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Journal of Chromatography A, 962 (2002) 105–115

JOURNAL OF CHROMATOGRAPHY A

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Automated capillary liquid chromatography for simultaneous determination of neuroactive amines and amino acids

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Received 13 June 2001; received in revised form 19 March 2002; accepted 25 April 2002

Abstract

A method for the separation and quantitative determination of neuroactive amino acids (aspartate, glutamate, citrulline, arginine, glycine, taurine, γ-aminobutyric acid) and neuroactive amines (noradrenaline, dopamine and serotonin) in a single chromatographic analysis is presented. The method is based on pre-column derivatization with *o*-phthalaldehyde and *tert*.-butyl thiol, on-column preconcentration and separation using 50 μm I.D. packed capillary columns, and detection by amperometry. Mass limits of detection are 80–900 amol for all neurotransmitters with RSDs of 0.71 and 4.6% or better for retention time and peak area, respectively. The method was demonstrated by application to the determination of neurotransmitters in microdialysis samples collected from striatum of live rats and tissue samples extracted from butterfly brains.

Keywords: Neurotransmitters; Amines; Amino acids; Catecholamines

1. Introduction

Biogenic amines such as catecholamines [noradrenaline (NA) and dopamine (DA)] or indoleamines [5-hydroxytryptamine (5-HT)] and amino acids [aspartate (Asp), glutamate (Glu), γ-aminobutyric acid (GABA), and glycine (Gly)] are both important classes of neurotransmitter [1]. In addition, other amino acids such as taurine (Tau), arginine (Arg), and citrulline (Cit) act as neuromodulators or indicators of neuronal activity. Because of the importance of these compounds, they are frequently measured in tissue extracts or in fractions collected from in vivo microdialysis sampling probes for neuroscience applications.

Neurotransmitter amino acid analysis is often performed by liquid chromatography (LC) following derivatization of the amino group with *o*-phthalaldehyde (OPA) and a thiol [2–5]. The OPA–thiol derivatization reaction with amines forms 1-alkylthio-2-alkyl-substituted isoindoles which are both fluorescent [6] and easily oxidized allowing both fluorescence and electrochemical detection (ED) [7]. Although 2-mercaptoethanol (β-ME) was originally used as the thiol [6], variable stability of OPA–β-ME derivatives made the method difficult to use for quantification [8–10]. Subsequent research revealed that the stability of OPA-derived isoindoles is influenced by several factors including thiol

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00533-2

structure, thiol concentration, amine structure, solvent composition and pH [11–13]. For example, use of *tert*.-butyl thiol (*t*-BuSH) as the thiol in the OPA reaction yields stable derivatives for many amino acids with a good electrochemical response [12] making it a popular reagent for LC–ED based methods of neurotransmitter amino acid analysis [14–17]. (*t*-BuSH has not been widely used with fluorescence detection because it results in derivatives with low fluorescence yield compared to other thiols [12]). In addition to OPA, 2,3-naphthalenedicarboxaldehyde (NDA) has proven to be a useful reagent for detection of amino acids by either fluorescence or electrochemistry [18–20]. More recently, the fluorogenic reagent 5-furoylquinloine-3-carboxaldehyde (FQ) has been utilized with capillary electrophoresis and laser-induced fluorescence detection (CE–LIF) for determination of amino acids in dialysis samples [21].

Catecholamines and indoleamines are naturally electroactive and usually are measured by amperometry following separation by LC or CE [4,22–24]. The amine functionality of these compounds has also been exploited for derivatization and fluorescence detection [25,26].

It is often of interest to quantify both biogenic amines and amino acids in neurological samples for studies of interactions of neurotransmitters or drug effects on multiple targets; therefore, it would be useful to develop a method for simultaneous determination of neuroactive amines and amino acids. While CE–LIF with NDA derivatization has been used to detect both amino acids and catecholamines, the conditions used are different necessitating two separate analyses to detect both classes of compounds [25–29]. FQ has been utilized with CE–LIF for simultaneous determination of dopamine and amino acids [21]; however, the detection limit achieved for dopamine in the initial report was not sufficient to measure this compound in dialysate samples.

In this work, we have investigated use of capillary LC–ED with OPA–*t*-BuSH derivatization for simultaneous determination of catecholamines and amino acids in neurological samples. In a previous report, we demonstrated that OPA–*t*-BuSH derivatization coupled with capillary LC–ED is well-suited for amino acid neurotransmitter analysis because of its high mass sensitivity and the ease with which dilute samples can be preconcentrated [30]. (The use of *t*-BuSH in this reaction is especially useful as it results in a hydrophobic derivative which is easily preconcentrated on the column). With on-column preconcentration, as well as care in eliminating background peaks, detection limits less than 1 n*M* in 250 nl samples has been achieved for all of the neuroactive amino acids [30]. It was found that only small modifications were necessary to extend this method to include catecholamines and indoleamines.

2. Experimental

2.1. Reagents and buffers

Boric acid, OPA, *t*-BuSH, amino acid and biogenic monoamine standards were obtained from Sigma (St. Louis, MO, USA). Iodoacetamide (IAA) and tetrabutylammonium perchlorate (TBAP) was from Fluka (St. Louis, MO, USA). Phosphate buffer salts, sodium hydroxide, ammonium acetate and ethylenediamine-tetraacetic acid (EDTA) were enzyme or ACS grade and were obtained from Fisher Scientific (Atlanta, GA, USA). Water, acetonitrile and methanol were HPLC grade and obtained from Burdick and Jackson (Muskegon, MI, USA).

