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Journal of Chromatography A, 828 (1998) 383–395

JOURNAL OF CHROMATOGRAPHY A

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

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Abstract

A sensitive method for quantitatively determining submicromolar levels of neurotransmitter amino acids (e.g. Asp, Glu and γ-aminobutyric acid) in microdialysates from brain and cerebrospinal fluids is reported. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was employed as the derivatization reagent, followed by HPLC separation and fluorescence detection of the derivatives. The derivatization was conducted simply by mixing the AQC directly with the microdialysis samples. The reaction was complete within seconds after mixing at room temperature. Separation development optimizing the gradient profile, eluent pH and column temperature resulted in an excellent separation of the required amino acids in less than 30 min. Other resolved amino acids in the same profile include Gly, taurine, and Pro. Recoveries for the amino acids of interest spiked into high salt containing perfusion buffers were greater than 97%. The sensitivity of the method was increased by employing a 16-μl flow cell in the detector and analyzing 20-μl aliquots of the derivatization mixtures. With the optimized conditions, the detection limits were 3–7 n*M* (fmol/μl). Typical reproducibility (%R.S.D.) for quantitation of these amino acids at submicromolar levels was approximately 2%. Excellent linearity (r^2>0.999) was achieved over the range 0.2–20 μ*M*. The low detection limits permitted the analysis of a number of different microdialysate samples including those from cerebrospinal fluid, as well as substantia nigra and hypothalamus from brain samples, even at basal levels where γ-aminobutyric acid concentration may be <50 n*M*. The excellent sensitivity made it easy to distinguish basal from stimulated levels of neurotransmitter amino acids, even from sample sizes as small as 10 μl.

Keywords: Amino acids; Neurotransmitter amino acids; γ-Aminobutyric acid; Glutamate

1. Introduction

The role of neurotransmitter amino acids (NAAs) in the function of the nervous system has been the focus of increasingly intense research over the past several years. Among the most studied NAAs are Glu, Asp, γ-aminobutyric acid (GABA), taurine (Tau) and Gly. Glu and Asp are excitatory neurotransmitters within the central nervous system and

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00836-X

are responsible for normal synaptic neurotransmission. GABA is the major inhibitory neurotransmitter in mammalian brain [1,2]. Due to their essential roles in numerous neurochemical events, Glu and GABA are the most frequently monitored amino acids. They are metabolically linked as Glu serves as a precursor to GABA via the enzymatic pathway involving glutamic acid decarboxylase. There are also several proposed pathological roles for Glu and GABA in various disease states ranging from Alzheimer's disease [3] and epilepsy [4] to schizophrenia [5].

One of the more popular methods for monitoring small molecule neurochemicals, including amino acids, is microdialysis sampling [6–9], a technique that has opened new avenues of research by allowing continuous monitoring of neurotransmitters. To maximize the time resolution, sample volumes are necessarily kept at a minimum. Problems in detecting NAAs are compounded due to their low concentrations in most samples. As a result, detection can challenge the mass detection limits of many analytical methods. For example, basal levels in typical microdialysate samples for Asp and Glu usually range from 0.3 to 2.0 μM and from 1.4 to 4.0 μM, respectively [10–14]. With GABA analysis, the analytical demands are even more daunting with basal levels being reported in the range of 0.02–0.40 μM [10,13,15]. As the sample volume available for each analysis is at most 10–30 μl, an appropriate method for NAA quantification in microdialysates must be capable of detecting sub-picomole quantities of NAAs.

Traditional methods for amino acid analysis rely on the separation of free amino acids on ion-exchange columns, followed by post-column derivatization with ninhydrin [16,17] or, more recently, with *ortho*-phthalaldehyde [18] (OPA). Unfortunately these are not suited to NAA studies as the sensitivity limits, even for the fluorescent OPA method, are only in the low picomole range. In the last 10 years, pre-separation derivatization methods have dominated the field of NAA analysis. In particular, fluorescent labeling procedures using OPA [10,14,19,20], the OPA-like compounds naphthalenedialdehyde [11,21–23] (NDA) and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [13,24] and fluorescein isothiocyanate [25,26] have found utility in NAA investigations. OPA has also been extensively used in conjunction with electrochemical detection [8,9,15,27–30]. Pre-column OPA derivatization has been the most widely used method for NAA studies. OPA adduct stability can be improved by substituting the more common B-mercaptoethanol with tert-butylthiol, sulfite or *N*-acetyl cysteine [28,29]. The best detection limits are realized via electrochemical detection (ED), but fluorescence has proven useful for some applications.

Separation of derivatized amino acids is accomplished by either HPLC or, increasingly commonly today, capillary electrophoresis (CE). Using HPLC, resolution of a number of amino acids can be accomplished using a gradient separation system. Unfortunately, ED systems cannot be easily used with gradient analysis and thus they may not be suitable for simultaneous detection of Asp, Glu and GABA. Most often when GABA analysis time is acceptable ($<$30 min), Asp retention is insufficient to resolve it from interferences due to injection artifacts. ED systems are also considerably less rugged than fluorescence detectors and can require constant monitoring to ensure consistent analyte response. In contrast, fluorescence detection, while more reliable, is approximately 10-fold less sensitive than ED. While sufficient for analyzing Asp and Glu, basal GABA concentrations are near or below the fluorescence detection limit.

