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Procedure for the sample preparation and handling for the determination of amino acids, monoamines and metabolites from microdissected brain regions of the rat

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Abstract

A method is described for the analysis of amino acids, monoamines and metabolites by high-performance liquid chromatography with electrochemical detection (HPLC–ED) from individual brain areas. The chromatographic separations were achieved using microbore columns. For amino acids we used a 100×1 mm I.D. C_8, 5 μm column. A binary mobile phases was used: mobile phase A consisted of 0.1 *M* sodium acetate buffer (pH 6.8)–methanol–dimethylacetamide (69:24:7, v/v) and mobile phase B consisted of sodium acetate buffer (pH 6.8)–methanol–dimethylacetamide (15:45:40, v/v). The flow-rate was maintained at 150 μl/min. For monoamines and metabolites we used a 150×1 mm I.D. C_{18} 5 μm reversed-phase column. The mobile phase consisted of 25 m*M* monobasic sodium phosphate, 50 m*M* sodium citrate, 27 μ*M* disodium EDTA, 10 m*M* diethylamine, 2.2 m*M* octane sulfonic acid and 10 m*M* sodium chloride with 3% methanol and 2.2% dimethylacetamide. The potential was +700 mV versus Ag/AgCl reference electrode for both the amino acids and the biogenic amines and metabolites. Ten rat brain regions, including various cortical areas, the cerebellum, hippocampus, substantia nigra, red nucleus and locus coeruleus were microdissected or micropunched from frozen 300-μm tissue slices. Tissue samples were homogenized in 50 or 100 μl of 0.05 *M* perchloric acid. The precise handling and processing of the tissue samples and tissue homogenates are described in detail, since care must be exercised in processing such small volumes while preventing sample degradation. An aliquot of the sample was derivatized to form the *tert*.-butylthiol derivatives of the amino acids and γ-aminobutyric acid. A second aliquot of the same sample was used for monamine and metabolite analyses. The results indicate that the procedure is ideal for processing and analyzing small tissue samples.

Keywords: Amino acids; Monoamines

1. Introduction

There are many methods in the literature describing the conditions for measuring biogenic monoamines/metabolites [1–7] and neurotransmitter amino acids/γ-aminobutyric acid (GABA) [7–12] in brain tissue samples using high-performance liquid chromatography with electrochemical detection (HPLC–ED). Since the development of microbore HPLC columns and instrumentation, the ability to

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detect very small amounts (femtomoles) of these analytes has become readily available [[13–15] for review]. Therefore, it is possible to detect both the biogenic monoamines and amino acids in a single, very small tissue sample. The ability to measure both classes of analytes in a single sample is important because: (1) it allows the researcher to more fully understand the interplay of these two neurotransmitter types, (2) the effects of drugs or other manipulations on these two classes of neurotransmitters can be simultaneously evaluated in a single animal, and (3) separate experiments i.e. rats do not have to be used in order to measure both the biogenic monoamines and amino acids. However, with the ability to measure small amounts of analytes in small samples, it is necessary to be able to dissect the tissue samples, homogenize the samples in very small volumes of liquid, then carry extremely small volumes (less than 100 μl) through a number of preparatory steps (e.g. filtrations, derivatizations, etc.), without allowing the samples to degrade, while conserving enough sample to inject into two separate chromatographic systems.

This paper describes the methodology, including the sampling and sample handling procedure, by which both biogenic monoamines/metabolites and amino acids/GABA can be measured in a single micropunched and other very small samples of brain tissue.

2. Experimental

2.1. Standards and chemicals

All amino acid and biogenic monoamine standards were obtained from Sigma (St. Louis, MO, USA). Glacial acetic acid, phosphoric acid, perchloric acid, sodium citrate 1-octane sulfonic acid, and sodium chloride were obtained from Mallinkrodt (Paris, KY, USA). Sodium hydroxide, sodium phosphate, monobasic, EDTA, isopentane and dimethylacetamide were obtained from J.T. Baker (Phillipsburg, NJ, USA). Methanol was obtained from Burdick and Jackson (Muskegon, MI, USA) and diethylamine HCL was obtained from Aldrich (St. Louis, MO, USA).

The amino acid derivatization kit for amino acids (via microbore) was obtained from Bioanalytical Systems (W. Lafayette, IN, USA). This kit contains all necessary reagents (premixed) which can be used without any further modification.

2.2. Animals and sample handling

Sprague–Dawley rats (350–450 g) were anesthetized with isoflurane (5% with N_2O–O_2, 4:1, v/v) and sacrificed by rapid decapitation. The brains were rapidly removed within 2 min and were frozen at −40°C by immersion into an isopentane–dry ice mixture. The brains were then wrapped in aluminum foil, placed in a freezer bag and an air-tight container and then stored in a freezer at −70°C. Prior to tissue slicing with a microtome, the brains were placed for 2–3 days in a cryostat at −6°C. The brains were then sliced into 300-μm sections, thaw mounted onto glass slides, wrapped in aluminum foil, then placed in a freezer bag and an air-tight container prior to being stored in a freezer at −70°C. Thaw mounting was performed by spraying the brain slices with tissue freezing aerosol (Stephens Scientific, Riverdale, NJ, USA) immediately after the brain slices had thawed.

2.3. Tissue dissection and preparation

Brain samples were taken by either free hand dissection with a microknife (Fine Science Tools, Foster City, CA, USA) or with micropunches (Stoelting, Wood Dale, IL, USA) while the glass slides were maintained at −25°C on a cold plate (Stoelting). The tips of the microknife and brain punches were kept frozen by keeping them in a mixture of isopentane–dry ice while not in use.