Stock solutions (10 m*M* stored at −20 °C) of neurotransmitter standards were prepared in 100 m*M* HCl containing 0.5 *M* ascorbic acid and diluted prior to use. Dilutions of the standards to 10, 50, 100, 400 n*M* or other concentration were prepared daily using HPLC-grade water as the diluent. Excess ascorbic acid was added to the stock solution to prevent auto-oxidation of NA, DA and 5-HT before derivatization. This allowed the same stock solution of neurotransmitters, when stored at −20 °C to be used up to 5 days. 50 m*M* phosphate-buffered mobile phase at pH 6.5 was prepared by dissolving 4.22 g Na_2HPO_4 and 4.73 g NaH_2PO_4 in 1 l water. 0.6 *M* boric acid solution adjusted to pH 10.5 was used as the derivatization buffer. Buffers were prepared using HPLC-grade water and filtered using 0.22-μm carbon activated PTFE membranes (Alltech, Deerfield, IL, USA) and a glass vacuum filtration system.

2.2. Derivatization procedure

40 m*M* OPA–50 m*M* *t*-BuSH reagent was made by dissolving 26.8 mg OPA in 2.5 ml filtered methanol and adding 28.1 μl *t*-BuSH and 2.5 ml borate buffer (0.6 *M*). The solutions were stored at room temperature in darkened borosilicate glass vials that had been cleaned using 1 *M* HCl followed by rinses with HPLC-grade water and absolute ethanol [30]. Derivatization was performed using a Famos autosampler (LC Packings, San Francisco, CA, USA) to deliver reagents. Unless stated other otherwise, derivatization was performed by adding 0.6 μl 40 m*M* OPA–50 m*M* *t*-BuSH to 2 μl samples, mixed and allowed to react for 5 min. For each sample derivatized, the ratio of OPA to *t*-BuSH was 1:1.25. The final concentration of OPA (9.2 m*M*) allowed for at least a 10-fold excess over the total amine concentration. For example, a mixture of 20 amino acids, each at a concentration of $8 \cdot 10^{-6}$ *M* corresponds to a total amine concentration of $1.6 \cdot 10^{-4} M$. Excess thiol was removed by adding 0.4 μl of 1 *M* IAA (935 mg in 5 ml methanol) and allowed to react for 3 min. All samples were derivatized in a precleaned, 250-μl tapered polypropylene microvials [30].

2.3. Capillary liquid chromatography

The capillary LC–ED system is similar to that previously described [30–32]. Capillary LC columns consisted of 30–34 cm×50 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) slurry-packed with 5 μm Alltima C_8 particles (Alltech) by a previously described technique [33]. Mobile phase was delivered at 40 μl/min using two high-pressure syringe pumps (100 DM, ISCO, Lincoln, NE, USA) with approximately 90% of the flow being carried to waste by a splitter (60 cm×25 μm I.D.) thus generating a backpressure of approximately 3500 p.s.i. (1 p.s.i.=6894.76 Pa). Injections were performed by the autosampler (Famos), which contains a six-port injection valve (Valco C2) fitted with a 1-μl injection loop. To reduce the dead volume of the system, the capillary column was threaded through the injection valve port to the rotor. Mobile phase A was 50 m*M* phosphate buffer, pH 6.5 containing 1 m*M* EDTA while mobile phase B was phosphate buffer–acetonitrile (35:65). Mobile phase solutions were degassed prior to loading the syringe pumps by sparging with He for at least 10 min.

2.4. Electrochemical detection

The working electrode was a carbon fiber microelectrode (1 mm×9 μm diameter) fabricated using previously described methods [34]. The electrode was inserted using a micropositioner into the outlet of the capillary column mounted in an electrochemical cell containing 0.1 *M* KCl as supporting electrolyte. Working electrodes were poised at +0.75 V versus Ag/AgCl reference electrodes and were pretreated to improve reproducibility and reduce background current by sweeping the potential from 0 to 1.8 V at 1 V/s for 30 s [35]. Current was amplified using a Stanford SR-570 current amplifier (Sunnyvale, CA, USA) set at 1 Hz low pass filter. The signal was digitized using a 16-bit AT-MIO data acquisition board (National Instruments, Austin, TX, USA) in a 486 DX computer with 5 Hz collection rate.

Cyclic voltammograms were collected with an EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN, USA) interfaced to an IBM-compatible personal computer via a multifunction board. The current was measured in a two-electrode system with a Ag/AgCl reference electrode and a carbon fiber disk microelectrode (9 μm diameter). Cyclic voltammograms were recorded from −500 to 1100 mV at a scan rate of 40 V/s in a partial aqueous background electrolyte solution of CH_3CN–50 m*M* TBAP (50:50) with 0.10 *M* acetate buffer, pH 5.75 by flow injection analysis (FIA). Background subtraction was used to remove the large charging currents associated with the use of high scan rates [36].

2.5. Microdialysis

Male Sprague–Dawley rats (250–350 g) were anesthetized with a 1.0 ml subcutaneous injection of 0.1 g/ml chloral hydrate (Sigma) and mounted in a stereotaxic frame before surgery. Microdialysis sampling was performed using side-by-side 3 mm long probes constructed in the laboratory using methods described elsewhere [37]. Probes were inserted at a

rate of 500 μm/min to minimize tissue damage and were implanted at +0.02 AP, −0.30 ML and −0.65 DL from bregma to sample the striatum [38]. Artificial cerebral spinal fluid (aCSF) (145 m*M* NaCl, 2.68 m*M* KCl, 1.01 m*M* $MgSO_4$ and 1.22 m*M* $CaCl_2$) was perfused through the probe at 0.3 μl/min using a microsyringe pump (CMA/102, Acton, MA, USA). Sample collection began after basal levels were constant, about 2 h after probe implantation. Fractions were collected at 7-min intervals (2.1 μl per fraction) and stored at −50 °C. To quantify the concentration of the dialysate, a standard curve consisting of 10, 50, 100, 400 and 1000 n*M* neurotransmitters was constructed. Standards and samples were treated identically and analyzed on the same day. In vitro microdialysis probe recovery was determined before an in vivo experiment at 0.3 μl/min.

