

High-performance liquid chromatographic determination of selected amino acids in rat brain by precolumn derivatization with phenylisothiocyanate

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SUMMARY

We describe here a simple, sensitive, selective and reproducible assay method for quantitative determination of aspartate, glutamate, serine, glutamine, glycine and γ -aminobutyric acid in rat brain using reversed-phase high-performance liquid chromatography. The method is based upon formation of phenylthiocarbamyl derivatives of the amino acids. Good resolution of the six amino acids and the internal standard norvaline is achieved within 40 min. Other amino acids which have been reported to be present in rat brain do not interfere with the analysis. Standard curves for each of the amino acids exhibited good linearity ($r > 0.9993$) over the range 0.5–20 nmol. The coefficient of variation for the intra-day and inter-day determinations ranged from 0.4% at the highest to 11% at the lowest concentration limit. Storage of whole brains at -0°C for up to 8 weeks did not affect mean concentrations of the six amino acids.

INTRODUCTION

The role of amino acids in mammalian central nervous system synaptic transmission is an area of increasing scientific interest. γ -Aminobutyric acid (GABA) has been shown to be the primary inhibitory neurotransmitter in the mammalian brain, while glutamate and aspartate have excitatory actions at many sites^{1–3}. Glycine acts as an inhibitory transmitter in the spinal cord, but in brain it facilitates excitation arising from N-methyl-D-aspartate receptor activity⁴. Glutamine, while not thought to play any direct role in synaptic transmission, is a precursor of both glutamate and GABA. As investigations into these amino acids have increased, so too has interest in means of assaying their concentrations in physiological samples.

Automated amino acid analysis based on separation with sulfonated cation-exchange resins, postcolumn derivatization with ninhydrin and colorimetric detec-

tion⁵ has been widely used for over 20 years. A need for expensive dedicated equipment, long assay times and large sample volumes have led investigators to search for other amino acid assay methods.

Heinrikson and Meredith⁶ introduced a liquid chromatographic method for the separation and quantification of several amino acids after precolumn derivatization with phenylisothiocyanate (PITC). This method has been used for the analysis of primary and secondary amino acids in protein hydrolysates^{7,8} and for the determination of asparagine and glutamine in physiological fluids and cells⁹. However, aspartate, glutamate, serine, glutamine, glycine and GABA in rat brain extracts cannot be separated adequately when using methods described for protein hydrolysates^{7,8}. In another method based on PITC derivatization, the running time necessary to achieve separation was 80 min⁹.

This paper describes an analytical assay method for the quantitative determination of aspartate, glutamate, serine, glutamine, glycine and GABA in rat brain using precolumn derivatization with PITC and separation by reversed-phase high-performance liquid chromatography (HPLC). We developed this assay method to study changes in amino acid neurotransmitter concentrations in rat brain during experimental status epilepticus¹⁰.

EXPERIMENTAL

Materials

Amino acid and PITC were purchased from Pierce (Rockford, IL, U.S.A.). Triethylamine was obtained from Aldrich (Milwaukee, WI, U.S.A.). Glacial acetic acid was purchased from Fisher (Fairlawn, NJ, U.S.A.), sodium acetate trihydrate from Sigma (St. Louis, MO, U.S.A.) and ethanol from EM Science (Cherry Hill, NJ, U.S.A.). Acetonitrile (HPLC grade) was purchased from Mallinckrodt (Paris, KY, U.S.A.) and HPLC-grade water was obtained using a Nanopure reagent-grade water system from Barnstead (Boston, MA, U.S.A.).

Preparation of standard solutions

Aspartate, glutamate, serine, glutamine, glycine, taurine and GABA (0.5 mM each) were dissolved in 0.1 M hydrochloric acid and stored at 4°C for no longer than 1 week. The internal standard, norvaline (1 mM), was dissolved in 0.1 M hydrochloric acid and stored at 4°C for no more than 3 months. Norvaline was chosen as the internal standard because it is less hydrophilic than most other amino acids and, therefore, elutes late enough not to interfere with the endogenous amino acids in the brain.

Solvent A was 0.1 M sodium acetate buffer (pH 5.7) prepared by adding 13.61 g of sodium acetate trihydrate to 1 l of water, followed by 0.5 ml of triethylamine, 0.7 ml of glacial acetic acid and 5.0 ml of acetonitrile. Solvent B was acetonitrile–water (60:40).