More recently, a popular alternative to the HPLC–ED method for NAA analysis has been the use of OPA [14], NDA [12,22,23] or CBQCA [13,25] derivatization in conjunction with CE and laser-induced fluorescence (LIF) detection. These systems provide extremely low detection limits, and are suitable for studies requiring good temporal resolution due to the low volume of sample required for the analysis. Detection limits in the low nanomolar range have been reported for Asp, Glu and GABA, with as little as 50 amol injected per analysis. Currently, however, CE–LIF systems are not readily accessible and the systems are relatively expensive. CE has also been used with ED [9], but these systems are not commercially available yet. In addition, CE systems, in general, are characteristically more prone to retention reproducibility problems in comparison to HPLC methods.

A recent addition to the choices for pre-column

derivatization is the highly fluorescing aminoquinolyl derivatives formed by the reaction of primary and secondary amines with 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) [31]. As with the other fluorescent methods, subpicomole detection limits are easily achieved, and following its introduction, there have been numerous papers demonstrating its utility for a variety of samples [32–36]. Several key advantages include reaction with secondary as well as primary amines, excellent derivative stability and high derivative yields in the presence of common buffer salts.

In the present investigation, improvements in analyzing AQC derivatized amino acids are reported that permit the analysis of Asp, Glu and GABA from microdialysate samples in a single run. A number of modifications to the originally reported method have been made to significantly boost both the concentration and mass detection limits which, in turn, provided sufficient sensitivity to analyze typical microdialysate samples both at basal and stimulated levels.

2. Experimental

2.1. Chemicals

AccQ·Fluor reagent kit (consisting of AQC, acetonitrile for dissolving the reagent powder and 0.2 m*M* sodium borate buffer, pH 8.8) and AccQ·Tag Eluent A concentrate were acquired from Waters (Milford, MA, USA). The concentrate was 1.4 *M* sodium acetate, 77 m*M* triethylamine with 10 m*M* calcium disodium EDTA, adjusted to pH 5.05 with phosphoric acid. Acetonitrile, phosphoric acid, sodium hydroxide were from Baker (Phillipsburg, PA, USA). Amino acid standards were from Pierce (Rockford, IL, USA) or Sigma (St. Louis, MO, USA). HPLC-grade water for all applications was supplied by a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Chromatographic instrumentation

The HPLC system used for the separation of derivatized neurotransmitter amino acids consisted of a Waters 625 LC system equipped with a column heater, a 717 auto sampler and a 474 scanning fluorescence detector. A Waters Millennium 2010 Chromatography Manager was used to control system operation and results management.

2.3. Standards and samples

Aqueous 2.5 m*M* stock solutions of GABA, Asn, Gln, citrulline (Cit) and Tau were prepared in water. Pierce's H amino acid standard was also used for mixing with some or all of the stock solutions with the appropriate volume of water added to make up standards with the appropriate concentrations and compositions. Alternatively, to investigate the effects of salt in the microdialysis buffer on derivatization, water was replaced with the buffer as the diluent.

In the experiments measuring GABA, microdialysate samples were obtained from adult random cycling female rats (Sprague-Dawley, from Zivic Miller Laboratory) weighing 250 g. The microdialysate probe (CMA probe/12 fitted with a 2-mm membrane, 200 000 molecular mass cutoff) was placed in the paraventricular nucleus of the hypothalamus. Artificial cerebrospinal fluid (CSF) was pumped at a flow-rate of 1.25 μl/min. Samples were stored at −70° prior to use. Reproducibility studies were conducted with samples collected from male, 250 gram Sprague Dawley rats, collected on ice at intervals of 20 min, and stored at −70°C prior to use. These samples were collected and prepared as previously described by Yadid et al. [37]. Briefly, the probe (2×0.5 mm) was inserted in the thalamus of freely moving animals and the CSF was pumped at 2 μl/min for 30 min and collected in a microtube containing 10 μl of preservative (0.1 *M* perchloric acid, 1% ethanol, and 0.02% EDTA). In the time-course study, the subject animal was also a Sprague-Dawley male rat weighing 358 g. A 2-mm concentric microdialysis probe (40 000 M_r cutoff) was located in the substantia nigra reticulata. Sample collection was initiated 1 h after the probe was positioned and the microdialysate flow was set at 1.5 μl/min. A total of 20 samples were collected at 15-min intervals, for a total volume of 22.5 μl each. After 120 min, before sample nine, a 15-min pulse of 1 *M* NaCl was introduced into the probe to increase release of amino acids.

2.4. Derivatization of standards and samples

Usually 10–30 μl of standard or sample was pipetted into a clean 50×6 mm sample tube, or in the case where the sample volume was limited, and shape of the sample vial was suitable for derivatization, the entire sample volume was kept in the vial for reaction. AccQ·Fluor Borate Buffer (10–20 μl) was then added to the tube or vial, thus bringing the total volume to approximately 30 μl. The derivatization was carried out by adding 10 μl of AccQ·Fluor reagent (3 mg/ml in acetonitrile) to the buffered mixture, and the sample was thoroughly vortexed to complete the derivatization procedure. Derivatization was essentially instantaneous [31] and the samples could be injected immediately.

2.5. Chromatography

Separations were carried out using a 20×3.9-mm Sentry guard column (Nova-Pak C_{18} bonded silica) connected to a 150×3.9 mm AccQ·Tag reversed-phase column (both from Waters). A quaternary elution system was employed as indicated in the tables and figure legends. Both the eluent A and eluent B of the system were aqueous acetate phosphate buffer prepared by diluting AccQ·Tag Eluent A concentrate with water to a ratio of 1:10, and then adjusting the pH of the eluents to appropriate values with dilute phosphoric acid or sodium hydroxide. Eluent C was HPLC-grade acetonitrile, and eluent D was water. Different gradients developed for various applications are described in the Section 3. Helium sparge was set at 20 ml/min for degassing the eluents.

Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm. The output of the signals was adjusted by setting the gain value of the detector at 10 or 100. Injections were made every 33 min, using an injection volume of 4–20 μl.

3. Results and discussion

3.1. Optimization of separation conditions for the neurotransmitter amino acids

A standard solution of 22 amino acids including 17 protein compositional amino acids, plus Asn, Gln, GABA, Tau and Cit was prepared and derivatized with the procedure described in the Section 2. The derivatives were used for the optimization of the conditions for separating neurotransmitter amino acids. Initially, the HPLC conditions described previously for the separation of 17 amino acids [31] were used for the analysis of these neurotransmitter amino acids. However, the resolution of GABA from Pro, Tau from Arg and Cit from Thr was not complete under these conditions. To improve the separation of these components, several HPLC parameters, such as column temperature, pH of the eluents and gradient profile were optimized. Each of these parameters influenced the relative retention of one or more amino acids of interest to various extents.

Column temperature has a significant effect on the separation of GABA/Pro and Arg/Tau, with little impact on the isolation of other amino acids of interest (Asp, Glu and Gly) (Fig. 1). The separation of Thr/Cit was also influenced by the column temperature in a similar way as for Arg/Tau: the higher the temperature, the greater the resolution (data not shown). What is also worth noting is the intersection of the GABA and Pro curves in Fig. 1, indicating that the elution order of these two amino acids may be reversed by changing the temperature.

A system consisting of two aqueous eluents, eluent A with pH at 4.80 and eluent B pH at 5.20, was used to optimize the separation pH. Adjusting the pH of the mobile phase was accomplished by varying the ratio of eluents A and B. As expected, the pH of the eluents substantially affected the retention of the two acidic amino acids, Asp and Glu, and thus affected their resolution from other amino acids, including Gly. The resolutions of Tau from Arg and GABA from Pro were also affected by changes in the eluent pH, and interestingly, as with temperature, such changes can reverse the elution order of GABA and Pro. On the other hand, the resolution of Cit from Thr was not significantly influenced by the changes in eluent pH (Fig. 2).

Optimized HPLC conditions were determined for the resolution of the NAAs based on the above experiments. As shown in Fig. 3a, all the amino acids of interest were well separated within 25 min using a column temperature of 50°C. Another set of HPLC conditions was optimized for the analysis of Asp, Glu, Gly and GABA, with the column tempera-

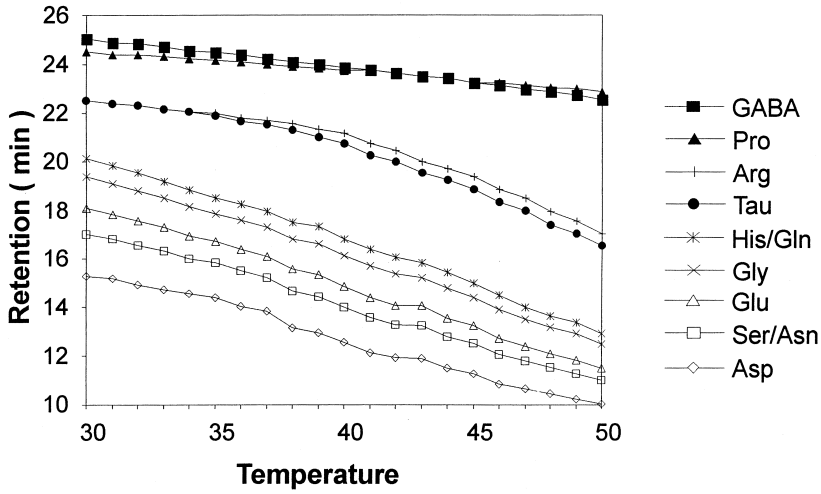

Fig. 1. Effects of column temperature on the separation of NAAs. Solvents were the same as in Table 1 except that solvent B was not utilized. From the initial step to 29.5 min, only solvent A and solvent C were used. Gradient: initial=0% C, 0.5=1% C, 18=5% C, 19=9% C, 29.5=17% C, hold for 3.5 min, wash with 60% acetonitrile in water for 3 min, equilibrate with 100% A for 9 min before subsequent injection. No guard column was used. A 4-μl aliquot of 20 nmol/ml derivatized amino acids was injected.

ture set at 40°C (Fig. 3b). This latter system does not separate Thr and Cit, and has lower resolution of Arg and Tau. However, many analyses do not require the quantification of these amino acids and these conditions can be used for these samples. With both separation systems, additional coelutions are Ser with Asn and His with Gln. Tables 1 and 2 list the gradients used for these two sets of conditions.