2.6. Butterfly brain tissue preparation

Whole brains including optic lobes of *Agraulis vanillae* L. (Lepidoptera, Nymphalidae) were removed and homogenized in 0.5 ml water and centrifuged for 2 min at high speed. The supernatant was diluted with distilled water to a volume of 1.5 ml, filtered through sterile acrodisc 0.2 nm syringe filters (Fisher Scientific) and stored at −50 °C prior to analysis.

3. Results and discussion

3.1. Detection and derivatization conditions

Previously we have demonstrated the separation and high-sensitivity detection of 16 amino acid neurotransmitters using 50 μm I.D. capillary columns packed with C_8 5 μm particles [30]. A two-step derivatization process was used. In the fist step, the OPA–*t*-BuSH (1:1.25 molar ratio) reagent was allowed to react with the amino acids to form electroactive 1-alkylthio-2-alkylisoindoles. IAA was added after the first reaction as this step has previously been found to reduce background peaks [12,32]. Amino acid derivatives were separated using an initial mobile phase of A–B (65:35) with a linear gradient of 2% B/min over 5 min, then 1% B over 10 min. After elution of the amino acids, the mobile phase was stepped to 100% B. In preliminary experiments using the above conditions, we found that OPA–*t*-BuSH derivatives of NA, DA, histamine and 5-HT eluted well after the most hydrophobic amino acids when the mobile phase was stepped to 100% mobile phase B. A chromatogram illustrating detection of the amines with the amino acids under these conditions is illustrated in Fig. 1. The lower trace is a blank, which shows the presence of background peaks. (Further experiments did not include histamine as this peak proved difficult to reliably resolve from a substantial background peak).

Using hydrodynamic voltammetry (HDV) in the potential range of 0.50–0.90 V, it was found that the detection potential that gave the best signal-to-noise ratio (*S*/*N*) for NA, DA, and 5-HT was +0.75 V, which was the same as that found previously for amino acids [30,32]. The time required for OPA–*t*-BuSH to react with these amines (500 n*M* of each) was determined by varying the reaction time (prior to adding the IAA) from 1 to 10 min while keeping all other conditions the same as in the Experimental section. Above 2.5 min the peak area for all the compounds was independent of reaction time indicat-

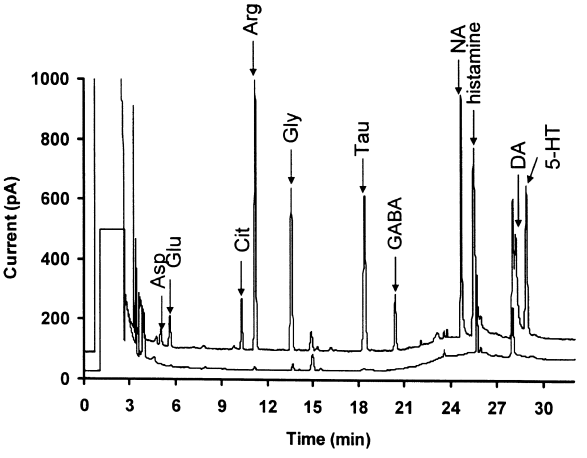

Fig. 1. Chromatogram illustrating the separation of target amino acids and NA, histamine, DA and 5-HT. Derivatives were separated at 3 nl/s using the mobile phase gradient described in the text. Standards are at 100–300 n*M*. A 100-nl volume of sample was injected. Analytes were derivatized as described in text with a final concentration 33 m*M* borate in the derivatization buffer. Lower trace is a blank (derivatization solution with no analytes).

ing that the reaction was complete within this time. The stability of the DA derivative was tested by comparing the peak areas obtained for 500 n*M* DA immediately after completion of the derivatization procedure (performed as in the Experimental section) and after allowing the derivatized solution to sit for 40 min. Peak areas obtained from the two chromatograms were within 5% of each other ($n=5$) indicating good stability of the derivatives. Thus, the DA derivative that is detected is stable for at least 40 min.

3.2. Problems with initial conditions

While good signals were obtained for NA, DA, and 5-HT at 100 n*M* (Fig. 2A), it was impossible to identify peaks corresponding to these compounds at 10 n*M* due to the highly irregular baseline (Fig. 2B). In contrast, the S/N for the amino acids even at 5 n*M* was still high (Fig. 2C) primarily due to a more regular baseline as seen by comparing the insets of Fig. 2B and C. (For separations shown in Fig. 2A, B, and C, a gradient optimized by applying the linear solvent strength (LSS) theory was used [39]. The result was a minor modification of the gradient elution method to 4% B/min over 8.5 min followed by a step change to 100% B).