Animal procedure and sample preparation

Male Sprague–Dawley rats (130–170 g) were decapitated. The brains were rapidly removed and frozen in acetone and dry ice (interval from decapitation to freezing averaged 60.7 s with a standard deviation of 17.1 s). Brains were weighed and homogenized in cold 80% ethanol (18 ml/g brain weight). Homogenization was

carried out in glass tissue grinders (Corning Pyrex No. 7725, New York, NY, U.S.A.), using a power drive for 5 min. The homogenate was centrifuged at 15 000 g for 15 min at 4°C. The supernatant was transferred to a glass culture tube and stored on ice. The pellet was rehomogenized and centrifuged as before. The combined supernatants from both extractions were vortexed thoroughly and stored at 4°C overnight. Supernatant samples of 50 μ l were derivatized and assayed after extraction as described in the next section.

To study the stability of brain amino acids, whole brains, extraction supernatants and phenylthiocarbonyl (PTC) derivatives were stored at -70°C for 0, 1, 2, 4 and 8 weeks before assay. Six brains were stored for each of the stated times, then extracted, derivatized and assayed. Six brains were immediately extracted and the extraction supernatant was divided into five aliquots for storage for the stated times. Six brains were immediately extracted and six 50- μ l aliquots of each were derivatized, with assay following storage for the stated times.

Derivatization of brain extract and standard curves

The internal standard, norvaline (5 nmol), was added to 50 μ l of brain extract in a 75 \times 10 mm borosilicate test tube and dried under vacuum. The residue was dissolved in 20 μ l of ethanol-water-triethylamine (2:2:1) and evaporated to dryness under vacuum. A 30- μ l volume of ethanol-water-triethylamine-PITC (7:1:1:1) was added to the residue and allowed to react for 20 min at room temperature to form the PTC derivatives of the amino acids⁷. Excess reagent was then removed under vacuum. For injection into the chromatograph, the PTC amino acid derivatives were dissolved in 300 μ l of solvent A.

Standard curves for aspartate, glutamate, serine, glutamine, glycine and GABA were determined in a similar manner using mixtures of amino acids in six different amounts (0.5, 1, 2, 5, 10 and 20 nmol). The concentration of individual amino acids in brain samples was determined from the standard curve by calculating the ratio of the peak area of each amino acid to that of the internal standard.

Apparatus and chromatographic conditions

An Altex (Berkeley, CA, U.S.A.) HPLC system which consisted of two Model 110A pumps, a Model 421 controller and a Shimadzu (Columbia, MD, U.S.A.) Model C-R6A integrator was used. Absorbance was monitored with a Gilson (Middleton, WI, U.S.A.) Model 116 variable-wavelength detector at 254 nm, with sensitivity set at 0.1 a.u.f.s. A Rheodyne (Cotati, CA, U.S.A.) 7125 valve with a 20- μ l loop was used for injection. The separation was carried out in a reversed-phase system with a Rainin (Emeryville, CA, U.S.A.) Dynamax C₁₈ Microsorb column as the stationary phase (250 \times 4.6 mm I.D., particle size 5 μ m) and a mobile phase gradient system modified from Cohen *et al.*⁸ and described in Table I. The flow-rate of the mobile phase was 0.6 ml/min. A total running time of 60 min is needed between sample injections to allow the system to equilibrate adequately.

RESULTS

Separation of selected amino acids

Fig. 1 shows a typical chromatogram of a standard mixture containing 5 nmol each of aspartate, glutamate, serine, glutamine, glycine, taurine and GABA after

TABLE I
GRADIENT PROGRAM FOR PTC AMINO ACID ANALYSIS

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
2.0	85	15
8.0	85	15
12.0	75	25
20.0	75	25
24.0	55	45
32.0	55	45
37.0	0	100
42.0	0	100
43.0	100	0
60.0	100	0

derivatization with PITC. Fig. 2 shows a chromatogram of a PTC-derivatized sample formed from 50 μ l of rat brain extract. In both cases 5 nmol norvaline were added to the samples prior to derivatization with PITC. As can be seen from the chromatograms, good resolution of the six amino acids and the internal standard was achieved in less than 40 min. The standard curves for each of the six amino acids exhibited good linearity over the range 0.5–20 nmol. The resulting correlation coefficients ranged from 0.9993 (glycine) to 0.9999 (GABA). Each standard concentration mixture was assayed five times on the same day. Precision between days was determined from standards that were derivatized and assayed on five separate days. The coefficient of variation ranged from 0.4 to 11.1% for within-day and from 1.5 to 11.0% for between-day peak-area ratio determinations. Table II shows the precision for within-day and between-day determinations of peak-area ratios in samples containing 0.5 or 20 nmol of amino acid standards. When peak area was used, the coefficient of variation (C.V.) for the within-day and between-day determinations ranged from 13.7 to 27.8% and 10.8 to 19.4%, respectively.

