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ORIGINAL RESEARCH

Analysis of neuroactive amino acids from microdialysate samples by fluorescence detection using a modification of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate method

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ABSTRACT: A sensitive and rapid reversed-phase high-performance liquid chromatographic method using pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and fluorescence detection is reported. By directly derivatizing microdialysate samples with AQC, an automatic and rapid simultaneous measurement of aspartate, serine, glutamate, glycine and histidine was developed. Excellent linearity ($r^2 \geq 0.998$) was achieved for the standard mixture used for the validation experiments. Within-day and between-day precision was less than 6.2%, and the accuracy ranged from 95 to 105.2% in standards. This method is suitable for single run analysis of a high number of small volume microdialysate samples from rat hippocampus. Amino acids from microdialysate samples were quantified with RSD for reproducibility below 2%, and at approximately 0.1% for retention time. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: HPLC; AQC; amino acids; microdialysis; fluorescence detection

INTRODUCTION

Local changes in brain amino acid extracellular concentrations have been related to several neurological diseases. Previous studies have shown significant changes in glutamate, glycine and aspartate during experimental seizures (Rogawski, 1995; Chapman, 1997; Sierra-Paredes *et al*., 2000) in several brain areas such as the hippocampus, cerebral cortex and amygdala.

A good method for *in vivo* continuous monitoring of amino acid concentrations from discrete brain areas is microdialysis sampling in chronic animals. Using this technique, it is possible to perform long-term experiments and collect numerous samples from the same animal, thus allowing useful comparisons of modifications in amino acid concentrations and behavioral or electrophysiological data (Sierra-Paredes and Sierra-Marcuño, 1996). However, in order to maximize the efficiency of microdialysis experiments, a reproducible method of amino acid analysis, suitable for processing a high number of small-volume samples, is required.

HPLC systems, in conjunction with reversed-phase chromatography (RP-HPLC), have been used since the 1970s and not only have they provided the best results in amino acid analysis but also performed a faster analysis and can analyze low sample volumes. RP-HPLC with pre-column derivatization is preferred because it is less time-consuming than other procedures and the instrumentation used is simple.

The most popular and widely used method is *ortho*-phthalaldehyde (OPA) in the presence of a mercaptan (β-mercaptoethanol, *tert*-butylthiol or 3-mercaptopropionic acid) or *N*-acetyl-L-cysteine as derivatization reagents (Soto-Otero *et al*., 1994; Liu *et al*., 1998). OPA does not react with secondary amino acids, and Gly derivatives, for instance, are unstable (Cohen, 2000). The most notable inconvenience of this method is that the derivatization reagent must be prepared daily because of the slight loss in fluorescence response observed after 3–5 days of storage. Furthermore, detection limits lie only in the picomole range. OPA was the derivatization reagent most frequently used to measure neuroactive amino acids in microdialysate samples. OPA derivatization has also been used also in conjunction with electrochemical detection (ED). Although ED has shown the best detection limits, fluorescence detection has proven very useful for

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Abbreviations used: AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; AMQ, 6-aminoquinoline; OPA, *ortho*-phthalaldehyde.

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certain applications, such as analysis of amino acids in microdialysate samples (Liu *et al.*, 1998).

The use of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as pre-column derivatization agent was first described by Cohen and Michaud (1993). In this procedure, primary and secondary amines react (in seconds) with the AQC reagent, yielding stable and highly fluorescent urea-type compounds. Excess reagent is hydrolyzed to yield 6-aminoquinoline (AMQ), carbon dioxide and *N*-hydroxysuccinimide. The advantages of this method include a very simple derivatization procedure, stable derivatives (1 week at room temperature; Cohen and De Antonis, 1994), excellent separation, detection by either absorbance or fluorescence, and commercial availability of reagents. Fluorescence detection permits highly sensitive analyses with detection limits for amino acids ranging from 50 to 300 fmol (Cohen, 2000). Disadvantages are seemingly limited: relatively long chromatography, high solvent consumption and no possibility of on-line derivatization. Cohen and co-workers (Cohen and Michaud, 1993; Cohen and De Antonis, 1994; Liu, 1994; Strydom and Cohen 1994; van Wandelen and Cohen, 1997; Liu *et al.*, 1998) applied this method to the determination of amino acids in different matrices. Recently, Liu *et al.* (1998) developed a modification of this technique to determine submicromolar concentrations of neurotransmitter amino acids (aspartate, glutamate, glycine and γ-aminobutyric acid) in microdialysate samples from brain and cerebrospinal fluids. In order to improve resolution and retention time reproducibility, the separation conditions proposed by Liu *et al.* (1998) were optimized to achieve simultaneous quantification of neuroactive amino acids of interest during epileptic seizures, using new chromatographic conditions and chromatographic instrumentation. Moreover, the samples were collected from a different brain area and serine and histidine were analyzed.