It was hypothesized that increased noise in the baseline during elution of the amines was due in part to elution of hydrophobic compounds present in the sample or a mobile phase component. To ameliorate this problem, the mobile phase and derivatization solutions were cleaned using a filter containing activated carbon (see Experimental section). In addition, the mobile phase gradient was changed to 4% B/min for the first 8.5 min followed by 10% B/min until 100% mobile phase B was achieved thus eliminating the use of a step change prior to elution of NA, DA, and 5-HT. The use of a linear gradient lowered noise by reducing the detrimental effects of a sudden change in mobile phase composition as well as improving resolution of background components. With these modifications, the peak-to-peak noise was reduced ~fourfold (from 8 to 2 pA, see insets of Fig. 2). While an improvement, these noise levels were still over three times the levels during the elution window of the amino acids. A third change that improved the S/N was increasing the final borate

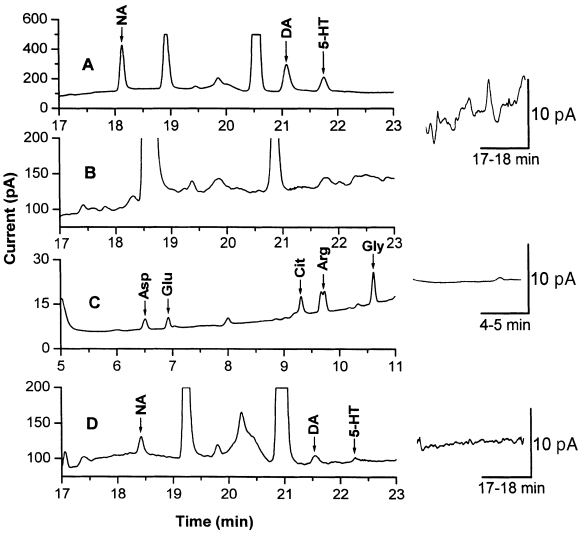

Fig. 2. Chromatograms illustrating improvements in detection limit. (A) Chromatogram showing elution of NA, DA and 5-HT at 100 n*M* (250 nl injection) derivatized as outlined in the text. Derivatives were separated at 3.2 nl/s by a gradient elution with a mobile phase initially consisting of 35% B, increasing by 4% B/min over 8.5 min followed by a step change to 100% B. (B) Same as (A) with analytes derivatized at 10 n*M*. Inset shows the irregular baseline that occurs in the elution region of the amines. (C) Detection of amino acids using the same conditions as (A) and (B) at 5 n*M*. Inset shows the lower noise of the baseline in this region of the chromatogram. (D) Chromatogram showing detection of derivatized NA, DA, and 5-HT at 10 n*M* using the modified method in which sample was filtered, gradient was modified (initial condition was 35% B, increasing by 4% B/min for the first 8.5 min followed by 10% B/min until 100% B was achieved), and borate in the derivatization media was increased to 70 m*M* as described in the text.

concentration in the derivatization media from 33 to 70 m*M* which improved the signal by ~twofold for the biogenic amines. (Above 70 m*M* no further improvements were obtained). A similar effect has been reported previously with NDA derivatization of NA and DA and may reflect improved derivatization yields [40]. These combined modifications improved the detection limit ~eightfold for the catecholamines and indoleamines. With these improved conditions, the 10 n*M* standards were readily detected (see Fig. 2D).

3.3. Reproducibility, linearity and detection limits

Fig. 3A, which depicts a chromatogram resulting

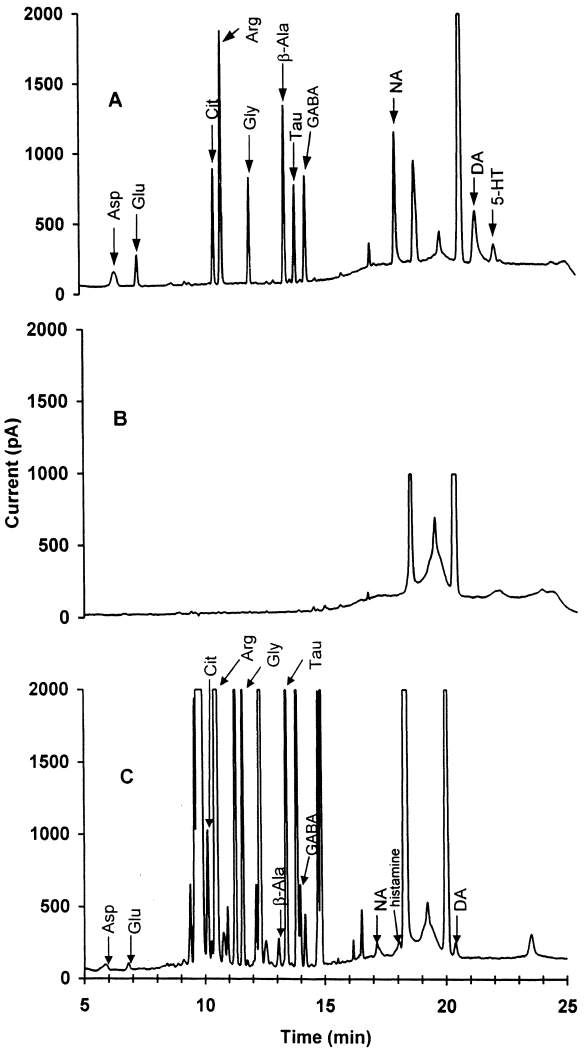

Fig. 3. Chromatograms resulting from analysis of: (A) amino acid and amine standards, each at 400 n*M*, (B) blank, and (C) a microdialysis sample obtained from a 7-min fraction (2.1 μl) collected from the striatum of an anesthetized rat. Samples (250 nl) were injected onto 50 μm I.D. capillary columns and separated at 3.3 nl/s using the gradient, derivatization, and sample preparation conditions as in Fig. 2D.

from injection of a mixture containing the neuroactive compounds Asp, Glu, Arg, Cit, Gly, Tau, GABA, NA, DA, and 5-HT demonstrates that these compounds were well resolved from each other and background peaks using the new separation conditions. Regression analysis using either peak height or area versus concentration demonstrated linearity from 10 n*M* to 10 μ*M*, with r^2 values 0.99 or better for all analytes. The precision of the method, evaluated using three consecutive analyses of a mixture 50 n*M* of standards on the same column, is summarized in Table 1. Consecutive chromatograms on the same column had excellent precision in retention time (Table 1); however, over the course of several hours of use the retention times could drift. Intercolumn comparisons of retention times also revealed larger variations. For example, for three different columns retention times had relative standard deviations of 3 to 7%.