The ten brain areas sampled included the somatosensory areas of the frontal and parietal cortices, motor area of the parietal cortex, cingulate cortex, striatum, substantia nigra, hippocampus, cerebellar folia, red nucleus and locus coeruleus. The sample locations were determined using established techniques [16,17]. The details of the brain levels and micropunch sizes are detailed in Fig. 1. All brain areas were sampled bilaterally (total=20 samples/brain) in order to compare the right and left sides.

Brain region tissue samples were placed into 500-

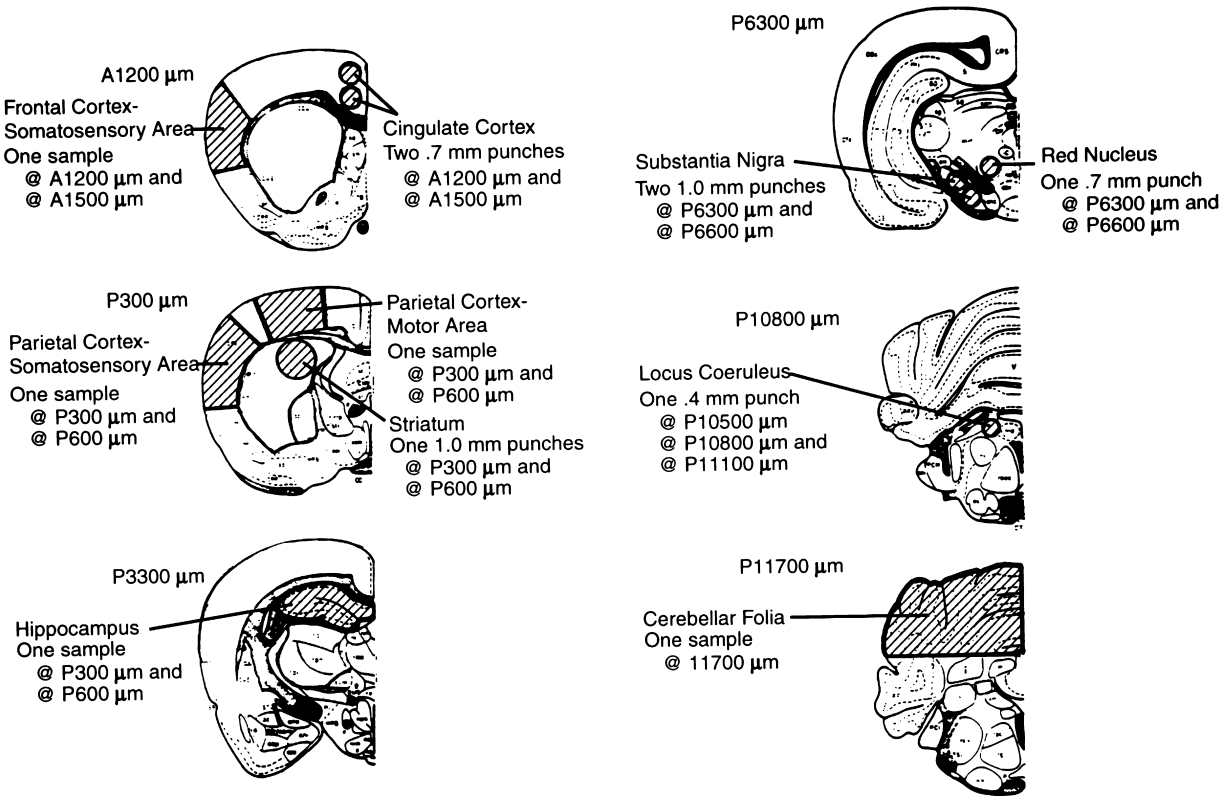

Fig. 1. Coordinates and microdissection location of sampled brain regions. A=anterior, P=posterior.

μl microcentrifuge tubes (Kontes, Vineland, NJ, USA), which were kept at −20°C in a labtop cooler (Nalgene, Rochester, NY, USA). Depending upon the brain region, the tissue samples were homogenized in 50–100 μl of ice cold 0.05 *M* perchloric acid using a battery operated PTFE pestle (Kontes). The homogenization volumes were: 100 μl for the somatosensory areas of the frontal and parietal cortices, motor area of the parietal cortex, striatum, hippocampus and cerebellar folia; 50 μl for the cingulate cortex, locus coeruleus, substantia nigra and red nucleus. Following centrifugation at 13 600 *g* at 4°C for 20 min the supernatant was removed, using a pipette fitted with a 100 μl pipette tip, and filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA) (Note: we tried other manufacturers' filter assemblies and determined that some other brands had a contaminant in the filter assembly and receiving tube which coeluted or interfered with desired biogenic amine analytes). Ten microliters of the filtered supernatant were set aside for amino acid determination while the remaining portion was used for the biogenic monoamine determinations (see Section 2.6).