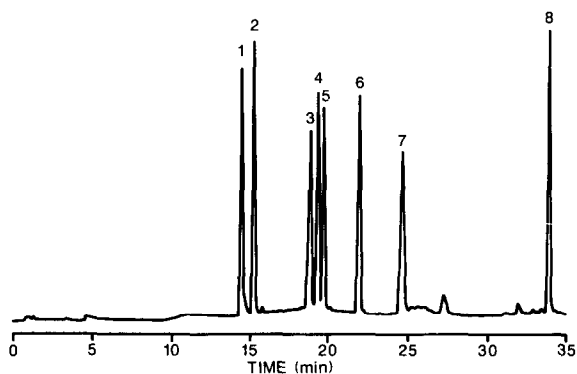


Fig. 1. Chromatographic separation of an amino acid standard mixture after derivatization with PITC. Peaks: 1 = aspartate; 2 = glutamate; 3 = serine; 4 = glutamine; 5 = glycine; 6 = taurine; 7 = GABA; 8 = norvaline. Each peak represents 5 nmol of compound.

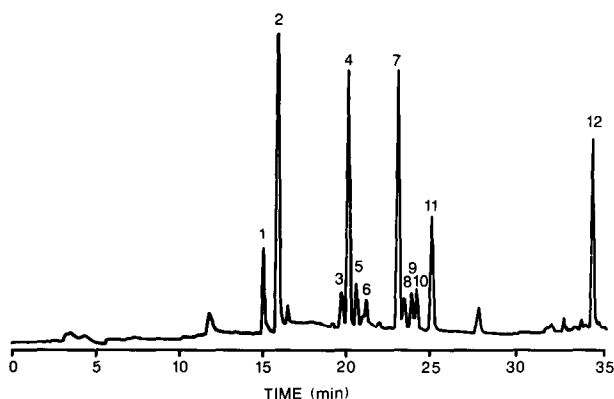


Fig. 2. Chromatographic separation of amino acids obtained from rat brain after extraction with 80% ethanol and derivatization with PITC. Norvaline (5 nmol) was added as internal standard. Peaks: 1 = aspartate; 2 = glutamate; 3 = serine; 4 = glutamine; 5 = glycine; 6 = glycerophosphoethanolamine; 7 = histidine and taurine; 8 = glutathione; 9 = threonine and proline; 10 = alanine and arginine; 11 = GABA; 12 = norvaline.

Interference

Interference studies were performed using other amino acids found in rat brain¹¹ plus cysteine, urea and ammonia.

Fig. 3 shows a chromatogram of 31 compounds after derivatization with PITC. A complete separation of all compounds was not attempted, but none of the compounds exhibited interference with aspartate, glutamate, serine, glutamine and glycine. Ammonia did not form a detectable PTC derivative. Histidine coeluted with taurine. Phosphoethanolamine formed a broad peak which eluted together with taurine, histidine, glutathione, threonine and proline. Methionine and cystathionine were not baseline-resolved from the internal standard and homocarnosine eluted closely to GABA. However, the concentrations of methionine, cystathionine and homocarnosine in rat brain are sufficiently low¹¹ so that interference which might be caused by these amino acids would be negligible. We confirmed this assumption by a separate experiment, in which two 50- μ l aliquots from the same rat brain extract sample were prepared and derivatized with PITC, one without (Fig. 4A) and one with (Fig. 4B) the addition of 5 nmol norvaline as the internal standard. Methionine and cystathionine were present in negligible amounts in the sample without norvaline. Fig. 5 shows two chromatograms obtained from rat brain extract, one without (Fig. 5A) and one with (Fig. 5B) the addition of 5 nmol homocarnosine. It can be seen that homocarnosine did not interfere with GABA and formed a negligible peak in the sample to which additional homocarnosine was not added.

