Chem. Pharm. Bull. 26(9)2684—2688(1978)

UDC 547.466.2.08 : 543.544.2.061

Liquid Chromatographic Determination of Amino Acids in the Rat Brain using *o*-Phthalaldehyde as Fluorogenic Reagent[1]

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(Received February 10, 1978)

Liquid chromatography with *o*-phthalaldehyde as a fluorogenic reagent and sodium citrate as an elution buffer was applied to the assay of taurine, aspartic acid, glutamic acid, glycine, and γ-aminobutyric acid in the rat brain. These amino acids were quantified at picomol level. This sensitive assay procedure and microdissection technique of Palkovits were used to measure their content in the substantia nigra and cerebral cortex in the brain of rats killed by decapitation or microwave exposure.

Keywords——taurine; aspartic acid; glutamic acid; glycine; GABA; *o*-phthalaldehyde; liquid chromatography; substantia nigra; cerebral cortex; microwave exposure

Taurine, aspartic acid, glutamic acid, glycine, and γ-aminobutyric acid (GABA) are putative neurotransmitters in a central nervous system. Examination of the regional distribution of these amino acids in the brain after the administration of a centrally acting agent should reveal their role in the brain function. Measurement of their content in a small amount of brain tissue requires a highly sensitive analytical method.

Because of high solubility in water, rapid reaction at room temperature, and high sensitivity, *o*-phthalaldehyde has been used as a fluorogenic reagent for the analysis of amino acids by high pressure liquid chromatography with a microbore column packed with a cation-exchange resin.[3]

Meek[4] succeeded in measuring less than 50 pmol of taurine, GABA, and 5-hydroxytryptophan in the rat brain by eluting the column with a constant pH perchloric acid solution, using high pressure liquid chromatography with *o*-phthalaldehyde.

In the present work, a liquid chromatography system with *o*-phthalaldehyde as a fluorogenic reagent and sodium citrate as an elution buffer was used for the analysis of taurine, aspartic acid, glutamic acid, glycine, and GABA. The standard amino acid mixture must be run at frequent intervals during quantitative analysis to compensate for the change of fluorescence intensity affected by aging of xenon lamp and *o*-phthalaldehyde reagent. It is desirable to shorten the time involved in one analysis. In addition, stable baseline on the chromatogram must be obtained at high sensitivity settings of the detector. Therefore they were divided into three groups of (1) taurine, (2) aspartic acid, glutamic acid and glycine, and (3) GABA, to be eluted separately at constant pH.

Optimum conditions for the separation and quantification were established for each group. The procedure and its application to their analysis in the substantia nigra and cerebral cortex are reported, herein.

Materials and Methods

Materials——*o*-Phthalaldehyde was obtained from E. Merck AG., Darmstadt, Germany. 2-Mercaptoethanol purified for biochemical analysis was from Wako Pure Chemical Industries, Osaka. Amino acids

1) Presented at the 3rd Symposium on Analytical Chemistry for Biological Substances, Tokyo, Japan, November, 1977.
2) Location: *1-5-8, Hatanodai, Shinagawa-ku, Tokyo, 142, Japan.*
3) *a*) J.R. Benson and P.E. Hare, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 619 (1975); *b*) M. Roth, *J. Clin. Chem. Clin. Biochem.*, **14**, 361 (1976); *c*) J.R. Cronin and P.E. Hare, *Anal. Biochem.*, **81**, 151 (1977).
4) J.L. Meek, *Anal. Chem.*, **48**, 375 (1976).

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were of the highest purity commercially available. EtOH was distilled twice and all other chemicals were used without further purification.