The remaining tissue pellets were left in the 500-μl microcentrifuge tubes and solubilized in 0.2 *M* NaOH and assayed for protein content using BCA protein assay reagent kits (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin as a standard. Depending on the brain area, either a standard (20–120 μg) or micro (1–20 μg) assay kit was used. The standard assay was used to determine the protein content of the somatosensory areas of the frontal and parietal cortices, motor area of the parietal cortex, striatum, substantia nigra, hippocampus and cerebellar folia. The micro assay was used for the cingulate cortex, locus coeruleus and red nucleus. All the tissue pellets solubilized in the 500-μl microcentrifuge tubes with 250 μl of 0.2 *M* NaOH using an ultrasonic homogenizer (Cole

Parmer, Vernon Hills, IL, USA). After complete solubilization an additional 250 μl of 0.2 *M* NaOH was added to all the tissue pellets except for the cingulate cortex, red nucleus, locus coeruleus, and substantia nigra. One hundred microliters of the solubilized tissue pellet solution were used in both the standard or micro assays.

2.4. Amino acid derivatization

Brain tissue homogenates contain large quantities of amino acids. In order to insure that all the amino acids are completely derivatized, it is necessary to dilute the supernatant from some of the samples. The amount of dilution (with 0.05 *M* perchloric acid) varied for each sample and was empirically predetermined. Using 10 μl of filtered supernatant, which was placed in a 500 μl tube, the following amounts of 0.05 *M* perchloric acid were added: 90 μl to the somatosensory area of the frontal and parietal cortices, motor area of the parietal cortex, hippocampus, and cerebellar folia; 30 μl to the caudate and substantia nigra; 20 μl to the cingulate cortex. No dilutions were necessary for the locus coeruleus or red nucleus.

Ten microliters of the filtered supernatant/diluted supernatant was placed in a glass 300-μl autosampler vial. Prior to derivatization, 20 μl of borate–methanol solution (diluent A in the derivatization kit) was added to the 10 μl of supernatant/diluted supernatant which was in a 300-μl autosampler vial. This was necessary in order to adjust the pH to about 8.5 so that the derivatization reaction would not be inhibited. Each vial was then sealed with a PTFE septum and inserted into the autosampler. *tert*.-Butylthiol derivatives of the amino acids and GABA were formed using microbore amino acid derivatization reagents and a CMA200 autosampler with an autoderivatization function (CMA, Acton, MA, USA). The computerized autoderivatization program was such that 10 μl of the *tert*.-butylthiol reagent (reagent A in the derivatization kit) was added to the sample solution, followed by 3 mixes and a 2-min reaction time, then 10 μl of the scavenging reagent (reagent B, iodoacetamide, in the derivatization kit) was added. After the derivatization reaction, five microliters, of the total 50 μl, were subsequently injected onto the column. If an autosampler with autoderivatization function is not available, the entire derivatization process can be carried out manually using small (10 μl) glass syringes, then manually injected onto the column.

A stock solution (2.5 m*M*) of 12 amino acids and GABA was made up in 0.05 *M* perchloric acid. Four external standard amounts were calculated and dilutions were made, using 0.05 *M* perchloric acid, so that 7, 14, 28 and 56 pmol were injected onto the column. Note that in making the standard dilution calculation it is necessary to allow for addition of the borate–methanol solution (20 μl), and the derivatizing and scavenging reagents (10 μl each).

2.5. Amino acid chromatography

Amino acid determinations were made using a dual channel electrochemical detection (potential=+700 mV vs. Ag/AgCl reference electrode) and a BAS 200 chromatograph (Bioanalytical Systems) equipped with a standard electrochemical cell and injector (Rheodyne, Cotati, CA, USA). Two channels were necessary because some amino acids, especially glutamate, occur in large quantities in relation to the other amino acids and GABA. For recording, the gain of one channel was set at 50 nA/V and the other was set at 200 nA/V full scale. Amino acid separations were achieved using a 100×1 mm I.D. microbore C_8, 5 μm chromatographic column (MF8901, Bioanalytical Systems) kept at 40°C and a linear gradient elution. Mobile phase A consisted of 0.1 *M* sodium acetate buffer (pH 6.8)–methanol–dimethylacetamide (69:24:7, v/v). The pH was adjusted with 6 *M* NaOH. Mobile phase B consisted of sodium acetate buffer (pH 6.8)–methanol–dimethylacetamide (15:45:40, v/v). The flow-rate was maintained at 150 μl/min. Since the pump in the BAS 200 is a conventional pump, a flow splitter (Bioanalytical Systems) was attached to the pump. Setting the pump at a flow-rate of 0.8 ml/min resulted in a flow-rate of about 150 μl/min through the analytical column. The proportioning of the two mobile phases was controlled by a microbore amino acid gradient program provided with the BAS200 liquid chromatograph. These conditions allow for the determination of aspartate (ASP), glutamate (GLU), glutamine (GLN), serine (SER), arginine (ARG), glycine (GLY), threonine (THR), tyrosine (TYR),

alanine (ALA), taurine (TAU), asparagine (ASN), histidine (HIS) and γ-aminobutyric acid (GABA) in 24 min, although the total run time takes about 40 min since the column must be cleaned between runs. Cleaning was achieved by letting mobile phase A pump through the system for an additional 16 min after completion of the analytical run.

2.6. Biogenic amine chromatography

The biogenic monoamines and metabolites were measured by HPLC–ED. These include the catecholamines, norepinephrine (NE), and dopamine (DA) and the indoleamine, serotonin (5-hydroxytryptamine; 5-HT). The metabolites included those of DA: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); and that of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA). The detector was a Unijet microbore electrochemical detector (Bioanalytical Systems) set at +700 mV which was equipped with a Unijet-compatible injector (Bioanalytical Systems). The gain of the recorder was set at 5 nA/V full scale. Five microliters of the filtered supernatant were injected onto a 150×1 mm I.D., C_{18} 5 μm reversed-phase microbore column (MF8912, Bioanalytical Systems).