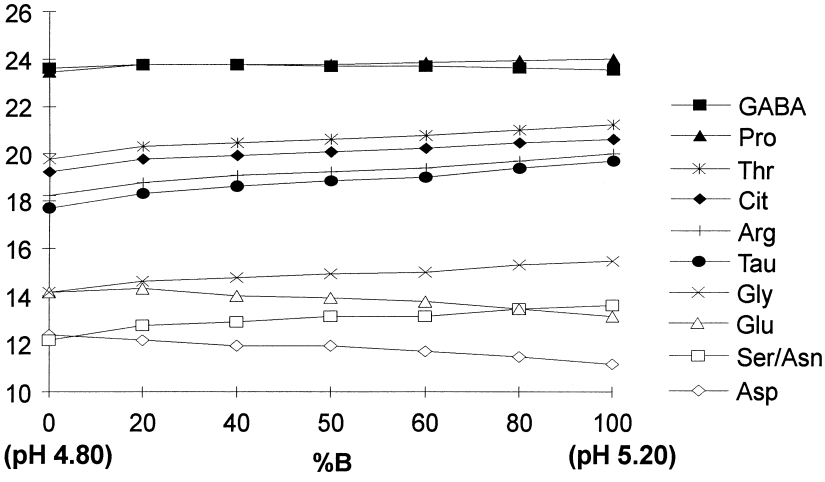

Fig. 2. Effects of eluent pH on the separation of NAAs. The pH of solvents A and B used were 4.80 and 5.20, respectively. The gradient profile for solvent C was the same as in Fig. 1. The sum of %A and %B was 100 of the aqueous portion of the eluent, with eluent C (acetonitrile) making up the remainder. The %B is indicated in the figure. A guard column was used and the column was equilibrated with initial conditions for 9 min before the next injection. The column temperature was set at 50°C. Other conditions were identical with Fig. 1.

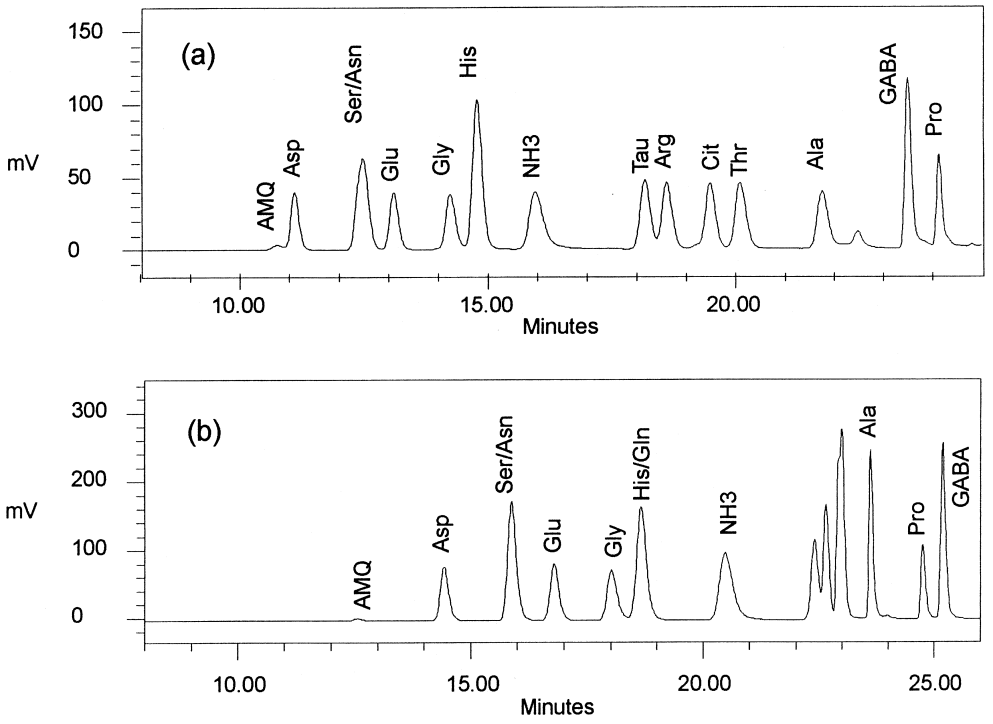

Fig. 3. Optimized separation of NAAs. A 4-μl aliquot of 10 nmol/ml (a) or 20 nmol/ml (b) of derivatized standard was injected. The column temperature was set at 50°C (a) or 40°C (b). Fluorescence detection: λ_{ex}=250 nm, λ_{em}=395 nm, gain=100, flow cell: 5 μl. See Tables 1 and 2 for the gradient used.

3.2. Effects of sample preparation and HPLC conditions on assay sensitivity

3.2.1. Optimizing buffer, sample and reagent ratios

AQC derivatized amino acids exhibit very strong fluorescence emission at 395 nm. The reported detection limits (signal/noise ratio of 3) for Asp, Glu, and Gly were 311, 315 and 293 fmol, respectively using a 5-μl flow cell [31]. Under the normal derivatization procedure, up to a 10-fold dilution of analyte concentration in the samples can be expected. Such a dilution results from the addition of borate buffer, which is added to keep the pH of the derivatization mixture within a desired range, and acetonitrile which is used to dissolve AQC. Therefore, when 4 μl of the final solution is injected, the

Table 1
Gradient profile for optimized separations of NAAs at 50°C

Time (min)	Flow ml/min	%A pH 5.05	%B pH 5.50	%C (Acetonitrile)	%D (Water)	Curve[a]
Initial	1.00	100	0	0	0	*
0.50	1.00	99	0	1	0	11
19.00	1.00	94.5	0	5.5	0	6
19.10	1.00	0	94.5	5.5	0	6
23.00	1.00	0	83	17	0	6
23.10	1.00	0	0	80	20	6
25.00	1.00	100	0	0	0	11

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

Table 2
Gradient profile for optimized separations of NAAs at 40°C

Time (min)	Flow ml/min	%A pH 5.05	%B pH 4.60	%C (Acetonitrile)	%D (Water)	Curve
Initial	1.00	90	10	0	0	*
0.50	1.00	90	9	1	0	11
18.00	1.00	86	9	5	0	6
19.00	1.00	0	91	9	0	6
25.00	1.00	0	86	14	0	6
25.10	1.50	0	0	80	20	6
28.00	1.50	90	10	0	0	11
33.00	1.00	90	10	0	0	11

concentration detection limits for these amino acids should be approximately 0.75 pmol/μl (0.75 μ*M*). This is usually insufficient for the quantitation of NAAs in microdialysates, especially for GABA. As a result, a number of steps were taken to increase the sensitivity of the method and to meet the sensitivity requirements for the trace level analysis of these NAAs.