Stability studies

Stability of amino acid concentrations when brain, extraction supernatant or PTC derivatives were stored at -70°C for up to 8 weeks was analyzed by least-squares analysis of variance (using a repeated measures design for extraction supernatant and PTC derivatives). When whole brains were stored, no statistically significant concentration differences were found secondary to storage time for the six selected

TABLE II
WITHIN-DAY AND BETWEEN-DAY PRECISION OF THE AMINO ACID ASSAY

Amino acid	Within-day precision			Between-day precision		
	Amount (nmol)	Mean peak-area ratio ^a	C.V. (%)	Amount (nmol)	Mean peak-area ratio ^a	C.V. (%)
Aspartate	0.5	0.07	11.1	0.5	0.06	11.0
	20.0	3.43	0.8	20.0	3.46	4.2
Glutamate	0.5	0.06	7.4	0.5	0.07	3.2
	20.0	3.47	0.5	20.0	3.54	5.4
Serine	0.5	0.07	2.1	0.5	0.06	4.3
	20.0	3.48	0.4	20.0	3.49	4.1
Glutamine	0.5	0.07	2.9	0.5	0.07	6.4
	20.0	3.61	0.5	20.0	3.62	1.5
Glycine	0.5	0.07	3.0	0.5	0.07	10.1
	20.0	3.71	0.6	20.0	3.77	5.2
GABA	0.5	0.07	5.1	0.5	0.07	9.5
	20.0	3.58	1.5	20.0	3.50	4.2

^a Defined as the ratio of amino acid peak area to that of the internal standard, norvaline. Each value is the mean of five assays.

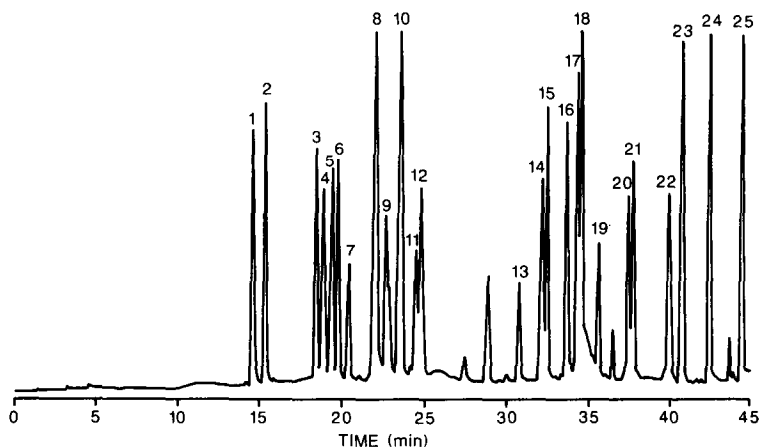


Fig. 3. Chromatogram of 31 amino acids and organic compounds found in rat brain after derivatization with PITC (5 nmol of each compound was used). Peaks: 1 = aspartate; 2 = glutamate; 3 = asparagine; 4 = serine; 5 = glutamine; 6 = glycine; 7 = glycerophosphoethanolamine; 8 = histidine and taurine; 9 = glutathione, threonine and proline; 10 = arginine and alanine; 11 = homocarnosine; 12 = GABA; 13 = hypotaurine; 14 = ethanolamine; 15 = tyrosine; 16 = valine; 17 = norvaline (internal standard); 18 = methionine and cystathionine; 19 = cysteine; 20 = isoleucine; 21 = leucine; 22 = phenylalanine; 23 = ornithine; 24 = lysine; 25 = urea. Phosphoethanolamine formed a broad peak and coeluted with taurine, histidine, glutathione, threonine and proline. Identities of unnumbered peaks is unknown.