The mobile phase consisted of 25 m*M* monobasic sodium phosphate, 50 m*M* sodium citrate, 27 μ*M* disodium EDTA, 10 m*M* diethylamine, 2.2 m*M* octane sulfonic acid and 10 m*M* sodium chloride with 3% methanol and 2.2% dimethylacetamide. The pH was adjusted to 3.2 using 85% *o*-phosphoric acid. The column was kept at ambient temperature and the flow-rate was kept at 70 μl/min (PM80 microbore pump, Bioanalytical Systems). The total run time was 24 min.

A stock solution (400 μg/ml) of the biogenic monoamines and metabolites were made up in 0.05 *M* perchloric acid. Three external standards (20, 40 and 80 pg were prepared using 0.05 *M* perchloric acid.

2.7. Assay precision and accuracy

Intra- and inter-assay ($n=5$ for each) were evaluated by spiking tissue homogenates with six different concentrations. The precision was expressed as a coefficient of variation (C.V.) by calculating the standard deviation as a percentage of the mean concentration found. The accuracy was expressed as a relative error (R.E.) by calculating the found concentration as a percentage of added concentration.

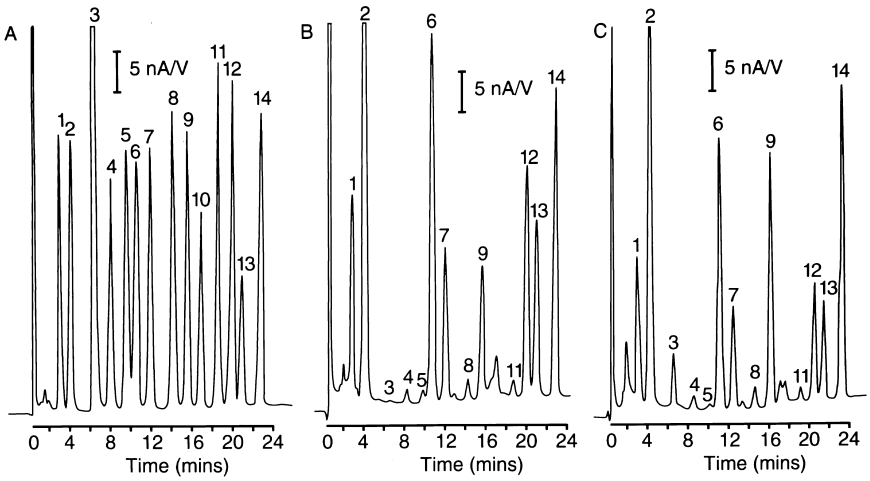

Fig. 2. Example of chromatograms of (A) amino acid/GABA standards (28.65 pmol each), (B) frontal cortex (somatosensory area) and (C) locus coeruleus. Tissue samples (B and C) were prepared as described in Section 2. All injections were 5 μl. 1=ASP, 2=GLU, 3=Thiol, 4=ASN, 5=HIS, 6=GLN, 7=SER, 8=ARG, 9=GLY, 10=THR, 11=TYR, 12=ALA, 13=TAU, 14=GABA.

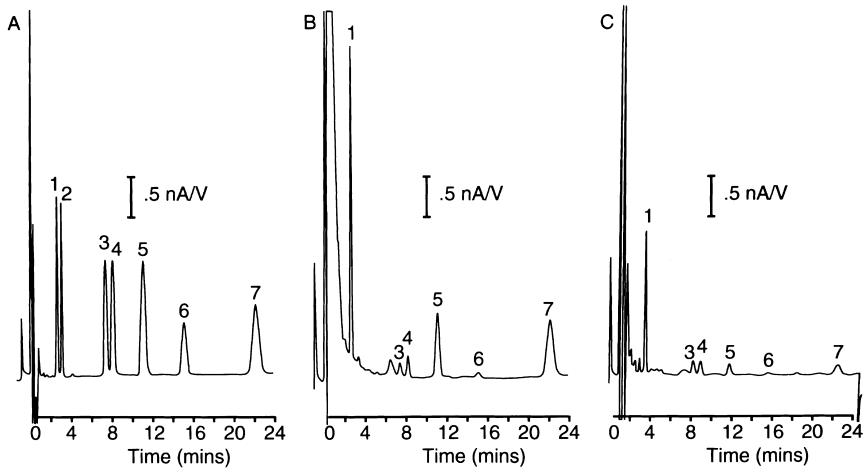

Fig. 3. Example of chromatograms of (A) biogenic monoamine standards (40 pg each), (B) frontal cortex (somatosensory area) and (C) locus coeruleus. Tissue samples (B and C) were prepared as described in Section 2. All injections were 5 μl. 1=NE, 2=EPI, 3=DOPAC, 4=DA, 5=5-HIAA, 6=HVA, 7=5-HT.