Liquid Chromatography——Liquid chromatographic system was assembled from two pumps, a water-jacketed column, a circulating water bath, and a fluorescence spectrophotometer. Hitachi Model 633 liquid chromatograph was used to pump the elution buffer. Samples were applied by a microsyringe through a sample injection valve (for high pressure; Hitachi) to a stainless steel column (2.6×250 mm) packed with 13 μm bead-diameter cation-exchange resin CK10S (Mitsubishi Kasei, Tokyo). The column temperature was maintained at 55° with a Hitachi Model 634 circulating water bath temperature controller. For detection of amino acids, the column effluent was mixed with *o*-phthalaldehyde reagent. The reagent was prepared as follows: To a solution of 50 mg of *o*-phthalaldehyde dissolved in 0.5 ml of EtOH, 100 ml of 0.5 M sodium borate buffer (pH 10.3) containing 200 μl of 2-mercaptoethanol was added. The reagent was driven by Kyowa Seimitsu mini-pump at a flow rate of 0.15 ml/min and mixed with the column effluent in a 3-way manifold. The mixture was allowed to react at room temperature in 1 m length of Teflon tubing (0.3 mm i.d., 1.5 mm o.d.). The fluorescence was monitored, with excitation at 330 nm and emission at 450 nm, using a Hitachi Model 204-A fluorescence spectrophotometer equipped with 75 μl quartz flow cell and an Ushio xenon discharge lamp.

Table I. Conditions for Liquid Chromatography

Group of amino acids	1 Taurine	2 Aspartic acid glutamic acid glycine	3 GABA
Elution buffer (sodium citrate)			
Na^+ concentration (M)	0.05	0.2	0.2
pH	2.20	3.24	5.14
flow rate (ml/min)	0.16	0.17	0.25
Column temperature (°C)	Room temp.	55	55

Table I shows conditions for the analysis. $HClO_4$ extract of up to 10 μl was applied to the column without further treatment. After four or five analyses were carried out, the elution buffer was allowed to run for about 1 hr to flatten the baseline. Then next samples were analyzed. When the elution buffer was changed to analyze the amino acids in another group, the resin was regenerated with 0.2 N NaOH containing 1 mM EDTA.

Individual amino acids were quantitated by relating their chromatographic peak height to the peak height from known amount of standard amino acid.

Tissue Preparation——Rat Whole Brain: After decapitation, the brain was immediately removed, weighed, and homogenized in 20 volumes of cold 0.4 N $HClO_4$ solution containing 1 mM EDTA with Polytron (20 vol. homogenate). After the homogenate was centrifuged at 13000 rpm for 15 min in a refrigerated centrifuge, the supernatant was stored frozen until assayed. Preliminary studies did not show any changes in the content of amino acids for one month under storage. The content of amino acids was determined by assuming 80% of the weight of the brain to be liquid and expressed in μmol/g wet weight.

Rat Substantia Nigra and Cerebral Cortex: Male Wistar rats weighing 240—280 g were decapitated and their brain was immediately removed and frozen on dry ice. The microdissection technique of Palkovits was used.[5] Coronal sections, 300 μm in thickness, were made in a cryostat (Lipshow 1900) at −10 to −15°. The substantia nigra and cerebral cortex were identified by reference to the atlas of König and Klippel,[6] and punched out from sections using a sharpened stainless steel needle (0.7 mm i.d.). The tissue was blown out from the needle into 100 μl of cold 0.4 N $HClO_4$ solution containing 1 mM EDTA in a 6×50 mm sample tube and sonicated 5 sec. After being kept in an ice basket for 30 min, the homogenate was centrifuged at 10000 rpm for 12 min. The supernatant was stored frozen until assayed. According to the procedure described by Lowry, *et al.*,[7] the precipitate was dissolved in 100 μl of 1 N NaOH at 100° for 30 min and then the protein content was measured using crystalline bovine serum albumin as a standard. The content of amino acids was expressed in pmol/μg protein. In other studies, rats were killed by microwave exposure for 2.5 sec at 2 kW (Metabostat NJE 2601; New Japan Radio Co., Ltd.). The brain was cautiously removed from the skull and the serial procedure was carried out as mentioned above.