Detection limits of 0.3–0.9 n*M* (80–200 amol) were obtained for amino acids while detection limits for NA, DA and 5-HT were 1–4 n*M* (200–900 amol). The detection limit was calculated as the concentration required to give a signal-to-noise ratio of 3 where the noise is measured as peak-to-peak noise in the chromatogram in the region where the peaks elute. These detection limits reflect the lowest concentration that can be derivatized and detected. Although other techniques may offer better limits of detection for one or more of these compounds, this is the first technique that enables determination of both classes of neurotransmitters at low nanomolar levels without the need for sample splitting or column switching.

The sensitivity for amino acids and catecholamines were similar under the conditions used. Thus, injection of 30 fmol of Gly, GABA, and DA resulted in peak areas of 1.2, 1.1, and 1.2 nC, respectively. This peak area corresponds to conversion efficiencies of ~40% for the amino acids assuming that they reacted quantitatively with OPA–*t*-BuSH and yield a 1 e^- transfer reaction during detection. It was originally expected that the DA peaks would have a threefold higher sensitivity than the amino acids because in addition to the isoindole functionality, DA bears a catechol functional group that can be oxidized in a 2 e^- transfer reaction. Indeed, underivatized DA yields a better signal (3.3 nC for 30 fmol injection) than derivatized DA. (Underivatized DA was detected at +0.75 V vs. Ag/AgCl after isocratic elution with 7% CH_3CN in 50 m*M* phosphate buffer, pH 3.7, 1 m*M* EDTA using a 15 cm×50 μm I.D. column packed with Alltima C_{18} μm particles).

Several hypotheses could be advanced to explain the lower than expected signals for DA including:

Table 1
Detection limits[a], reproducibility of retention time, peak area and peak height for neuroactive amines ($n=3$)

Analyte	Retention time RSD (%)	Peak height RSD (%)	Peak area RSD (%)	Detection limit (n*M*)	Detection limit (amol)
Asp	0.71	2.9	2.9	0.8	200
Glu	0.75	3.1	1.3	0.9	200
Cit	0.10	2.3	1.2	0.4	100
Arg	0.12	4.2	3.2	0.3	80
Gly	0.21	1.4	1.8	0.4	90
Tau	0.15	1.6	1.1	0.7	200
GABA	0.19	2.1	1.6	0.6	100
NA	0.33	4.6	3.9	1	200
DA	0.45	6.2	4.6	2	400
5-HT	0.53	6.3	4.3	4	900

[a] Calculated as the concentration corresponding to a signal-to-noise ratio of 3 where noise is measured as peak-to-peak noise. Values are based on 250 nl injection volume.

(1) formation of multiple products during derivatization, (2) incomplete reaction of DA under the conditions used, and (3) chemical conversion of DA during derivatization to a product that does not generate a three-electron transfer reaction. As only a single peak for derivatized DA was detected, no evidence supporting the first hypothesis was found. The second hypothesis was eliminated by the observation that no underivatized DA could be detected after the derivatization reaction indicating that all of the DA was consumed under the conditions used. Evidence supporting the third hypothesis was obtained by cyclic voltammetry (CV) of DA before and after derivatization. CV of underivatized DA at 40 V/s gave the expected result with a peak at 700 mV on the anodic scan, corresponding to oxidation of DA, and a peak at −100 mV on the cathodic scan corresponding to reduction of quinone formed by dopamine oxidation (Fig. 4A). CV of derivatized dopamine revealed a different profile, with a peak on the anodic scan at −200 and 800 mV (Fig. 4B). No waves were observed on the reverse scan indicating an irreversible oxidation. The complete lack of a reverse (cathodic) wave for derivatized DA suggests that the product of OPA–*t*-BuSH reaction with DA does not contain a catechol group. Irreversible voltammetry is characteristic of the isoindole moiety and in fact the cyclic voltammograms are similar to those observed for OPA derivatives of amino acids [12]. Thus, the CV data are consistent with the hypothesis that the decrease in sensitivity observed for catecholamines upon derivatization is due to loss of the catechol functional group during derivatization resulting in amperometric detection of only the isoindole group. If the derivatization product of DA is an isoindole with no catechol group, as suggested by the CV data, then the peak area for comparable injected quantities would be the same for amino acids and the amines in agreement with our observation (see above). Chemical identification of the products by mass spectrometry and/or nuclear magnetic resonance (NMR) would be required to identify the species actually detected.

3.4. Applications

To demonstrate the utility of the method, dialysate collected in vivo from rat striatum and tissue samples (butterfly brain) were analyzed as examples of typical neurochemical applications. Fig. 3 compares chromatograms for standards at 400 n*M*, a blank and a striatal microdialysis sample. Peaks corresponding to Asp, Glu, Cit, Arg, Gly, Tau, GABA, Tau, NA, histamine, and DA were readily detected in the dialysate sample. (As mentioned above, the proximity of the histamine peak to a reagent peak precluded quantification on all samples; however, a peak matching its retention time was detected in many cases). Poor recovery of 5-HT from the dialysis probe (see Table 2) prevented its detection in dialysate. This problem may be solved by use of different dialysis probes. One difficulty with the simultaneous measurement resulted from the large range of concentrations (some amino acids have

