 1.0 m*M* $MgCl_2$ and 0.04 m*M* ascorbic acid) was infused through the probe at a flow-rate of 2 μl/min. The collection of microdialysis samples was started 2.5–3 h after the insertion of the probe. The average concentration of the first 3–4 stable samples was determined as baseline.

2.6. Pharmacological manipulations

To illustrate changes in GABA concentration in striatal perfusate of rat brain, the release of GABA was stimulated by high concentration of KCl. The perfusion medium was switched to modified Ringer solution containing 100 m*M* of KCl for 30 min. To illustrate the changes in striatal Tau output the rats were administered 1.5 mmol/kg of Tau intraperitoneally three times at 30-min intervals, the total dose of Tau being 4.5 mmol/kg.

3. Results

3.1. Separation of glutamate, glycine and taurine

Fig. 1 presents chromatograms of the separation of Glu, Gly and Tau in a standard mixture of amino acids (15 pmol each per injection; panel A) and in 5 μl of striatal perfusate (panel B). In striatal perfusate no β-alanine is detected, therefore it was introduced as an I.S. for Glu, Gly and Tau analysis (panel C). All amino acids of interest were clearly separated within 5 min. The retention times for Glu, Gly and Tau were about 0.7, 2.1, and 4.9 min, respectively. In Glu, Gly and Tau assay, other amino acids giving single peaks were aspartic acid, glutamine, threonine, arginine and L-alanine, which allows one to determine them if of interest. Serine was co-eluted with histidine and poorly separated from asparagine.

3.2. Separation of GABA

Fig. 2 illustrates the separation of GABA in an amino acid standard mixture containing 0.30 pmol of each amino acid per injection (panel A) and in 15 μl rat brain microdialysis perfusate (panel B). The

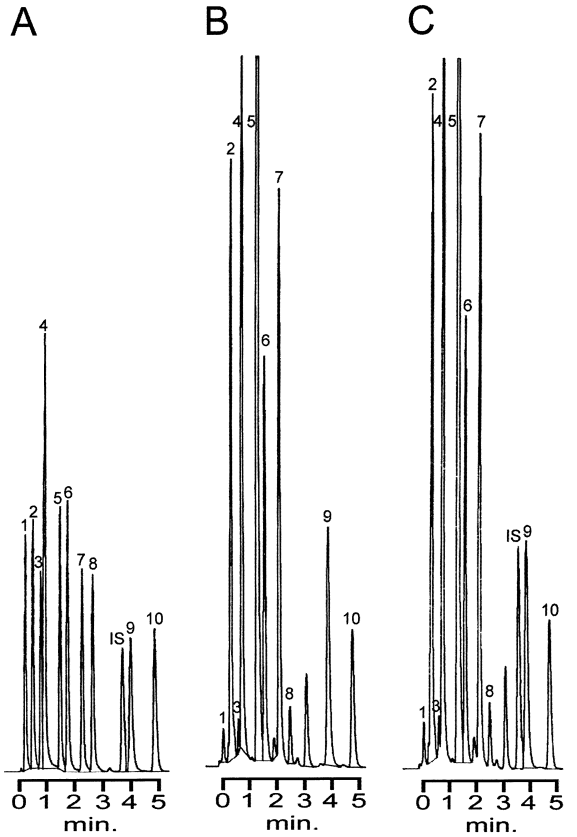

Fig. 1. Separation of OPA–2-MCE derivatives (A) in a standard mixture of amino acids (15 pmol of each amino acid injected) containing (1) aspartate, (2) glutamate, (3) asparagine, (4) serine+histidine, (5) glutamic acid, (6) glycine, (7) threonine, (8) arginine, (I.S.) β-alanine, (9) L-alanine, (10) taurine; (B) in 5 μl of rat striatal microdialysis perfusate without internal standard; (C) in 5 μl of rat striatal microdialysis perfusate+15 pmol of internal standard (I.S.). Assay conditions for Glu, Gly and Tau assay described in the Experimental section.