5) M. Palkovits, *Brain Res.*, **59**, 449 (1973).
6) J.F.R. König and R.A. Klippel, "The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem," R.E. Krieger Publishing Co., Inc., New York, 1967.
7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

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Results and Discussion

Figures 1, 2, and 3 show the separation profiles of a mixture of standard amino acids and perchloric acid extract of the whole brain.

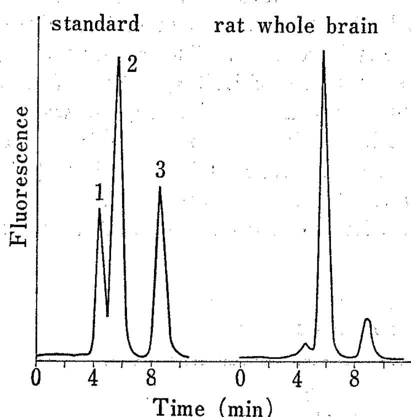

Fig. 1. Separation of Equimolar Mixture of Standard Cysteic Acid (1), Taurine (2), and Phosphoethanolamine (3) dissolved in 0.4 N Perchloric Acid containing 1 mM EDTA (left) and Perchloric Acid Extract of Rat Whole Brain (right)

The column was eluted with 0.05 M sodium citrate buffer (pH 2.20) at 0.16 ml/min at room temperature.

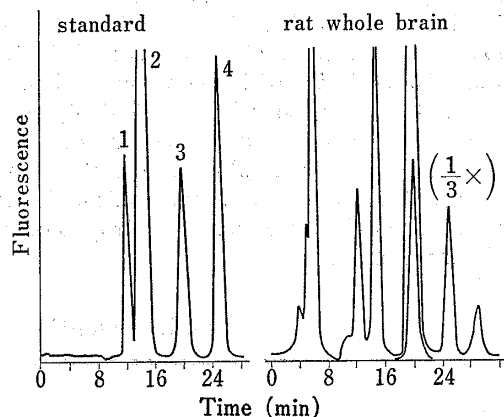

Fig. 2. Separation of Equimolar Mixture of Standard Aspartic Acid (1), Glutamine (2), Asparagine (2), Serine (2), Threonine (2), Glutamic acid (3), and Glycine (4) dissolved in 0.4 N Perchloric Acid containing 1 mM EDTA (left) and Perchloric Acid Extract of Rat Whole Brain (right)

The column was eluted with 0.2 M sodium citrate buffer (pH 3.24) at 0.17 ml/min at 55°.

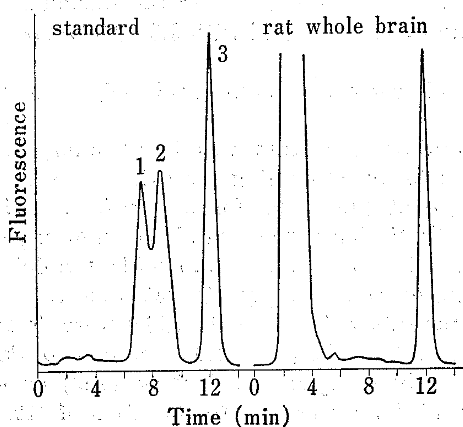

Fig. 3. Separation of Equimolar Mixture of Standard Tyrosine (1), Phenylalanine (2), and GABA (3) dissolved in 0.4 N Perchloric Acid containing 1 mM EDTA (left) and Perchloric Acid Extract of Rat Whole Brain (right)

The column was eluted with 0.2 M sodium citrate buffer (pH 5.14) at 0.25 ml/min at 55°.