The present investigation describes a sensitive and rapid RP-HPLC method, using AQC derivatization and fluorescence detection. The objective is to obtain an automatic and rapid simultaneous measurement of aspartate, serine, glutamate, glycine and histidine in a high number of small volume microdialysates of rat hippocampus during controls and under different experimental conditions. Serine has been included because of its involvement in synaptic transmission (Takeda *et al.*, 2004) and epileptic seizures (Engstrom *et al.*, 2001).

EXPERIMENTAL

Chemicals. AccQ-Fluor reagent kit and AccQ-Tag Eluent A concentrate were acquired from Waters (Milford, MA, USA). The derivatization reagent kit consists of AQC dry powder, acetonitrile for dissolving the reagent powder and 0.2 mM sodium borate buffer, pH 8.8; the concentrate was 1.4 M sodium acetate trihydrate, 17 mM triethylamine with 10 mM calcium disodium EDTA, 0.01% sodium azide, adjusted to pH 5.02 with phosphoric acid. Amino acid standards (Asp, Ser, Glu, Gly, His) were obtained from Sigma (St Louis, MO, USA). Acetonitrile (HPLC grade) was supplied by Romil Chemicals (Cambridge, UK). Ultrapure water was generated using a Milli-Q system purchased from Millipore (Bedford, MA, USA).

Table 1. Optimized gradient for the separation of amino acids at 37°C

Time (min)	Acetate buffer (%)	Acetonitrile (%)	Water (%)	Curve[a]
Initial	100.0	0.0	0.0	—
0.5	99.0	1.0	0.0	11
18.0	95.0	5.0	0.0	6
21.0	0.0	60.0	40.0	11
33.0	100.0	0.0	0.0	11

[a] Curve 6 is a linear segment; curve 11 is a step function.

Chromatographic instruments and conditions. The HPLC system used was a Waters Alliance consisting of a 2695 separation model, a thermostat-controlled column oven, a system interface module, and a 2475 scanning fluorescence detector (all Waters components, Millipore, Milford, MA, USA). A Waters Empower 5.0.0. Chromatography Manager was used to control system operation and results management.

Separation was carried out using a 20 × 3.9 mm Sentry guard column (Nova-Pak C_{18} bonded silica) connected to a 4 μm AccQ-Tag C_{18} column (150 mm × 3.9 mm I.D.; both from Waters).

Working eluents. Eluent A was aqueous acetate phosphate buffer prepared by diluting AccQ-Tag eluent A concentrate with water to a ratio of 1:10, eluent B was HPLC-grade acetonitrile and eluent C was water. The gradient program is shown in Table 1.

For column regeneration, 12 min with 60% acetonitrile and 40% water was sufficient to wash out the column and before the next injection, 100% acetate buffer for 10 min was needed to equilibrate the system.

The AccQ-Tag column was thermostated at 37°C and operated at a flow-rate of 1 mL/min. Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm. The signal output was adjusted by setting the gain at 10. Injections were made every 43 min (injection-to-injection), using an injection volume of 5 μL.

Microdialysis. Adult male Sprague–Dawley rats, obtained from the University of Santiago de Compostela and weighing 250–300 g, were used. The intracerebral guide for the microdialysis probe (CMA/12, CMA/Microdialysis AB, Stockholm, Sweden) was implanted vertically into the ventral hippocampus. Stereotaxis coordinates derived from the atlas of Paxinos and Watson (1982) were 5 mm posterior, 4.8 mm lateral and 4 mm ventral for the tip of the cannula relative to bregma and dural surface. The system used was a CMA/120

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for freely moving animals (CMA/Microdialysis AB, Stockholm, Sweden) and CMA/12 microdialysis probes with 4 mm of membrane length. The probe was connected via polyethylene tubing to a syringe selector (CMA/111), and to 1 mL syringes mounted on a microinjection pump (CMA/100). Ringer's solution (NaCl 147 mM, KCl 4.0 mM, $CaCl_2$ 2.4 mM) was perfused at a constant flow rate of 2 μL/min throughout the experiment. Samples were collected in a fraction collector (CMA/140) every 15 min starting 60 min after the beginning of perfusion, and stored at –30ºC (Sierra-Paredes *et al.*, 2000).