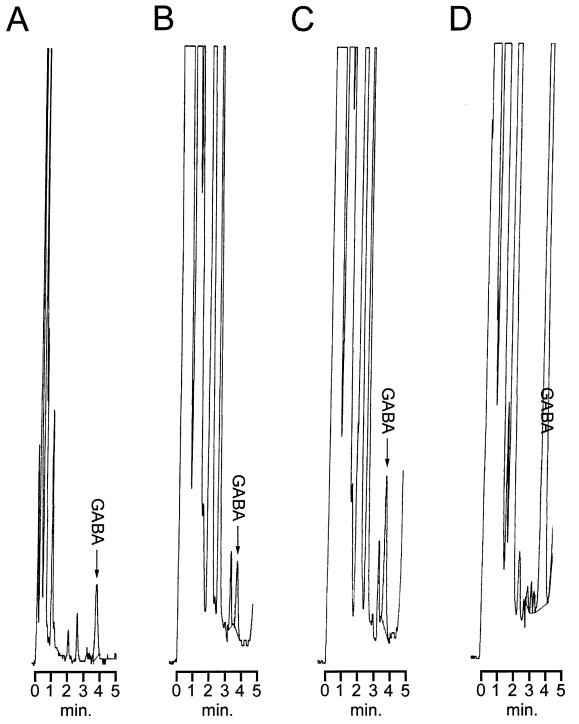

Fig. 2. Separation of GABA (A) in the amino acid standard mixture (0.30 pmol of each amino acid injected); (B) in 15 μl of rat microdialysate perfusate; (C) the same as in (B) but spiked with 0.30 pmol GABA; (D) in 15 μl of striatal perfusate obtained with intrastriatal perfusion of 100 m*M* KCl. Assay conditions for analysis of GABA as described in the Experimental section.

retention time for GABA was about 4.1 min. To verify the peak purity of GABA in striatal perfusate it was spiked with 0.30 pmol external GABA. As shown in panel C, this gives exact peak height summation.

3.3. Linearity of the detector response

The linearity of the detector response was verified in the concentration range from 15 to 120 pmol per injection for Glu, Gly and Tau and from 0.15 to 1.5 pmol per injection for GABA. The concentrations used correspond to concentrations found in striatal microdialysis perfusates. Linear response was obtained for all four amino acids in the concentrations used (Table 1).

3.4. Repeatability of the assay

Repeatability was tested by determining the concentrations of Glu, Gly, Tau and GABA in 13 samples taken from a pooled microdialysis perfusate and is expressed as relative standard deviation (RSD). The corresponding RSDs were 4.6% (Glu); 3.3% (Gly); 1.9% (Tau) and 4.2% (GABA). Introducing β-alanine as I.S. in the assay diminished the RSDs for Glu and Gly by approximately 30% (RSDs with internal standard 3.1% and 2.0% for glutamate and glycine, respectively) but had no effect on the RSD of Tau (1.9% with I.S.). The amino acid

Table 1
Linear regression equations and quality coefficients of detector response for glutamate (Glu), glycine (Gly), taurine (Tau) [15, 30, 60, 90 and 120 pmol of each injected, in relation to the internal standard (β-alanine; 15 pmol) area] and γ-aminobutyric acid (GABA; 0.15, 0.3, 0.6, 0.9, 1.2 and 1.5 pmol injected, as peak height in μV)

Amino acid	Regression equation	*R*	S.E. of coefficient	S.E. of intercept
Glu	$y=-3.560+47.072x$	0.9997	0.654	1.067
Gly	$y=-3.363+31.333x$	1.0000	0.099	0.243
Tau	$y=0.488+46.168x$	0.9999	0.212	0.488
GABA	$y=-0.006+0.003x$	0.9995	0.0001	0.017

S.E., Standard error.

concentrations found in this pooled striatal microdialysis perfusate were 4 pmol/5 μl for Glu, 21 pmol/5 μl for Gly, 10 pmol/5 μl for Tau and 0.31 pmol/15 μl for GABA (concentrations not corrected with in vitro recovery).