Table 1
Amino acids and GABA in selected brain regions[a]

Brain region	ASP	GLU	GLN	SER	GLY	ALA	TAU	GABA
Frontal cortex (Somatosen.)	46.7 ±2.2	125.3 ±4.9	63.9 ±4.8	17.7 ±1.0	15.0 ±0.9	14.7 ±0.8	58.6 ±2.6	29.2 ±0.8
Parietal cortex (Somatosen.)	43.5 ±1.9	128.5 ±3.3	71.6 ±4.8	17.0 ±1.0	14.7 ±0.8	14.6 ±0.5	73.4 ±4.0	30.7 ±1.2
Parietal cortex (Motor)	49.9 ±2.8	126.1 ±1.7	69.3 ±4.5	19.1 ±1.6	13.7 ±0.6	15.3 ±0.7	59.2 ±1.6	28.0 ±1.0
Cingulate Cortex	32.3 ±2.2	142.5 ±5.5	76.8 ±5.4	22.6 ±1.7	20.0 ±1.4	18.3 ±1.3	79.9 ±2.2	32.5 ±1.7
Striatum	28.3 ±1.8	135.9 ±6.3	98.5 ±3.4	33.2 ±3.3	25.7 ±2.7	21.7 ±1.3	103.0 ±1.9	28.7 ±1.0
Hippocampus	22.7 ±1.3	117.0 ±4.3	71.5 ±2.8	17.5 ±0.9	17.8 ±1.2	18.2 ±1.0	79.7 ±2.7	23.8 ±0.9
Substantia Nigra	35.8 ±1.9	65.2 ±1.7	62.0 ±3.8	15.1 ±1.2	28.3 ±1.8	9.5 ±0.5	46.2 ±1.8	130.2 ±4.3
Cerebellar Folia	24.2 ±1.7	117.5 ±4.3	82.0 ±6.1	13.9 ±1.1	11.5 ±0.5	11.1 ±0.6	67.3 ±1.8	28.3 ±1.8
Red nucleus	33.3 ±0.6	84.7 ±1.9	57.2 ±3.7	14.8 ±1.6	33.6 ±1.4	10.0 ±1.0	29.5 ±0.9	42.0 ±1.5
Locus Coeruleus	31.0 ±2.1	81.3 ±3.9	58.5 ±3.1	22.4 ±2.5	47.5 ±2.3	12.5 ±1.0	27.4 ±1.2	40.2 ±2.0

[a] $n=12$, concentrations (mean±S.E.M.)=nmol/mg protein.

3. Results

3.1. Chromatography

Examples of chromatographic separations of amino acids/GABA and biogenic monoamine/metabolite standards are shown in Fig. 2A and Fig. 3A respectively. Examples of separations of amino acids/GABA from tissue homogenates of the frontal cortex (somatosensory area) and the locus coeruleus are shown in Fig. 2B and C; and for biogenic monoamine/metabolite separations in Fig. 2C and Fig. 3C, respectively. There were no apparent interfering or coeluting peaks in the chromatograms except for THR in the brain homogenate samples.

Statistical evaluation (ANOVA) comparing the right and left side of each brain area indicated that there were no differences between the two sides among the amino acids and GABA (all $P>0.95$) or the biogenic amines/metabolites (all $P>0.89$). Since there were no differences between the right and left side tissue samples, the data from both sides were combined for each brain area for determination of means and standard errors [18]. The levels of the amino acids and GABA are presented in Table 1. Although all 12 amino acids were present in the brain regions sampled, five amino acids (ASN, HIS, ARG, THR and TYR) occurred in only trace amounts and so are not presented in the table. The levels of the biogenic amines and metabolites are presented in Table 2.

3.2. Assay validation

The intra- and inter-assay precision and accuracy for the amino acids are shown in Table 3. They were evaluated by five replicates of six different concentrations (114.60, 57.30, 28.65, 14.33, 7.16 and 1.80 pmol). The C.V. was generally below 6%. With few exceptions the R.E. was generally below 8% with the largest being about 14% at the lowest concentrations for some of the amino acids. These

Table 2
Biogenic amines and metabolites in selected brain regions[a]

Brain region	NE	DA	DOPAC	HVA	5-HT	5HIAA
Frontal cortex (Somatosen.)	5.527 ±0.207	0.415 ±0.021	0.286 ±0.029	0.351 ±0.033	3.953 ±0.139	4.312 ±0.168
Parietal cortex (Somatosen.)	5.353 ±0.110	0.600 ±0.060	0.221 ±0.028	0.297 ±0.056	3.115 ±0.142	3.381 ±0.183
Parietal cortex (Motor)	5.473 ±0.214	0.578 ±0.068	0.218 ±0.031	0.281 ±0.064	3.667 ±0.167	4.012 ±0.189
Cingulate Cortex	4.824 ±0.194	1.540 ±0.109	0.293 ±0.030	0.611 ±0.067	2.314 ±0.078	4.804 ±0.275
Striatum	N.d.	194.50 ±6.01	26.173 ±0.891	12.746 ±0.730	3.369 ±0.211	6.436 ±0.501
Hippocampus	6.304 ±0.183	0.608 ±0.101	n.d	n.d	3.965 ±0.197	5.308 ±0.211
Substantia Nigra	9.984 ±0.578	23.478 ±1.271	6.337 ±0.346	9.984 ±0.316	14.321 ±1.295	13.793 ±0.639
Cerebellar Folia	2.556 ±0.771	N.d	N.d.	N.d.	0.575 ±0.023	0.936 ±0.053
Red nucleus	12.187 ±0.405	3.288 ±0.403	2.205 ±0.189	2.328 ±0.180	13.846 ±0.904	18.604 ±0.945
Locus Coeruleus	49.745 ±1.755	5.447 ±0.562	2.934 ±0.341	1.687 ±0.365	8.564 ±0.273	8.261 ±0.489

[a] $n=12$, concentration±S.E.M.=ng/mg protein.
N.d.=not detectable.