Since the concentration detection limits can be improved by increasing the volume ratio of sample and/or decreasing the volume ratio of the other derivatization constituents (buffer and/or AQC solution), the effect of varying the volumes of sample, buffer and AQC solution was studied. However, several factors may affect the derivatization. First, it is important to maintain the pH of the resulting aqueous solution in the proper range (>8.0) to ensure quantitative derivatization. Second, the final AQC concentration must be maintained at an appropriate level (≥2 m*M*) to drive the derivatization reaction to completion. Another practical consideration is to ensure that there is adequate precision in pipetting small volumes. Consequently, 10 μl of AQC was used for derivatization for all experiments. Sample volumes were 10–30 μl, depending on the availability of sample, and 10–30 μl borate buffer was added to keep the volume of the final solution to 40–50 μl. Since the perfusion buffers used during microdialysis are normally neutral and have low buffering capacity, as little as 10 μl of borate buffer can be added to a sample with a volume as high as 30 μl while maintaining sufficient pH control. Approximate buffered sample pH was occasionally checked with a pH stick.

The chromatographic responses of the amino acids of interest in the derivative solution obtained by mixing 30 μl sample, 10 μl borate buffer and 10 μl AQC solution were compared with that obtained by adding 10 μl sample, 30 μl borate buffer and 10 μl AQC solution. With a theoretical ratio of three, the experimental values for response ratios of Asp, Glu, Gly, and GABA were 3.09, 3.17, 3.04, 3.15, respectively, indicating successful quantitative derivatization could be accomplished with a sample volume ratio as high as 60%. This procedure limits the dilution of the original sample to 1.7-fold.

3.2.2. Maximizing injection volume

Sensitivity can also be improved by increasing the injection volume, but excessive volume may adversely impact the chromatographic resolution of the analytes, due to the injection volume itself and the organic solvent in the sample. The maximum injection volume for the current system was investigated based on the compromise between sensitivity and resolution of the NAAs. Using the gradient described in Table 2, up to 20 μl of the derivatization solution containing 20–24% acetonitrile could be injected without noticeable distortion of the shape of the peaks of interest. When the injection volume exceeded 20 μl, however, significant deterioration of peak shape of the earlier eluting components (AMQ, Asp, Ser, Glu) was observed. However, the more strongly retained peaks, such as GABA, were not affected by injection volumes up to 30 μl, suggesting that large volumes can be analyzed if the determination of GABA is the only concern of the analysis. It may be likely that the strong retention of the analytes under the initial chromatographic

conditions is the main factor that allows large injection volumes of organic-containing samples.

3.2.3. Flow cell optimization

The flow cell of the fluorescence detector used for amino acid analysis is usually a small one (e.g. 5 μl) to improve the resolution of the analytes. In this work, the effects of flow cell size on the sensitivity and resolution of NAAs was evaluated by comparing the chromatographic results obtained with 16 μl and 5 μl flow cells. An approximately 4-fold sensitivity enhancement was found when the larger cell was employed. The decrease in resolution was small, with a loss of 5% typical for non-baseline resolved peak pairs (e.g. Gly/His).

It has been previously reported that AQC-derivatized amino acids have stronger fluorescence emission in a higher pH environment [30]. This effect was studied using a system similar to that described earlier for separation optimization, except that the pH of eluent A was 5.05 and that of eluent B was either 4.10 or 6.50. Again, final eluent pH was established by varying the ratio of A and B. In this study, a significant effect of eluent pH on the fluorescence response of GABA was observed (Fig. 4) with an increase in pH from 4.1 to 6.5 resulting in an approximately 7-fold increase in response. Although the eluent pH is one of the critical factors affecting the separation of amino acids of interest, and is usually optimized for resolution of the analytes, it can also be used to obtain higher sensitivity for GABA in studies where an ultra low detection limit is required and where the separation of GABA from other components is sufficient.

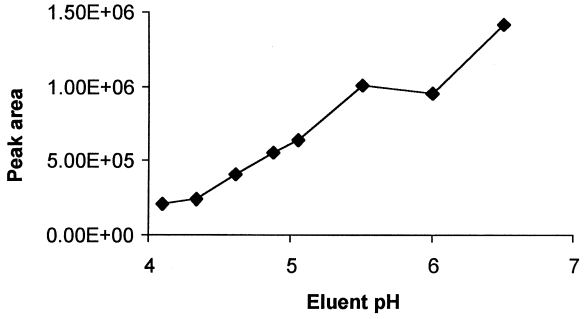

Fig. 4. Effect of eluent pH on the fluorescence response of GABA. The pH of eluent A was 5.05, and the pH of eluent B was adjusted with dilute NaOH or H_3PO_4. Ten μl of 0.2 nmol/ml derivatized GABA was injected. The gradient used from injection until 18 min, and from 25 min to 33 min was the same as in Table 2. Between 18 and 25 min the aqueous eluent pH was adjusted either by varying the ratio of eluent A to eluent B or by changing eluent B pH. The %C was the same as in Table 2 during this period of time.