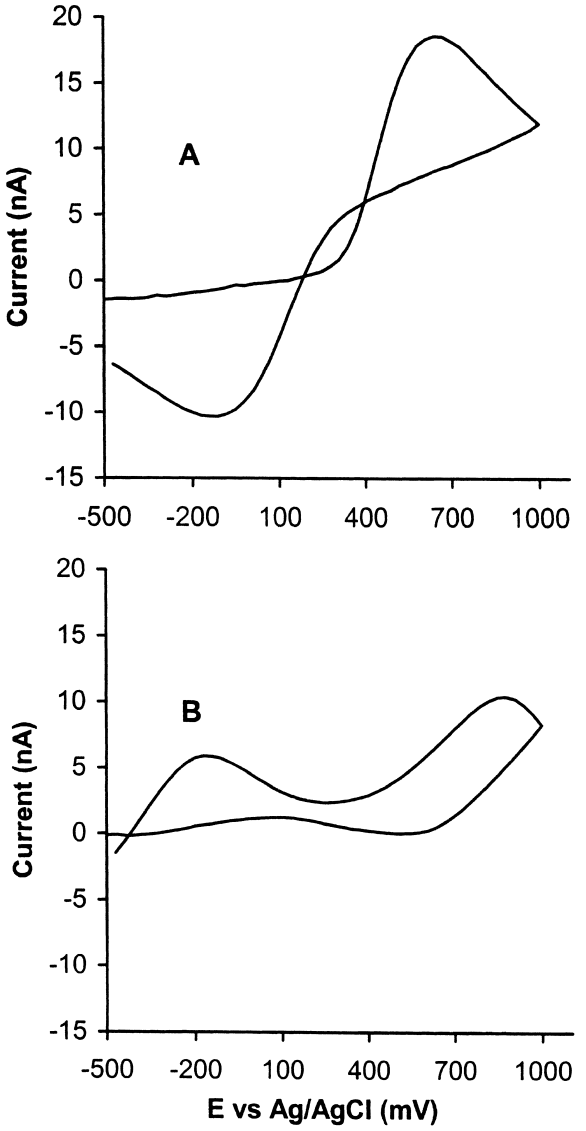

Fig. 4. Cyclic voltammograms of (A) underivatized DA and (B) derivatized DA at 500 μ*M* in a background electrolyte solution of CH_3CN–50 m*M* TBAP (50:50) with 0.1 *M* acetate at pH 5.75 at a scan rate of 40 V/s. Voltammograms were obtained in a flow injection apparatus and are background subtracted as described in the Experimental section.

levels over 1000-fold greater than NA and DA) observed. As a result, some amino acids such as Gly, Arg, and Tau are off-scale at the same gain settings needed to detect the lower level NA and DA. This problem can be readily solved by using a data acquisition system with a larger dynamic range or use of automatic gain control to adjust the gain as analytes elute.

Table 2
Basal concentrations ($n=8$) and probe recovery ($n=5$) of amine neurotransmitters in the rat striatum

	Dialysate basal concentration (n*M*)	In vitro probe recovery (%)
Asp	178±34	51±5
Glu	454±66	61±5
GABA	117±16	58±8
NA	31±6	77±3
DA	48±5	81±17
5-HT	ND	14±1

Values are given as mean±S.E.M.
ND: Not detected.

The basal dialysate concentrations obtained for Asp, Glu, GABA, NA and DA are listed in Table 2. The basal concentrations for Asp, Glu, GABA, and DA are consistent with those reported previously at similar flow-rates [32,41–44]. NA has not been measured previously in the striatum at this flow-rate; however, in a previous report in which NA and DA were both detected in the dialysate samples of the rat striatum, the levels were found to be present at a nearly 1:1 ratio which is similar to that observed here [45].

To demonstrate measurement during a pharmacological manipulation, the catecholamine uptake blocker nomifensine (30 μ*M*) was added to the perfused aCSF after basal levels were established. Following this treatment, considerable increases in DA and NA were observed while Asp, Glu and GABA levels were decreased (Fig. 5); however, the decrease in GABA was not statistically significant ($P>0.05$). The increase in DA dialysate levels with nomifensine treatment is well documented and is due to the increased recovery and overflow of DA [46–49]. These results are in good agreement with previous work wherein perfused nomifensine was shown to considerably enhance dialysate levels of DA and NA in the striatum (500 and 240% increases above basal for DA and NA, respectively) [50]. The changes in the amino acids are believed to be the consequence of the increased levels of DA.

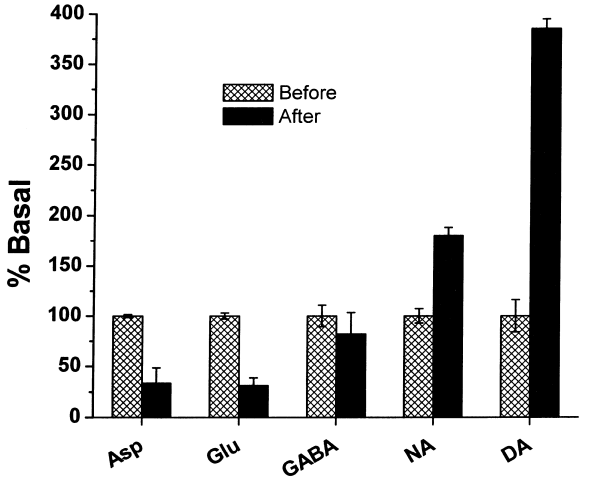

Fig. 5. Comparison of selected neurotransmitter levels during in vivo microdialysis in the striatum before and after perfusion of the DA uptake inhibitor nomifensine (30 μ*M*) for 45 min. Data are presented as the percent of the basal level (i.e., the level before addition of the drug) and represent the mean±S.E.M. for four rats.