Standards and derivatization. Stock standard solutions (10 mM) of amino acids (Asp, Ser, Glu, Gly, His) were prepared in sterile Ringer's solution and stored at –30°C. Different working standards were prepared by mixing and dilution of appropriate volumes of these stock solutions in Ringer's solution and were used exclusively for validation experiments.

In a typical analysis, 10 μL of standards or sample was buffered to pH 8.8 with 30 μL of AccQ-Fluor borate buffer. The derivatives were formed with 10 μL of AQC solution (3 mg/mL in acetonitrile). The reaction of AQC with all primary and secondary amines was rapid and excess reagent was hydrolyzed within 1 min.

RESULTS

Linearity and limits of detection

The linearity of the method was obtained by analyzing a series of dilutions of the standard mixture ranging from 2.5 to 100 μM (three points for each data).

Linearity data were calculated by examining the area vs concentration of amino acid plots. All the correlation coefficients (r^2) were greater than 0.998 or better for each amino acid, and $r \geq 0.999$, which demonstrates an excellent linearity of the calibrations.

The detection limits (signal-to-noise ratio = 3:1) for all of the amino acids were less than 5 nM (fmol/μL).

Figure 1(a) shows a typical chromatogram from a standard mixture (25 μM) containing the assayed amino acids. As can be seen, the resolution is good for all the amino acids studied, and shows an excellent signal-to-noise ratio, allowing easy quantification. Under these conditions, Asp, Ser, Glu, Gly, His, were well separated, and, moreover background noise [Fig. 1(b)] did not interfere in the analysis.

Validation experiments

Precision was expressed as the relative standard deviation (%RSD). The precision and accuracy of the method were assessed by repeatedly injecting working standard solutions on the same day and over a period of five consecutive days. The working standard solutions (2.5, 25 and 100 μM) were derivatized and analyzed according to the procedures described previously. Five replicates of each concentration were injected on the same day, and the precision of the responses ranged from 0.9 to 2.6%, while the accuracy ranged from 95.3 to 98%. The between-day RSD (n = 5) was from 4.5 to 6.2% and recoveries ranged from 95 to 105.2% (Table 2).

The retention time of different amino acids (standards) was used for peak identification (Table 3). These results show an excellent reproducibility of all the amino acids.

Different replicates (n = 3) of a microdialysate sample obtained from rat hippocampus were analyzed to determinate reproducibility, using the same chromatographic conditions as standards (Table 4). All amino acids of interest were quantified with RSD below 2%, and the retention time RSD was excellent

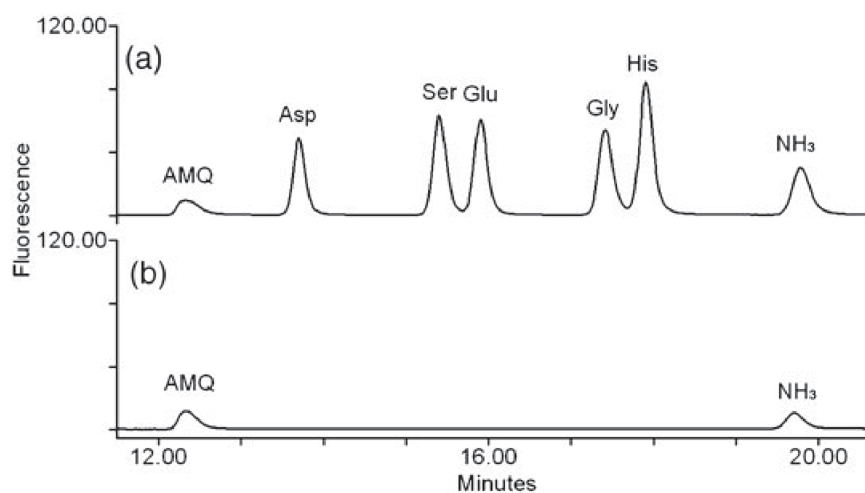

Figure 1. (a) Optimized chromatography for derivatized amino acid standard mixture (25 μM). (b) Chromatogram for derivatized blank. The injected volume was 5 μL. See text for the gradient used. Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm.