3.2.4. Detection limits

A standard NAA mixture in water containing 20 n*M* of each amino acid of interest was derivatized by adding 10 μl of borate and 10 μl of AQC to 30 μl of standard. The detection limit of the method was determined by injecting 20 μl of the derivatives and a derivatization blank (Fig. 5). The noise level was estimated to be ca. 0.02 mV, and the detection limits for Asp, Glu, Gly and GABA were 7.6, 7.0, 4.1 and 3.3 n*M* (signal-to-noise ratio=3), respectively.

3.3. Analysis of NAAs in microdialysis samples

3.3.1. Separation of NAAs in microdialysis samples

Initial studies used samples obtained from a microdialysate probes placed in the paraventricular nucleus of normal adult female rats. Fig. 6a shows a typical chromatogram for these samples using the conditions optimized for both resolution and sensitivity of the amino acids of interest, namely Asp, Glu and GABA. A 20-μl aliquot of the derivatized solution was injected, accounting for approximately 40% of the total amount of the derivatized sample. A 16-μl flow cell was installed in the fluorescence detector, and the pH of eluent B was adjusted to 4.6, a compromise between resolution and sensitivity. With these conditions, Asp, Glu, Gly and GABA were well separated from other components. Gln and NH_3, which are usually in far greater concentration than other amino acids, did not interfere with the quantitation of the amino acids of interest. Fig. 6b shows a blank chromatogram obtained by derivatization of the perfusate used for microdialysis, demonstrating a clean background lacking significant Asp, Glu or GABA. Although the concentration of GABA is quite low (ca. 135 n*M*), it is readily quantified despite the low response (Fig. 6c, d).

3.3.2. Quantitation

Perfusion buffers usually contain high concen-

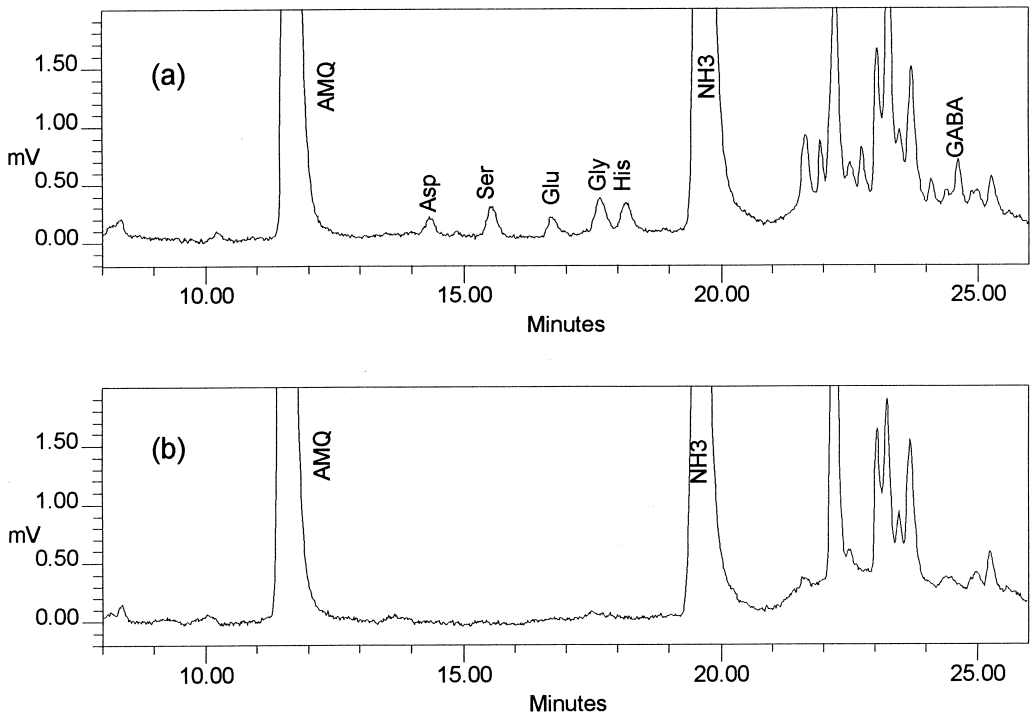

Fig. 5. High sensitivity analysis of derivatized NAAs. A 20 n*M* NAA solution (30 μl) was mixed with 10 μl of borate buffer and derivatized with a 10-μl aliquot of AQC solution. The derivatized amino acid mixture (a) is shown in comparison to a derivatized blank (b). The injection volume was 20 μl. Fluorescence detection: λ_{ex}=250 nm, λ_{em}=395 nm, gain=10, flow cell: 16 μl. See Table 2 for the gradient used.

trations of salts that may interfere with the reaction of amino acids with derivatization reagents [38]. If phosphate is present in the buffer, it can also react with the derivatization reagent and consume some of the reagent. To investigate this effect, a series of standard solutions were prepared by spiking different amounts of standard into a dialysis buffer consisting of 127.6 m*M* NaCl, 2.5 m*M* KCl, 1.4 m*M* $CaCl_2$, 1.0 m*M* $MgSO_4$ and 12.0 m*M* sodium phosphate (pH 7.4). The resulting calibration curves exhibited a linear fluorescence response as a function of amino acid concentration with correlation coefficients (R^2)>0.999 (Fig. 7), indicating little interference by salt present in the buffer with the derivatization reaction. Another perfusion buffer, similar in composition (no phosphate) with the above one, was used to dilute an amino acid standard to 1/10 of its original concentration, and the recovery was compared with that of another amino acid standard at the same concentration but without buffer. The calculated recoveries for Asp, Glu, Gly and GABA in the buffer were 101%, 97.2%, 99.9% and 98.8%, respectively.