In another application, this method was used to measure the amine-containing neurotransmitters present in butterfly brains. The knowledge of neurotransmitters in insects is limited; however, over 60 neurotransmitter receptor sub-types have been documented including receptors for DA, 5-HT, GABA, and Glu [51–53]. Fig. 6A illustrates a chromatogram of a butterfly brain diluted 100-fold and Fig. 6B the same extract without dilution. All the neuroactive amino acids (Glu, Asp, GABA, Gly, Tau, Arg, Cit) as well as NA, DA, and 5-HT were detected in the samples. Unlabeled peaks observed in the chromatograms likely correspond to other amino acids (e.g., methionine, valine, leucine, phenylalanine, ornithine, lysine, β-aminobutyric acid and α-aminobutyric acid); however, as these other compounds were not included in our standards they were not quantitated. As with the dialysate samples, a larger dynamic range than available with this system would be required to quantify amino acids and amines in a single chromatographic run. Nevertheless, reproducible data were obtained for analysis of all the detectable amines and amino acids as shown in Table 3 which compares the levels found in brains of wild and naïve (i.e., housed in cages) butterflies.

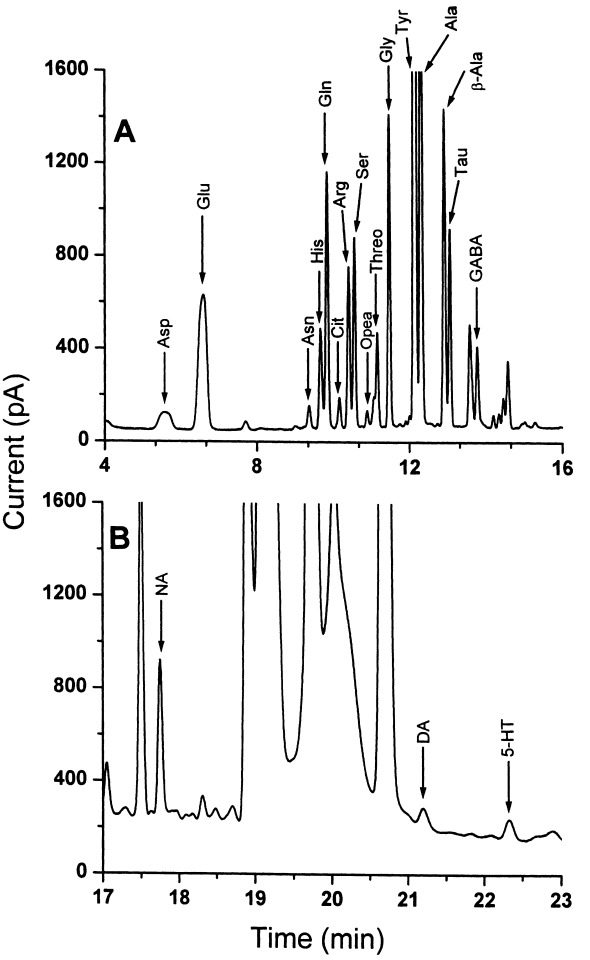

Fig. 6. Chromatograms resulting from analysis of butterfly brain tissue samples. (A) 1:100 dilution of sample for determination of amino acids. (b) Chromatogram for detection of catecholamines and indoleamines without dilution. Peak labels are: asparagine (Asn), histidine (His), glutamine (Gln), serine (Ser), *o*-phenylethanolamine (Opea), threonine (Threo), tyrosine (Tyr), and alanine (Ala). Separation and analysis conditions as in Fig. 2D.

4. Conclusion

The method presented allows detection of amine and amino acid neurotransmitters with detection limits from 0.3 to 4 n*M* in 250 nl samples. Only minor modifications of existing methods for amino acid analysis were required. The technique is useful for analyzing small samples obtained from different

Table 3
Neurotransmitter levels in butterfly brain samples

	Wild male	Naïve male
Asp	80±9	32±5
Glu	100±19	56±7
GABA	7.8±0.4	3.2±0.9
NA	142±21	306±41
DA	14±1	27±4
5-HT	26±1	24±3

Five brains homogenized in water were used for each analysis. Concentrations are reported in micromolar (μM) for Asp, Glu and GABA and nanomolar (nM) for NA, DA and 5-HT. Values are the mean±S.E.M. for four samples.

biological sources. The reproducibility of derivatization, injection, migration and detection limits are satisfactory for biological or pharmacological studies requiring simultaneous monitoring of amine-containing neurotransmitters. The main limitation of the method is the lack of dynamic range to cover large differences in concentration typically found for amino acids and amines; however, this problem can be readily solved. In addition, the detection limit for 5-HT may not be sufficient to detect this compound in some dialysate samples; however, improvements in the dialysis recovery of 5-HT may improve this situation. Nonetheless, these results illustrate the potential for monitoring multiple neurotransmitter classes by a single technique.

Acknowledgements

This work was supported by a grant from NIH (NS38476). We thank Dr. Vadim Kroutov for supplying the butterfly brains.