3.5. Stability of the amino acid derivatives

The stability of OPA–2-MCE derivatives of amino acids were tested by comparing a reaction time of 1 min with a reaction time of 5 min. There were no differences in detector responses between the two reaction times tested, except for the Tau–OPA–2-MCE derivative which showed a 5% loss in detector response using a 5-min reaction time (data not shown).

3.6. Detection limits

The detection limit was only determined for GABA, because Glu, Gly and Tau concentrations in cerebral striatal perfusates are far above their detections limits. This limit for GABA was 80 fmol on the column at a signal-to-noise ratio 3:1.

3.7. Effect of pharmacological manipulations on the concentrations of taurine and GABA

Perfusion by 100 m*M* KCl dramatically (about 20-fold) increased the concentration of GABA in striatal perfusates (Fig. 2D). Similarly, administration of Tau (totally 4.5 mmol/kg i.p. within 1 h) increased the extracellular concentration of Tau maximally by about 10-fold (Fig. 3B). However, the concentrations of Glu or Gly were not altered by the administration of Tau. The intraperitoneal administration of saline did not affect the extracellular concentrations of Tau, Glu or Gly (Fig. 3A).

4. Discussion

The two methods described here allow the separation of Glu, Gly, Tau and GABA in cerebral microdialysates with good resolution and sensitivity in less than 5 min. This was achieved by using a new kind of non-porous silica packed HPLC column. Even with the wash-out step the analysis time remains less than 12 min, although the concentrations of organic solvents in the mobile phases used were very low. The unstability of OPA–2-MCE amino acid derivatives has been a concern, especially for late-eluting amino acids [16]. Because of short retention times of amino acid derivatives in our chromatographic system there were no problems with the stability of OPA–2-MCE amino acid derivatives.

The detection limit of the assay for GABA (80 fmol) in our method is higher than reported in the microbore HPLC amino acid assay method (5 fmol; [14]) but less than that observed with common reversed-phase porous silica packed HPLC columns [5,7,9,17] with the exception of the method of Rowley et al. [12] where the detection limit of 10 fmol for GABA was achieved by using electrochemical detection. This is not surprising given that electrochemical detection is generally more sensitive than fluorescence detection. However, the use of gradient HPLC analysis with electrochemical detection is difficult or even impossible, and even in step-gradient analysis such as described here the equilibrium interval after column wash-out would be