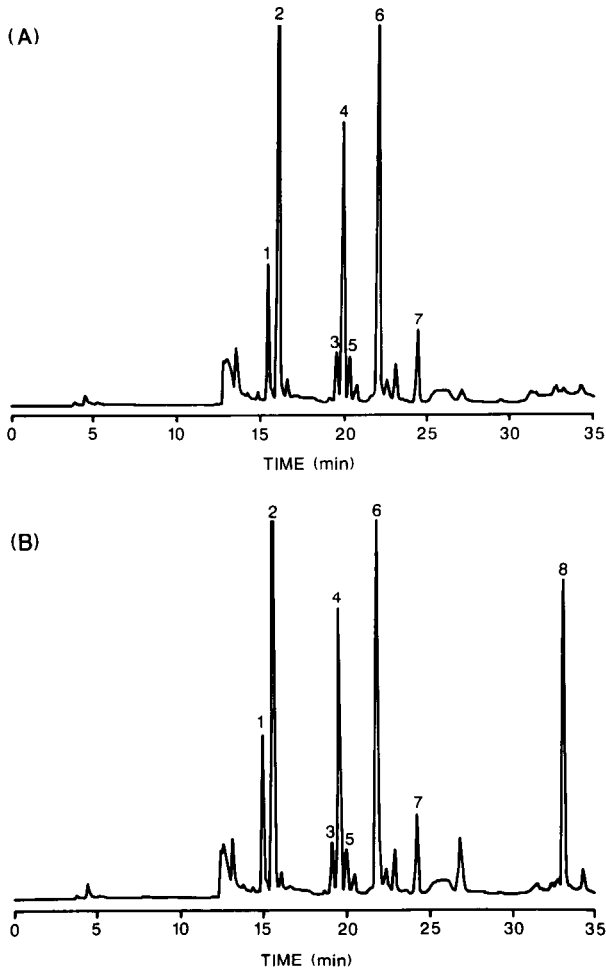


Fig. 4. Chromatograms obtained from rat brain extract after derivatization with PITC, one without (A) and one with (B) the addition of 5 nmol norvaline as internal standard. Peaks: 1 = aspartate; 2 = glutamate; 3 = serine; 4 = glutamine; 5 = glycine; 6 = histidine and taurine; 7 = GABA; 8 = norvaline.

amino acids ($p > 0.05$ in all cases). These results are shown graphically in Fig. 6. Stability was poorer when PTC derivatives or extraction supernatants were stored. Serine increased significantly when PTC derivatives were stored while glutamine decreased ($p < 0.05$). Aspartate and GABA rose significantly during storage of extraction supernatant ($p < 0.001$ and 0.05 , respectively), while glycine decreased ($p < 0.05$).

The mean amino acid concentrations found in the brains of the combined control (no storage) groups are presented in Table III.

DISCUSSION

Concentrations of amino acids in rat brain determined by the method described

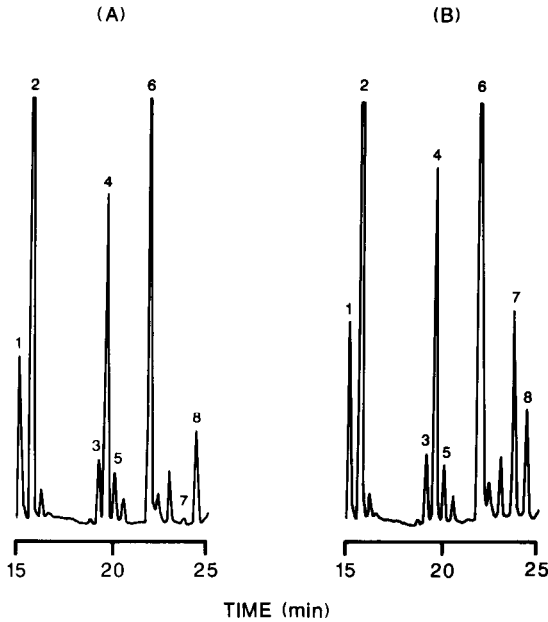


Fig. 5. Chromatograms obtained from rat brain extract after derivatization with PITC, one without (A) and one with (B) the addition of 5 nmol homocarnosine. Peaks: 1 = aspartate; 2 = glutamate; 3 = serine; 4 = glutamine; 5 = glycine; 6 = histidine and taurine; 7 = homocarnosine; 8 = GABA.

here were similar to values which have been reported by other method, including automated ion-exchange analysis and HPLC using other precolumn derivatization techniques. Table III shows how similar mean amino acid concentrations in rat brain determined by our method are compared to the classical technique using postcolumn derivatization with ninhydrin in an amino acid analyzer^{11,12}. In Table IV amino acid concentrations in rat hippocampus are compared with reports from the literature using other derivatization methods. Results using our method of PITC derivatization¹⁰ are similar and comparable to those obtained by HPLC methods using other precolumn derivatization techniques^{13,14}. It is difficult to compare results across experiments because, in addition to differences in analytical technique, variation in anatomic sample definition, sample preparation and species used can also change brain amino acid concentrations.

We believe our methodology has several advantages. The equipment required is relatively inexpensive and can be used for numerous other analytical procedures besides amino acid analysis. The derivatization is rapid and simple. Since the coupling reagent and solvent are volatile and readily removed by evaporation under reduced pressure, interference from reagent peaks is minimized. By employing a UV-absorbing rather than a fluorescence-active coupling reagent the instability as well as quantification difficulties which are associated with these derivatives is avoided.