Table 3
Precision and accuracy of amino acid assays

Analyte	Amount added (pmol)	Amount found (Mean±S.D) (pmol)	C.V. (%)	R.E. (%)
Intra-assay variability (n=5)				
ASP	114.60	113.33±4.62	4.1	−1.1
	57.30	54.13±2.19	4.0	−5.0
	28.65	28.58±0.17	0.6	−0.4
	14.33	14.05±0.27	1.9	−1.7
	7.16	6.85±0.29	4.2	−4.9
	1.80	1.75±0.14	8.0	−2.8
GLU	114.60	110.30±3.34	3.0	−3.8
	57.30	54.33±2.03	3.7	−5.2
	28.65	29.45±0.33	1.1	2.6
	14.33	14.23±0.39	2.7	−0.5
	7.16	7.18±0.09	1.3	−0.3
	1.80	1.70±0.06	3.5	−5.6
ASN	114.60	109.03±3.64	3.3	−4.9
	57.30	63.55±2.32	3.7	−10.9
	28.65	31.25±1.39	4.4	8.9
	14.33	15.08±0.73	4.8	5.5
	7.16	7.28±0.21	2.9	1.1
	1.80	1.68±0.11	6.5	−6.7
HIS	114.60	110.73±4.48	4.0	−3.4
	57.30	59.98±1.08	1.8	4.7
	28.65	29.75±1.49	5.0	3.7
	14.33	14.55±0.84	5.8	1.7
	7.16	6.63±0.38	5.7	−7.9
	1.80	1.73±0.05	2.9	−3.9
GLN	114.60	112.00±2.69	2.4	−2.3
	57.30	61.30±1.43	2.3	7.0
	28.65	30.06±1.01	3.4	4.7
	14.33	14.88±0.56	3.8	4.1
	7.16	7.23±0.23	3.2	0.4
	1.80	1.68±0.09	5.4	−6.7
SER	114.60	110.43±3.91	3.5	−3.6
	57.30	61.08±1.61	2.6	6.6
	28.65	31.75±1.05	3.3	10.6
	14.33	15.70±0.68	4.3	19.8
	7.16	7.33±0.24	3.3	1.8
	1.80	1.80±0.11	6.1	0.0
ARG	114.60	119.30±2.61	2.2	4.1
	57.30	60.28±1.55	2.5	5.2
	28.65	31.63±1.03	3.3	10.2
	14.33	14.55±0.48	3.3	1.7
	7.16	7.05±0.30	4.3	−2.1
	1.80	1.78±0.06	3.4	−1.1
GLY	114.60	121.43±3.36	2.8	6.0
	57.30	61.25±1.81	3.0	6.9
	28.65	31.93±1.20	3.8	11.3
	14.33	15.33±0.68	4.4	7.2
	7.16	7.23±0.23	3.2	0.4
	1.80	1.85±0.06	3.2	2.8

Table 3 (continued)

Analyte	Amount added (pmol)	Amount found (Mean±S.D) (pmol)	C.V. (%)	R.E. (%)
THR	114.60	101.90±0.84	0.8	−11.1
	57.30	58.55±0.65	1.1	2.2
	28.65	29.38±1.30	4.4	2.4
	14.33	13.93±0.71	5.1	−2.6
	7.16	6.58±0.24	3.6	−8.6
	1.80	1.65±0.12	7.3	−8.3
TYR	114.60	107.95±4.04	3.7	−5.9
	57.30	61.45±1.44	2.3	7.2
	28.65	30.95±0.89	2.9	7.8
	14.33	14.28±0.27	1.9	−0.1
	7.16	7.08±0.23	3.2	−1.7
	1.80	1.60±0.07	4.4	−11.1
ALA	114.60	115.50±1.03	0.9	0.7
	57.30	62.03±1.98	3.2	8.3
	28.65	29.58±1.44	4.9	3.2
	14.33	15.20±0.59	3.9	6.3
	7.16	7.20±0.25	3.5	0.0
	1.80	1.63±0.14	8.6	−9.4
TAU	114.60	117.03±2.52	2.2	2.0
	57.30	59.90±1.04	1.7	4.5
	28.65	30.13±0.67	2.2	5.0
	14.33	14.70±0.30	2.0	2.8
	7.16	7.35±0.18	2.4	2.1
	1.80	1.65±0.06	3.6	−8.3
GABA	114.60	122.00±2.63	2.2	6.4
	57.30	61.45±1.51	2.5	7.2
	28.65	30.85±0.78	2.5	7.5
	14.33	14.65±0.20	1.4	2.4
	7.16	7.35±0.16	2.2	1.4
	1.80	1.70±0.06	3.5	−5.6
Inter-assay variability (n=5)				
ASP	114.60	110.58±4.84	4.4	−3.5
	57.30	56.00±2.89	5.2	−2.3
	28.65	28.88±0.57	2.0	0.6
	14.33	14.85±0.49	3.3	3.8
	7.16	7.45±0.19	2.6	3.5
	1.80	1.60±0.09	5.6	−11.1
GLU	114.60	112.40±4.34	3.9	−2.0
	57.30	54.58±2.21	4.0	−4.7
	28.65	29.05±0.31	1.1	1.2
	14.33	14.60±0.47	3.2	2.1
	7.16	7.35±0.19	2.6	2.1
	1.80	1.70±0.04	2.4	−5.6
ASN	114.60	109.25±3.76	3.4	−4.7
	57.30	62.70±1.81	2.9	9.4
	28.65	28.90±0.30	1.0	0.7
	14.33	14.65±0.25	1.7	2.4
	7.16	7.10±0.31	4.4	−1.4
	1.80	1.55±0.09	5.8	−13.9

(Cont.)