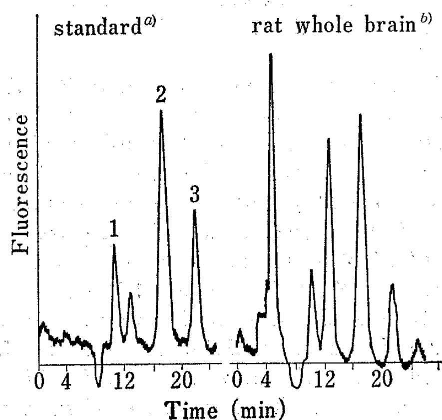

Fig. 4. Chromatogram of Small Amounts of Amino Acids

a) A mixture of 10 pmol of standard aspartic acid (1) and glycine (3) and 30 pmol of glutamic acid (2) dissolved in 10 μl of 0.4 N perchloric acid containing 1 mM EDTA.
b) The supernatant of 20-vol. homogenate of rat whole brain was diluted 80 fold and 5 μl of it was applied to the column.

Figure 4 shows the chromatograms for a standard mixture containing small amounts of aspartic acid, glutamic acid, and glycine and for the diluted extract of the whole brain. Because of the high S/N ratio, about 10 pmol each of amino acids was sufficiently measured.

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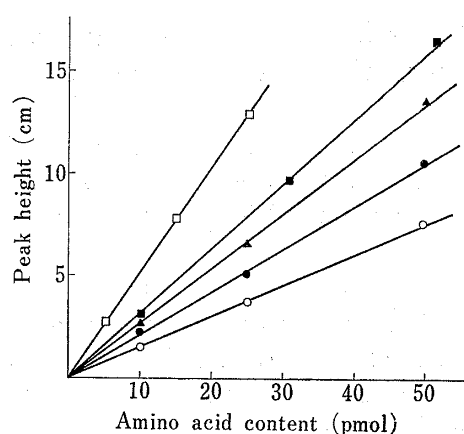

Fig. 5. Plot of Content of Standard Amino Acid *vs.* Peak Height

Each point represents the mean value of three assays.
□ taurine, ■ GABA, ▲ aspartic acid, ● glycine, ○ glutamic acid.

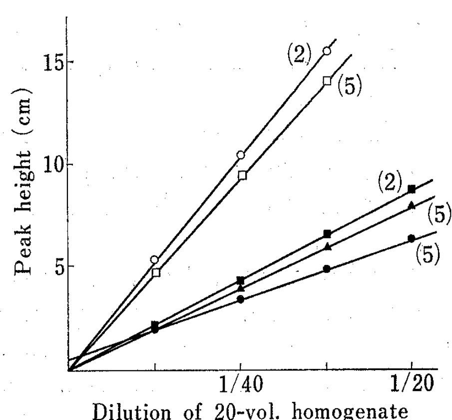

Fig. 6. Peak Height of Amino Acid in Various Dilutions of Homogenate

Each point represents the mean value obtained from the number of assays indicated in parentheses. Five-μl supernatant was used for the analysis of taurine, 10 μl for others. The peak heights of amino acids found in 1/40 dilution correspond to 31.4, 41.5, 124.8, 21.6, and 28.3 pmol for taurine, aspartic acid, glutamic acid, glycine, and GABA, respectively.
□ taurine, ■ GABA, ▲ aspartic acid, ● glycine, ○ glutamic acid.

Cronin and Hare[3c] reported that fluorescence yield with amino acids declined with increasing age of xenon lamp and *o*-phthalaldehyde reagent. In the experiment to find the relationship between peak height and the amino acid content, the samples were analyzed by using all the same reagents within a day. Figure 5 shows the plot of the standard amino acid content *vs.* peak height. A linear relationship was obtained for 10 to 50 pmol of aspartic acid, glutamic acid, glycine, and GABA, and for 5 to 25 pmol of taurine.