Histidine and phosphoethanolamine elute closely enough to taurine that separation is not possible using this method. However, by changing the triethylamine concentration¹⁵ or altering the pH of the buffer used, it may be possible to optimize the system with regard to quantification of taurine or other amino acids which may be of

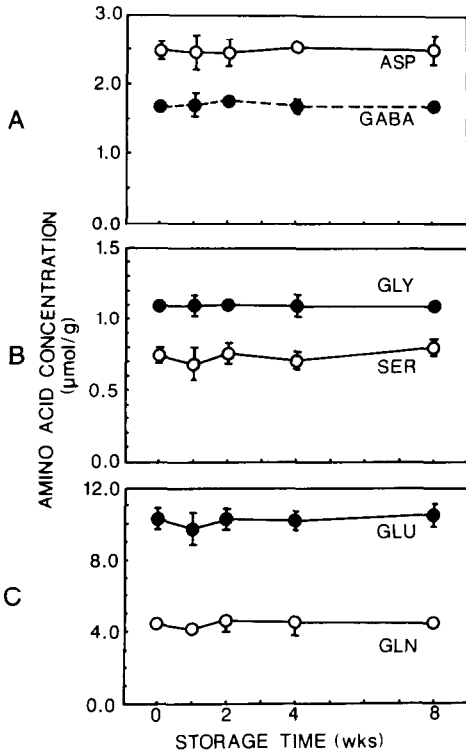


Fig. 6. Amino acid concentrations following storage of rat brains at -70°C prior to extraction, derivatization and assay. Each point is the mean of six brains \pm standard deviation.

interest. Although methionine and cystathionine may interfere with norvaline (see Fig. 3), their concentrations in rat brain, as reported by Perry *et al.*¹¹, are too low to interfere significantly. This is supported by the two chromatograms shown in Fig. 4. By using a relatively high concentration of the internal standard, norvaline (5 nmol), interference from methionine and cystathionine became negligible. None of the other amino acids reported to be present in rat brain, or urea or ammonia, interferes with the amino acid analysis described here.

Although Henrikson and Meredith⁶ and Bidlingmeyer *et al.*⁷ reported that PTC derivatives of protein hydrolysates are stable when stored dry and frozen, we found only intact rat brain (but not brain extracts or PTC derivatives) to be stable during storage at -70°C . Biological samples may include compounds which reduce stability when stored.

The methodology described here provides a relatively simple, rapid and inexpensive method for study of changes in brain amino acid levels associated with experimental variables of interest to the investigator. As is true with any analytical method applied to physiological problems, care in design of experiments is required to tease effects of interest out of the normal variability found in physiological samples.

TABLE III

CONCENTRATIONS OF SELECTED AMINO ACIDS IN RAT BRAIN AFTER DERIVATIZATION WITH PITC COMPARED TO METHODS USING NINHYDRIN WITH AMINO ACID ANALYZER

Amino acid	Concentration (mean \pm standard error of the mean) ($\mu\text{mol/g brain}$)		
	PITC (n=18)	Ninhydrin ¹¹ (n=7)	Ninhydrin ¹² (n=4)
Aspartate	2.42 \pm 0.04	2.60 \pm 0.13	3.73 \pm 0.13
Glutamate	10.42 \pm 0.14	11.29 \pm 0.37	9.77 \pm 0.22
Serine	0.73 \pm 0.02	0.84 \pm 0.03	1.12 \pm 0.05
Glutamine	4.51 \pm 0.07	4.51 \pm 0.15	3.91 \pm 0.26
Glycine	1.10 \pm 0.02	1.06 \pm 0.04	1.04 \pm 0.05
GABA	1.75 \pm 0.03	1.79 \pm 0.07	1.26 \pm 0.05

TABLE IV

CONCENTRATIONS OF SELECTED AMINO ACIDS IN RAT HIPPOCAMPUS AFTER VARIOUS PRECOLUMN DERIVATIZATION METHODS

Amino acid	Concentration (mean \pm standard error of the mean) ($\mu\text{mol/g brain}$)		
	PITC ¹⁰ (n=6)	<i>o</i> -Phthalaldehyde ¹³ (n=8)	Dansyl chloride ¹⁴ (n=5)
Aspartate	1.98 \pm 0.10	1.28 \pm 0.06	3.89 \pm 0.40
Glutamate	9.23 \pm 0.35	7.36 \pm 0.21	11.33 \pm 0.63
Serine	0.96 \pm 0.07	—	—
Glutamine	3.85 \pm 0.12	3.92 \pm 0.17	4.64 \pm 0.60
Glycine	0.81 \pm 0.02	0.37 \pm 0.12	—
GABA	1.83 \pm 0.07	2.45 \pm 0.21	2.03 \pm 0.08

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