Table 3 (continued)

Analyte	Amount added (pmol)	Amount found (Mean±S.D) (pmol)	C.V. (%)	R.E. (%)
HIS	114.60	109.48±3.76	3.4	−4.5
	57.30	60.18±1.22	2.0	5.0
	28.65	27.93±0.85	3.0	−2.7
	14.33	14.28±0.31	2.2	0.1
	7.16	7.03±0.08	1.1	−2.4
	1.80	1.55±0.09	5.8	−13.9
GLN	114.60	112.30±2.85	2.5	−2.0
	57.30	60.15±1.13	1.9	5.0
	28.65	29.05±0.28	1.0	1.2
	14.33	14.58±0.29	2.0	2.0
	7.16	7.30±0.14	1.9	1.4
	1.80	1.73±0.03	1.7	−3.9
SER	114.60	110.58±3.99	3.6	−3.5
	57.30	62.59±1.75	2.8	9.2
	28.65	29.00±0.20	0.7	1.0
	14.33	14.63±0.24	1.6	2.3
	7.16	7.35±0.18	2.4	2.1
	1.80	1.68±0.06	3.6	−6.7
ARG	114.60	119.30±2.61	2.2	4.1
	57.30	61.33±1.38	2.3	7.0
	28.65	28.85±0.06	0.2	0.5
	14.33	14.45±0.13	0.9	1.0
	7.16	7.20±0.11	1.5	0.0
	1.80	1.68±0.06	3.6	−6.7
GLY	114.60	121.28±3.43	2.8	5.8
	57.30	62.43±1.72	2.8	9.0
	28.65	29.08±0.39	1.3	1.3
	14.33	14.65±0.30	2.0	2.4
	7.16	7.30±0.17	2.3	1.4
	1.80	1.68±0.05	3.0	−6.7
THR	114.60	103.60±8.16	7.9	−9.6
	57.30	59.58±0.90	1.5	4.0
	28.65	26.80±1.51	5.6	−6.6
	14.33	13.95±0.43	3.1	−2.4
	7.16	6.95±0.18	2.6	−3.5
	1.80	1.85±0.03	1.6	2.8
TYR	114.60	109.20±4.85	4.4	−4.7
	57.30	60.80±1.53	2.5	6.1
	28.65	28.85±0.27	0.9	0.5
	14.33	14.70±0.30	2.0	2.8
	7.16	7.23±0.13	1.8	0.4
	1.80	1.63±0.08	4.9	−9.4
ALA	114.60	115.28±1.04	0.9	0.6
	57.30	63.03±1.93	3.1	10.0
	28.65	29.15±0.26	0.9	1.6
	14.33	14.70±0.23	1.6	2.8
	7.16	7.20±0.11	1.5	0.0
	1.80	1.68±0.14	8.3	−6.7

Table 3 (continued)

Analyte	Amount added (pmol)	Amount found (Mean±S.D) (pmol)	C.V. (%)	R.E. (%)
TAU	114.60	115.23±1.55	1.3	0.5
	57.30	57.23±2.54	4.4	0.1
	28.65	29.38±0.35	1.2	2.4
	14.33	14.98±0.23	1.5	4.8
	7.16	7.28±0.08	1.1	1.1
	1.80	1.60±0.07	4.4	−11.1
GABA	114.60	119.98±3.24	2.7	4.7
	57.30	58.38±1.53	2.6	1.9
	28.65	29.08±0.32	1.1	1.3
	14.33	14.83±0.21	1.4	3.7
	7.16	7.23±0.11	1.5	0.4
	1.80	1.63±0.06	3.7	−9.4

results indicate that the assay as performed here has acceptable error limits. The limit of quantification was about 1.80 pmol. Amounts of 1.00–1.50 pmol although detectable, exceeded 20% accuracy for most of the amino acids.

The intra- and inter-assay precision and accuracy for the biogenic amines are shown in Table 4. They were evaluated by analyzing five replicates of six different samples (80, 40, 20, 10, 5 and 2.5 pg per 5-μl injection). The C.V. (precision) was generally below 5% for all concentrations tested and the R.E. (accuracy) was also below 5% for the higher concentrations and below 10% for the lower concentrations indicating acceptable levels of error. Inspection of the chromatograms indicated that the limit of quantification is about 2.0–2.5 pg per 5-μl injection. Amounts below this level, although detectable, were not able to be reliably quantified and exceeded 20% accuracy.