In a microdialysis time course study, the sample collection was started one hour after the probe was positioned in the substantia nigra of an adult male Sprague-Dawley rat (Fig. 8). A total of 20 samples was collected with a 22.5 μl in volume for each sample (collection time 15 min, flow-rate: 1.5 μl/min). In the middle of the study (sample 9) a 15-min pulse of 1 *M* NaCl was introduced through the probe to stimulate the release of amino acids. This precipitated a rapid increase in all NAA concentrations followed by a return to basal levels approximately 30 min later.

In quantitative studies, the derivatizing reagent added should be in sufficient excess to drive the reaction to completion. This is important for the quantitation of NAAs in dialysates as some NAAs, such as Asp and Glu, are very sensitive to in-

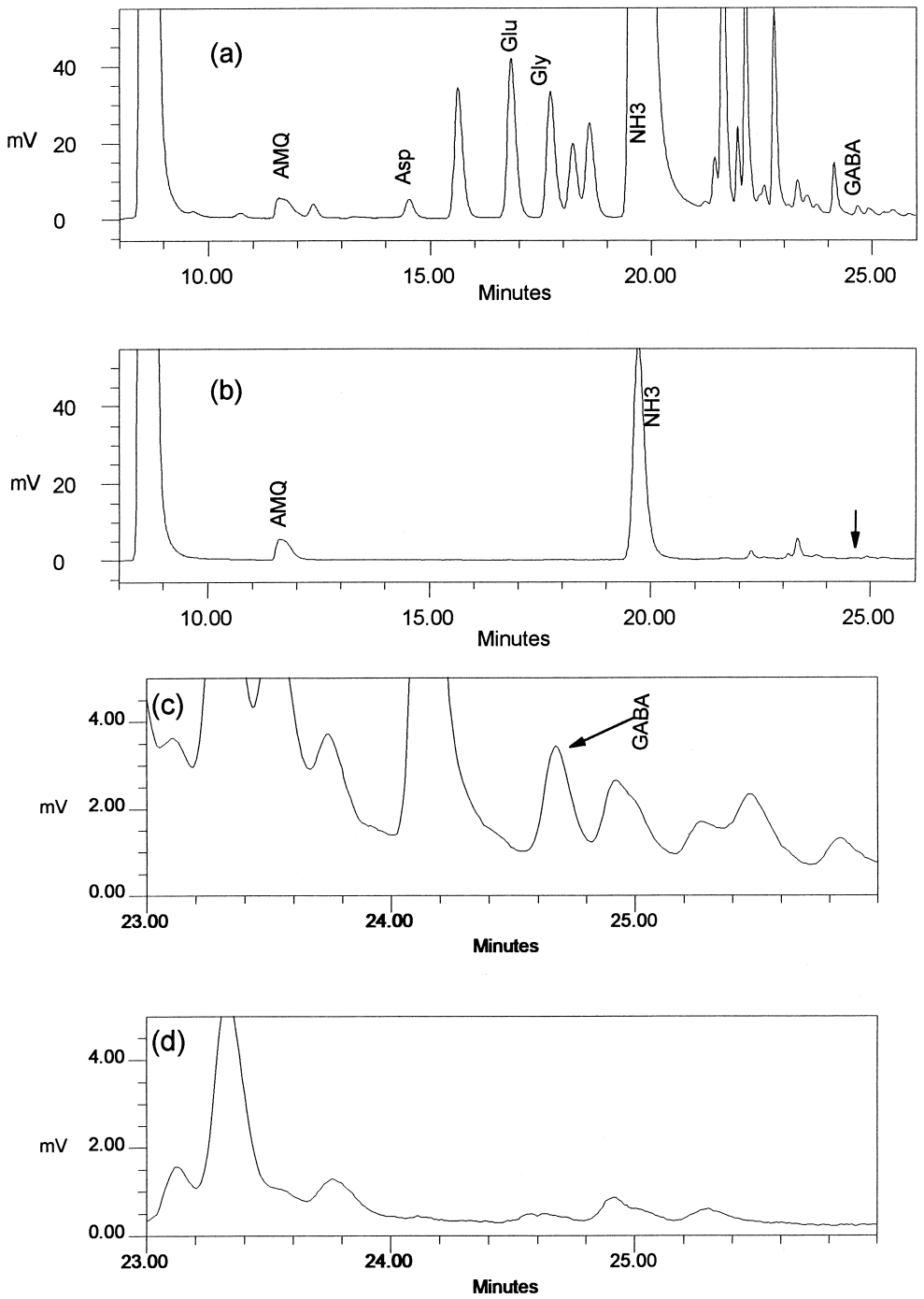

Fig. 6. Analysis of NAAs in a derivatized microdialysis sample. A 20-μl aliqout of the derivatized sample (a) or blank (b) was injected. Expanded views of the sample (c) and blank (d) are also shown. The blank was prepared with dialysis buffer using the same procedure as the sample. Fluorescence detection as in Fig. 5. See Table 2 for the gradient used.