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Table 2. Accuracy and precision of the method for the determination of the assayed amino acids

Amino acids/ nominal concentration (μM)		Within-day ($n = 5$) Area RSD (%)	Within-day ($n = 5$) Accuracy %	Between-day ($n = 5$) Area RSD (%)	Between-day ($n = 5$) Accuracy %
Asp	2.5	1.5	98	5.3	97.8
	25	1.1	96.7	4.5	104.2
	100	1.2	95.3	4.7	100.5
Ser	2.5	2.1	96.9	5.5	101.9
	25	0.9	97.4	4.6	95
	100	1.1	96.7	4.8	102.1
Glu	2.5	2.6	97	5.5	99.3
	25	1	96.9	4.7	104.3
	100	1.3	95.7	4.8	100.8
Gly	2.5	1.9	96.6	5.5	102.3
	25	0.9	97.4	4.9	105.2
	100	1.1	96.6	5	102.3
His	2.5	1.6	96.2	6.2	101.8
	25	1.2	97.6	4.5	104.6
	100	1.2	97	4.8	101.9

and similar to that found in standards. The low concentrations observed in samples from rat hippocampus were correctly determined with the technique described.

Analysis of microdialysate samples

The method described in this paper was applied for amino acid analysis in microdialysate samples from rat hippocampus. The seven standard mixture concentrations used for sample quantification were based on the concentrations found in the microdialysate samples during previous studies. The standard mixture for calibration runs was prepared in sterile Ringer's solution, then perfused and collected as samples. Thus, standards for calibration and samples were processed equally. A linear response was obtained with the concentrations used (data not shown).

Figure 2 shows a typical chromatogram for microdialysate samples using the conditions optimized for both resolution and sensitivity of amino acids of interest. Using this method, 10 μL of sample is sufficient for derivatization, and 5 μL aliquot of the derivatized solution is injected.

An 8 μL flow cell was used in the fluorescence detector. The pH of the eluent A was not adjusted because the pH (5.02) of the concentrate permits good resolution and sensitivity.

Table 4. Results of reproducibility data of microdialysis samples in the rat hippocampus ($n = 3$)

Amino acids	Concentration (μM)	Retention time RSD (%)	Area RSD (%)
Asp	0.6 ± 0.01	0.21	0.42
Ser	11.3 ± 0.01	0.12	0.21
Glu	12.5 ± 0.20	0.13	1.40
Gly	40.5 ± 0.10	0.11	0.15
His	10.1 ± 0.01	0.10	0.30

CONCLUSIONS

The method described here, using a pre-column derivatization with AQC, is a rapid, sensitive, selective

Table 3. Retention Times and RSD (%) of amino acids within-day ($n = 5$) and between-day ($n = 5$)

Amino acids/ nominal concentration (μM)		Within-day Retention Time (min)	Within-day Mean	Within-day RSD (%)	Between-day Retention Time (min)	Between-day Mean	Between-day RSD (%)
Asp	2.5	13.6	13.6	0	13.7	13.6	0.36
	25	13.6			13.7		
	100	13.6			13.6		
Ser	2.5	15.4	15.3	0.32	15.4	15.3	0.32
	25	15.3			15.4		
	100	15.3			15.3		
Glu	2.5	15.8	15.8	0	15.9	15.8	0.31
	25	15.8			15.9		
	100	15.8			15.8		
Gly	2.5	17.4	17.4	0	17.4	17.4	0
	25	17.4			17.4		
	100	17.4			17.4		
His	2.5	17.9	17.8	0.28	17.9	17.8	0.28
	25	17.8			17.9		
	100	17.8			17.8		

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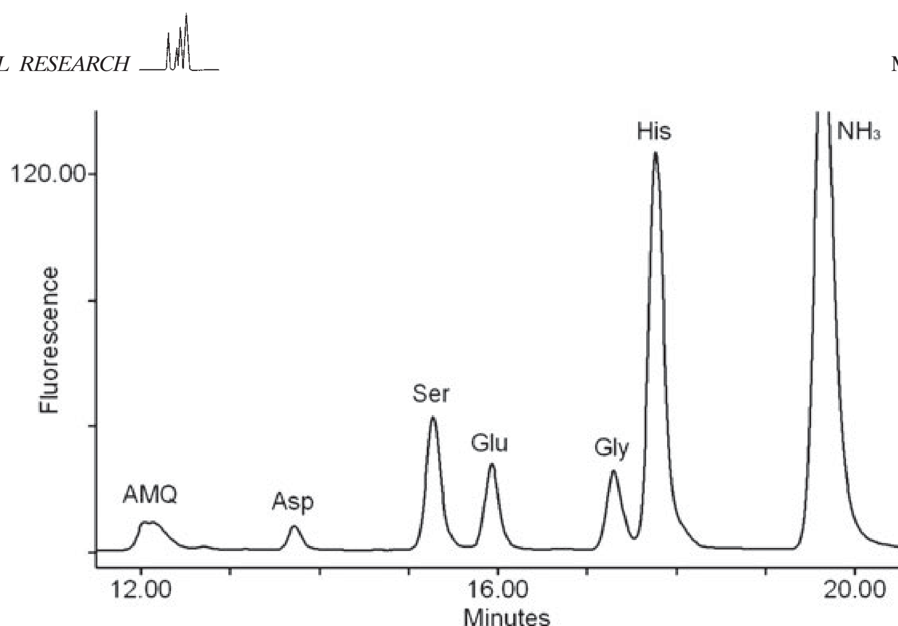