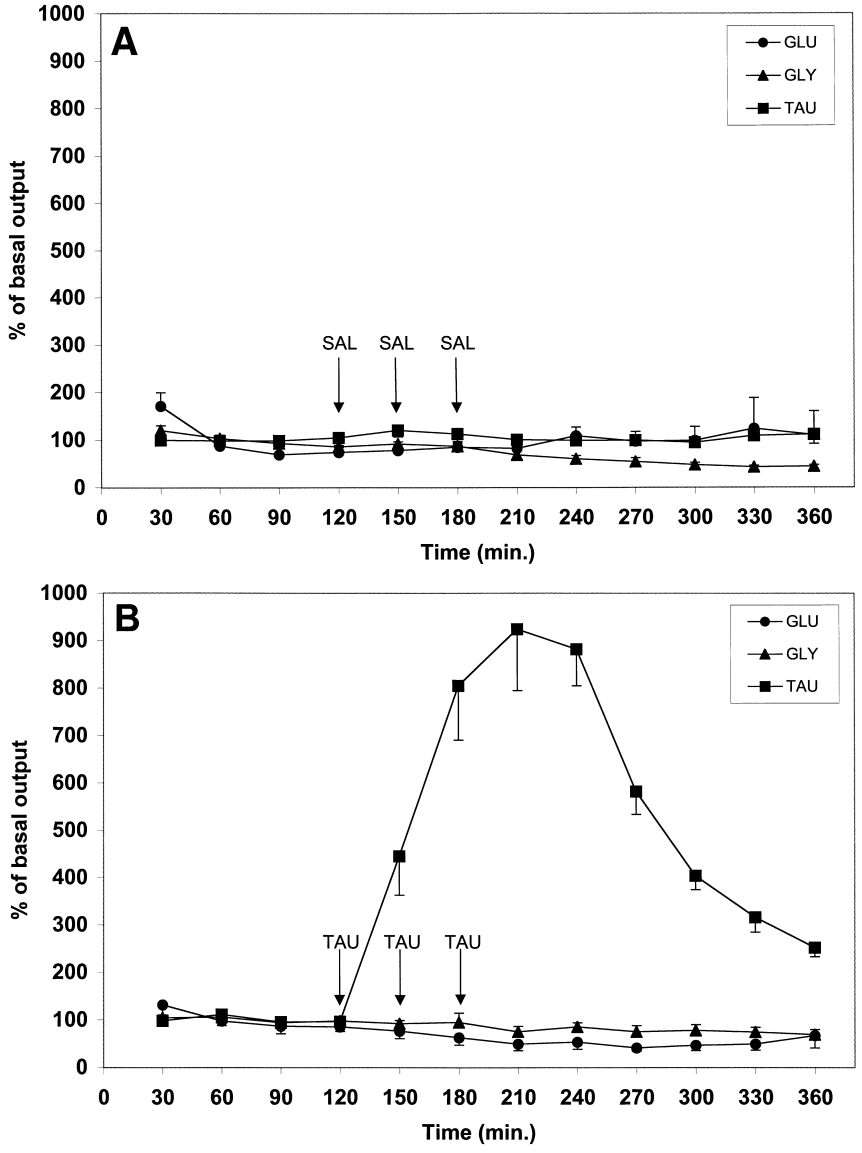

Fig. 3. Effects of three consecutive intraperitoneal injections of saline (Sal; panel A) and taurine (1.5 mmol/kg/injection; panel B) on striatal output of glutamate (Glu), glycine (Gly) and taurine (Tau) in rats. Given are means±standard errors (n=4). The basal levels of Glu, Gly and Tau were 2.42±2.24, 11.87±6.37 and 15.09±4.08 pmol in 5 μl sample, respectively (mean±standard deviation).

significantly longer. Alternatively, when using isocratic analysis one has to wait for the late-eluting compounds to come off the column, which makes the overall analysis time significantly longer. Indeed, the overall analysis times for GABA in the method of Rowley et al. [12] was about 25 min.

Several groups have developed rapid isocratic HPLC methods for the determination of amino acids from microdialysis perfusates or from cerebral homogenates [5,7,17,18]. However, in these methods there was no wash-out of the column at the end of the chromatographic run. Thus, the fate of the late-eluting amino acids in these methods remains open. At least in striatal perfusates there are large peaks

appearing in our chromatograms even at 30 to 40 min after the beginning of the run. Therefore, the wash-out step after the run is obligatory.

Tetrahydrofuran (1%, v/v) was introduced to all three mobile phases. Actually it was important only for Glu, Gly and Tau assay in order to separate β-alanine (I.S.) and Tau from L-alanine. Since the same column was used both for Glu, Gly, Tau and GABA analyses and tetrahydrofuran-containing systems require considerable equilibration times, it was used in all three mobile phases.

To illustrate the validity of our assay the effects of KCl perfusion on the release of GABA as well as the effects of intraperitoneal administration of Tau on the extracellular concentrations of Gly, Gly and Tau were studied. As expected, intrastriatal perfusion with 100 m*M* KCl profoundly increased the concentration of GABA in the microdialysate. Intraperitoneally administered Tau (total amount of 4.5 mmol/kg) selectively increased the concentration of Tau in striatal perfusates without affecting Glu or Gly levels. This clearly shows that peripherally administered Tau is able to cross the blood–brain barrier and penetrates the brain. The extracellular concentrations found for Gly, Tau and GABA were more or less stable among different rats, but in agreement with previously reported results [19] we found a considerable interindividual variation in the basal levels of extracellular glutamate (data not shown).

In summary, the methods presented here are sensitive, rapid, reproducible and relatively simple, and provide some advantages that were previously only associated with microbore HPLC systems. For neurochemists these methods offer one additional choice for a method for determination of amino acids.

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