The 20 vol. homogenate of the whole brain was diluted 20 to 80 fold. After the diluted homogenate was centrifuged, 5 or 10 μl of each supernatant was assayed. Straight lines were obtained by plotting the peak height of the amino acid *vs.* degree of dilution of the homogenate (Fig. 6). Only the straight line for glycine had a small value of *y*-intercept. The linearity allowed determination of the content of each amino acid in the brain tissue by comparing its peak height with that of known amount of standard amino acid, which was

Table II. Amino Acid Content (mean ± S.E.) of the Rat Whole Brain and Recovery of Known Amount of Standard Amino Acid added to the Homogenate

(*n*)	Rat whole brain (μmol/g wet weight) (3)	Recovery (%)[a] 40-vol. (6)	Recovery (%)[a] 100-vol. (5)
Taurine	5.27±0.14	94.7±0.7	95.2±1.6
Aspartic acid	2.73±0.17	85.1±3.1	89.9±2.1
Glutamic acid	10.60±0.15	90.9±2.2	95.3±3.7
Glycine	1.44±0.08	89.4±2.5	89.0±3.6
GABA	3.02±0.05	93.6±3.0	91.1±2.2

a) Homogenates of 40-vol. and 100-vol. are prepared by diluting 20-vol. homogenate of the whole brain with 0.4 N perchloric acid solution containing 1 mM EDTA, 2-fold and 5-fold, respectively. Amounts of standard amino acids added to 1 ml of 40 vol. were: taurine 125 nmol, aspartic acid 62.5 nmol, glutamic acid 125 nmol, glycine 37.5 nmol, and GABA 62.5 nmol. To 1 ml of 100-vol., two-fifths of those in 40-vol, were added.

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injected every three or four runs of samples. In determining the glycine content, a standard mixture which had almost the same concentration of glycine as the sample was used to minimize the error.

Table II shows the content of amino acids in the whole brain and the recovery of known amounts of standard amino acids added to the homogenate of the whole brain. The recovery was almost independent of the dilution of homogenates and showed little adsorption of amino acids to proteins.

To elucidate the utility of this assay, amino acids in the substantia nigra and cerebral cortex were measured. The steady state values of GABA in the brain depend on the way of killing rats and the microwave exposure seems to be the best method to inactivate enzymic processes and to prevent postmortem changes. Thus, two methods of sacrifice, decapitation and microwave exposure, were employed for the determination of the content of amino acids in the substantia nigra and cerebral cortex. The results are given in Table III.

Table III. Mean Amino Acid Contents (pmol/μg protein ± S.E.) in the Substantia nigra and Cerebral Cortex of Rats killed by Decapitation or Microwave Exposure

(n)	Substantia nigra		Cerebral Cortex	
	Decapitation (5)	Microwave exposure (7)	Decapitation (5)	Microwave exposure (7)
Taurine	63.3±7.0	52.8± 2.7	87.6±7.4	86.4±4.0
Aspartic acid	47.3±4.0	38.8± 1.7	42.1±1.2	34.9±2.2[a]
Glutamic acid	63.4±2.7	93.8± 3.0[b]	155.8±6.5	141.6±5.5
Glycine	36.3±4.4	43.7± 4.1	26.9±2.9	30.0±2.5
GABA	162.3±4.2	67.4±12.8[b]	26.5±1.6	21.7±2.6

a) $0.01 < p < 0.05$.
b) $p < 0.01$.

A significant difference was found in GABA and glutamic acid content in the substantia nigra. The decapitation method gave a lower content of glutamic acid than microwave exposure, whereas GABA content was about 2.5-fold higher by the decapitation method. Tappaz, *et al.*[8] stated that GABA content found in the substantia nigra pars reticularis was 116±5 or 25±3 pmol/μg proteins in the rats killed by decapitation or microwave exposure, respectively, and that the increase in the content of GABA was directly related to the activity of L-glutamate decarboxylase. Our results substantiated their report, indicating that the increase of GABA content was accompanied with the decrease of glutamic acid, precursor of GABA, in the substantia nigra. Accordingly, we conclude that the microwave exposure method is suitable for the analysis of amino acids in the brain, especially GABA and glutamic acid.

Because of much simplicity and high sensitivity, the present method is applicable to the quantitation of amino acids in small brain tissues.

8) M.L. Tappaz, M.J. Brownstein, and I.J. Kopin, *Brain Res.*, **125**, 109 (1977).

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