4. Discussion

The chromatograms from both assays (Figs. 2 and 3) display that all the analytes of interest can be separated rapidly. The reproducibility and linear response of tissue samples spiked with varying amounts of calibration standards indicated that there were probably no significant interfering or coeluting substances from brain tissue homogenates (data not shown). The tissue levels of the amino acids/GABA and the biogenic monoamines/metabolites deter-

Table 4
Precision and accuracy of biogenic amine assays

Analyte	Amount added (pg/5 μl)	Amount found (mean±S.D.) (pg/5 μl)	C.V. (%)	R.E. (%)
Intra-assay variability (n=5)				
NE	80.0	79.40±0.72	0.9	−0.8
	40.0	39.86±0.30	1.0	−0.4
	20.0	20.88±0.71	3.4	0.4
	10.0	9.16±0.33	3.6	−8.4
	5.0	5.16±0.18	3.5	3.2
	2.5	2.32±0.07	3.0	−7.2
EPI	80.0	78.70±0.52	0.6	−1.6
	40.0	38.90±0.79	2.0	−2.8
	20.0	19.24±0.40	2.1	−3.8
	10.0	9.04±0.39	4.3	−9.6
	5.0	4.88±0.21	4.3	−2.4
	2.5	2.28±0.12	5.3	−8.8
DA	80.0	79.24±0.52	0.6	−1.0
	40.0	39.32±0.33	0.8	−1.7
	20.0	19.84±0.35	1.8	−0.8
	10.0	9.78±0.21	2.1	−2.2
	5.0	4.98±0.12	2.4	−0.4
	2.5	2.36±0.05	2.1	−5.6
DOPAC	80.0	76.52±0.94	1.2	−4.4
	40.0	38.60±0.47	1.2	−3.5
	20.0	19.22±0.29	1.5	−3.9
	10.0	9.34±0.23	2.5	−6.6
	5.0	5.10±0.22	4.3	2.0
	2.5	2.40±0.15	6.3	−4.0
HVA	80.0	75.70±1.16	1.5	−5.4
	40.0	38.60±0.44	1.1	−3.5
	20.0	19.36±0.20	1.0	−3.2
	10.0	9.42±0.16	1.7	−5.8
	5.0	4.74±0.17	3.6	−5.2
	2.5	2.44±0.10	4.1	−2.4
5-HT	80.0	76.42±1.48	1.9	−4.5
	40.0	38.68±0.76	2.0	−3.3
	20.0	19.16±0.33	1.7	−4.2
	10.0	9.56±0.38	4.0	−4.4
	5.0	5.00±0.20	4.0	0.0
	2.5	2.36±0.07	3.0	−5.6
5-HIAA	80.0	75.92±1.00	1.4	−5.1
	40.0	38.08±0.82	2.2	−4.8
	20.0	18.76±0.35	1.9	−6.2
	10.0	9.66±0.12	1.3	−3.4
	5.0	4.80±0.07	1.5	−4.0
	2.5	2.40±0.09	3.8	−4.0

Table 4 (continued)

Analyte	Amount added (pg/5 μl)	Amount found (mean±S.D.) (pg/5 μl)	C.V. (%)	R.E. (%)
Inter-assay variability (n=5)				
NE	80.0	82.48±0.83	1.0	3.1
	40.0	41.20±0.87	2.1	3.0
	20.0	21.56±0.43	2.0	7.8
	10.0	9.66±0.29	3.0	−3.4
	5.0	5.20±0.07	1.3	4.0
	2.5	2.38±0.07	2.9	−4.8
EPI	80.0	79.46±0.52	0.7	−0.7
	40.0	39.70±0.68	1.7	−0.8
	20.0	19.24±0.73	3.8	−3.8
	10.0	9.12±0.36	3.9	−8.8
	5.0	4.74±0.14	3.0	−5.2
	2.5	2.27±0.12	5.3	−9.2
DA	80.0	79.76±0.67	0.8	−0.3
	40.0	39.70±0.45	1.1	−0.8
	20.0	20.10±0.24	1.2	0.5
	10.0	9.96±0.12	1.2	−0.4
	5.0	5.06±0.07	1.4	1.2
	2.5	2.44±0.19	7.8	−2.4
DOPAC	80.0	74.92±1.34	1.8	−6.4
	40.0	38.84±0.37	1.0	−2.9
	20.0	18.72±0.38	2.0	−6.4
	10.0	9.44±0.18	1.9	−5.6
	5.0	5.26±0.07	1.3	5.2
	2.5	2.28±0.09	3.9	−8.8
HVA	80.0	74.96±1.32	1.8	−6.3
	40.0	38.88±0.78	2.0	−2.8
	20.0	19.10±0.36	1.9	−4.5
	10.0	9.30±0.22	2.4	−7.0
	5.0	5.04±0.07	1.4	−0.8
	2.5	2.29±0.10	4.4	−8.4
5-HT	80.0	77.52±0.98	1.3	−3.1
	40.0	39.10±0.56	1.4	−2.3
	20.0	18.80±0.33	1.8	−6.0
	10.0	9.30±0.29	3.2	−8.0
	5.0	4.82±0.17	3.5	−3.6
	2.5	2.38±0.10	4.2	−4.8
5-HIAA	80.0	76.68±1.13	1.5	−4.2
	40.0	39.38±0.82	2.5	−1.6
	20.0	18.20±0.74	4.1	−9.0
	10.0	9.18±0.24	2.6	−8.2
	5.0	4.86±0.13	2.7	−2.8
	2.5	2.44±0.10	4.1	−2.4

mined by our procedure are in agreement with others [6,9,16,18–23].

There is at least one other report on the chromatographic conditions by which biogenic monoamines/metabolites and amino acids/GABA can be simultaneously measured, but there were no details on tissue handling protocols [7]. The methodology described in this paper includes an easy-to-follow procedure for the handling of very small tissue samples and microliter quantities of homogenates, while conserving sample amounts and preventing sample degradation. The information for the derivatization of amino acids for HPLC–ED and protein analysis can serve as a reference point which can be generalized to the handling of other brain tissue samples.

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