Figure 2. Typical separation for a derivatized microdialysis sample. The injected volume was 5 μL. Fluorescence detection was as in Fig. 1. See text for the gradient used.

and reproducible technique with stable derivatives (1 week) for analyzing neuroactive amino acids. Chromatographic conditions allow an automatic determination of amino acids, thus permitting rapid quantification in small sample volumes, as well as shorter analysis and data processing times. This method was used to measure Asp, Ser, Glu, Gly and His in small volume microdialysates obtained from rat hippocampus.

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REFERENCES

Chapman AG. Microdialysis. In *Epilepsy: a Comprehensive Textbook*, Engel J Jr and Pedley TA (eds). Lippincott-Raven: New York, 1997; 1067–1072.

Cohen SA. Amino acid analysis using precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. In *Methods in Molecular Biology*. Humana Press, Totowa, NJ, 2000; 39–47.

Cohen SA and De Antonis KM. Applications of amino acid derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. *Journal of Chromatography A* 1994; **661**: 25–34.

Cohen SA and Michaud DP. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Analytical Biochemistry* 1993; **211**: 279–287.

Engstrom ER, Hillered L, Flink R, Kihlstrom L, Lindquist C, Nie JX, Olsson Y and Silander HC. Extracellular amino acid levels measured with intracerebral microdialysis in the model of posttraumatic epilepsy induced by intracortical iron injection. *Epilepsy Research* 2001; **43**: 135–144.

Liu H. Determination of amino acids by precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and high-performance liquid chromatography with ultraviolet detection. *Journal of Chromatography A* 1994; **670**: 59–66.

Liu H, Sañuda-Peña C, Harvey-White JD, Kalra S and Cohen SA. Determination of submicromolar concentrations of neurotransmitter amino acids by fluorescence detection using a modification of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate method for amino acid analysis. *Journal of Chromatography A* 1998; **828**: 383–395.

Paxinos G and Watson C. *The Rat Brain in Stereotaxic Coordinates.* Academic Press: New York, 1982.

Rogawski MA. Excitatory amino acids and seizures. In *CNS Neurotransmitters and Neuromodulators: Glutamate*, Stone TW. CRC Press, Boca Ratan, FL, 1995; 219–237.

Sierra-Paredes G and Sierra-Marcuño G. Microperfusion of picrotoxin in the hippocampus of freely moving rats through microdialysis probes: a new method of induce partial and secondary generalized seizures. *Journal of Neuroscience Methods* 1996; **67**: 113–120.

Sierra-Paredes G, Galán-Valiente J, Vazquez-Illanes MD, Aguilar-Veiga E and Sierra-Marcuño G. Effect of ionotropic glutamate receptors antagonist on the modifications in extracellular glutamate and aspartate levels during picrotoxin seizures: a microdialysis study in freely moving rats. *Neurochemistry International* 2000; **37**: 377–386.

Soto-Otero R, Méndez-Alvarez E, Galán-Valiente J, Aguilar-Veiga E and Sierra-Marcuño G. Quantitative analysis of neuroactive amino acids in brain tissue by liquid chromatography using fluorescent pre-column labelling with *o*-phthalaldehyde and *N*-acetyl-L-cysteine. *Biomedical Chromatography* 1994; **8**: 114–118.

Strydom DJ and Cohen SA. Comparison of amino acid analyses by phenylisothiocyanate and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate precolumn derivatization. *Analytical Biochemistry* 1994; **222**: 19–28.

Takeda A, Minami A, Seki Y, Nakajima S and Oku N. Release of amino acids by zinc in the hippocampus. *Brain Research Bulletin* 2004; **63**: 253–257.

van Wandelen C and Cohen SA. Using quaternary high-performance liquid chromatography eluent systems for separating 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate-derivatized amino acid mixtures. *Journal of Chromatography A* 1997; **763**: 11–22.

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