References

[1] J.R. Cooper, F.E. Bloom, R.H. Roth, in: The Biochemical Basis of Neuropharmacology, Oxford University Press, Oxford, New York, 1986, p. 126, Chapter 6.
[2] F. Cheng, L. Lang, F. Chang, L. Chai, J. Kuo, J. Chromatogr. 582 (1992) 19.
[3] J. Keating, L. Dractu, M. Lader, R.A. Sherwood, J. Chromatogr. 615 (1993) 237.
[4] B.R. Cooper, R.M. Wightman, J.W. Jorgenson, J. Chromatogr. B 653 (1994) 25.
[5] T.B. Jensen, P.D. Marley, J. Chromatogr. B 670 (1995) 199.
[6] M. Roth, Anal. Chem. 43 (1971) 880.
[7] M.H. Joseph, P.J. Davies, J. Chromatogr. 277 (1983) 125.
[8] R.F. Chen, C. Scott, E. Trepman, Biochim. Biophys. Acta 576 (1979) 440.
[9] C. Buteau, L. Duitschaever, G.C. Ashton, J. Chromatogr. 212 (1981) 23.
[10] K.S. Lee, D.G. Drescher, Int. J. Biochem. 9 (1978) 457.
[11] W.A. Jacobs, M.W. Leburg, E.J. Madaj, Anal. Biochem. 156 (1986) 334.
[12] L.A. Allison, G.S. Mayer, R.E. Shoup, Anal. Chem. 56 (1984) 1089.
[13] J.F. Stobaugh, A.J. Repta, L.A. Sternson, K.V. Garren, Anal. Biochem. 135 (1983) 495.
[14] S.S. Simons Jr., D.F. Johnson, Anal. Biochem. 82 (1977) 250.
[15] I. Smolders, S. Sarre, Y. Michotte, G. Ebinger, J. Neurosci. Methods 57 (1995) 47.
[16] Y. Qu, L. Arckens, E. Vandenbussche, S. Geraerts, F. Vandesande, J. Chromatogr. A 798 (1998) 19.
[17] W.A. Jacobs, Curr. Sep. 7 (1986) 39.
[18] P. de Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem. 59 (1987) 1096.
[19] M.C. Roach, M.D. Harmony, Anal. Chem. 59 (1987) 411.
[20] M.P. Oates, B.R. Cooper, J.W. Jorgenson, Anal. Chem. 62 (1990) 1573.
[21] Z. Chen, J. Wu, G.B. Baker, M. Parent, N.J. Dovichi, J. Chromatogr. A 914 (2001) 293.
[22] J. Qian, Y. Wu, H. Yang, A. Michael, Anal. Chem. 71 (1999) 4486.
[23] R.A. Wallingford, A.G. Ewing, Anal. Chem. 61 (1989) 98.
[24] A.P. Newton, J.B. Justice, Anal. Chem. 66 (1994) 1468.
[25] F. Robert, L. Bert, L. Denroy, B. Renaud, Anal. Chem. 67 (1995) 1838.
[26] S.D. Gilman, A.G. Ewing, Anal. Chem. 67 (1995) 58.
[27] P. Gamache, E. Ryan, J. Svendsen, J. Chromatogr. 614 (1993) 213.
[28] L. Bert, F. Robert, L. Denroy, L. Stoppini, B. Renaud, J. Chromatogr. A 755 (1996) 99.
[29] J.S. Soblosky, L.L. Colgin, C.M. Parrish, J.F. Davidson, M.E. Carey, J. Chromatogr. B 712 (1998) 31.
[30] B.W. Boyd, S.R. Witowski, R.T. Kennedy, Anal. Chem. 72 (2000) 865.
[31] M.D. Oates, J.W. Jorgenson, Anal. Chem. 61 (1989) 432.
[32] B.W. Boyd, R.T. Kennedy, Analyst 123 (1998) 2119.
[33] R.T. Kennedy, J.W. Jorgenson, Anal. Chem. 61 (1989) 1128.
[34] K.T. Kawagoe, J.B. Zimmerman, R.M. Wightman, J. Neurosci. Methods 48 (1993) 225.
[35] R.L. St. Claire, J.W. Jorgenson, J. Chromatogr. Sci. 23 (1985) 186.
[36] B.D. Bath, D.J. Michael, J. Trafton, J.D. Joseph, P.L. Runnels, R.M. Wightman, Anal. Chem. 72 (2000) 5994.
[37] H.O. Pettit, J.B. Justice, in: T.E. Robinson, J.B. Justice (Eds.), Microdialysis in the Neurosciences, Elsevier, Amsterdam, 1991, p. 117, Chapter 6.
[38] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, Orlando, FL, 1997.

[39] L.R. Snyder, J.W. Dolan, J.R. Gant, J. Chromatogr. 165 (1970) 3.
[40] L. Denroy, L. Bert, S. Parrot, F. Robert, B. Renaud, Electrophoresis 19 (1998) 2841.
[41] M. Moriari, W.T. O'Connor, U. Ungerstedt, K. Fuxe, J. Neurochem. 60 (1993) 1884.
[42] J. Semba, S. Kito, M. Toru, J. Neural Trans. [GenSect] 100 (1995) 39.
[43] P. Britton, P.S. Whitton, L.J. Fowler, N.G. Bowery, J. Neurochem. 67 (1996) 324.
[44] S.A. Wages, W.H. Church, J.B. Justice, Anal. Chem. 58 (1986) 1649.
[45] L.A. Dawson, H.Q. Nguyen, P. Li, Br. J. Pharm. 130 (2000) 23.
[46] N.R. Zahniser, G.A. Larson, G.A. Gerhardt, J. Pharmacol. Exp. Ther. 289 (2000) 266.
[47] C. Davidson, E.H. Ellinwood, S.B. Douglas, T.H. Lee, J. Neurosci. Methods 101 (2000) 75.
[48] W. Hauber, H. Fuchs, Behav. Brain Res. 111 (2000) 39.
[49] J.L. Peters, A.C. Michael, J. Neurochem. 74 (2000) 1563.
[50] M.A. Cenci, P. Kalen, R.J. Mandel, A. Bjorklund, Brain Res. 581 (1992) 217.
[51] N.V. Kulagina, M.J. Zigmond, A.C. Michael, Neuroscience 102 (2001) 121.
[52] T. Roeder, Comp. Biochem. Physiol. 107C (1994) 1.
[53] R.H. Osborne, Pharmacol. Ther. 69 (1996) 117.