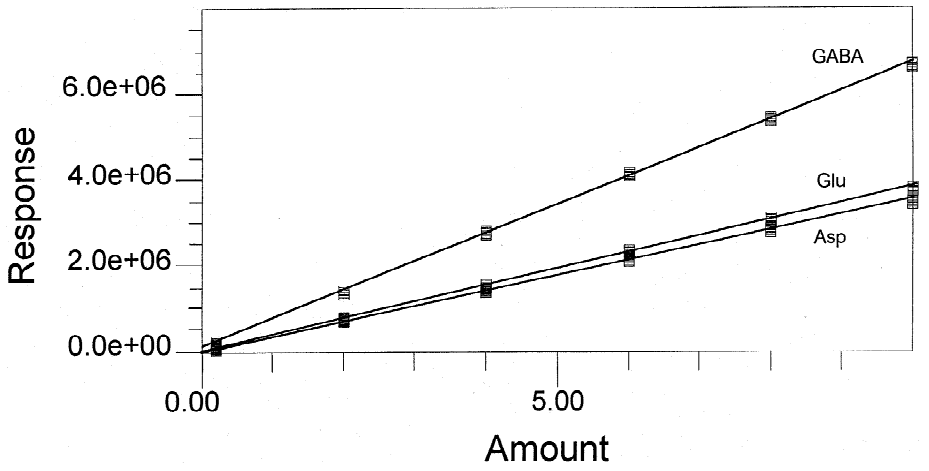

Fig. 7. Calibration curves for derivatized NAA standards. Standards were prepared by diluting the stock solutions with a microdialysis buffer. Ten μl of derivatized standard was injected. See Table 2 for the gradient used and Fig. 5 for the detection conditions.

sufficient reagent [31]. Although NAAs in dialysates are usually at very low concentration, there are other substances, such as NH_3, Gln and phosphate, which can react with AQC and may be at high concentrations in the dialysates. The presence of excess reagent can be estimated by comparing the peak response of AMQ, the major hydrolysis product of AQC, in the chromatogram of a sample with that in a blank chromatogram. For example, if the AMQ response in a sample chromatogram exceeds 80% of that in a blank, it can be estimated that the added molar excess of reagent is more than four times greater than the total molar amount of all the substances reacting with AQC at a rate comparable to that of NAAs.

This is a sufficient molar excess to produce maximal yields. In all studies with micro dialysate samples, the AMQ peak area has exceeded 80% of

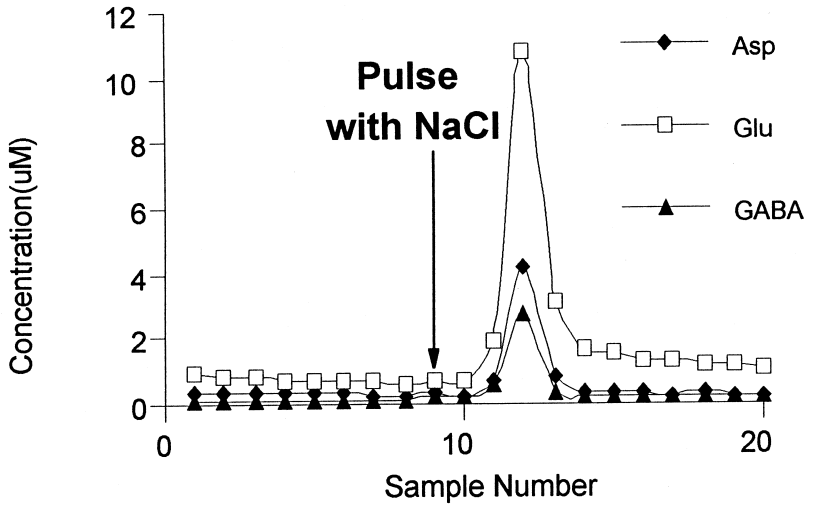

Fig. 8. Time course of NAAs from derivatized rat microdialysates. See Section 3 for sample collection. Ten μl of the collected sample was derivatized by mixing 30 μl of borate buffer and 10 μl of AQC. A 20-μl aliquot of the solution was injected. Fluorescence detection as in Fig. 5 except gain=100. See Table 2 for the gradient used.

Table 3
Reproducibility of derivatization and analysis of microdialysis samples ($n=5$)

NAA	Concentration (μM)	Relative standard deviation (%), retention time	Relative standard deviation (%), area
Asp	2.05±0.04	0.19	2.1
Glu	1.16±0.02	0.27	2.0
Gly	4.45±0.06	0.24	1.4
GABA	0.044±0.003	0.08	6.0

the AMQ area in derivatization blanks, indicating that sufficient reagent was present.

3.3.3. Reproducibility

A dialysate sample obtained from rat hypothalamus was used for the investigation of the analytical method reproducibility. Five replicates of the sample were prepared by adding 30 μl of borate buffer and 10 μl of AQC to 10 μl of dialysate. With a concentration greater than 1 μM, most of the amino acids of interest (Asp, Glu and Gly) were quantified with relative standard deviations of approximately 2%, and for GABA which had a concentration of less than 50 nM, the R.S.D. was approximately 6% (Table 3).

4. Conclusions

Analysis of the neurotransmitters Glu, Asp and GABA in microdialysates by HPLC with fluorescence detection has been demonstrated. Optimization of chromatographic separation and fluorescence detection of AQC-derivatized amino acids provides sufficient sensitivity for their quantification at basal levels as well as allowing the monitoring of concentration changes during stimulation of NAA release. Chromatographic conditions can be easily modified to allow the simultaneous analysis of all three major NAAs, or optimized for the analysis of a single NAA. Further enhancements to lower the detection limits for GABA are desirable to enhance the response at basal levels and permit the collection of smaller microdialysis fractions to increase the temporal resolution.

Acknowledgements

The work of S.K. was supported by NIH grant No. DK37273.

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