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DETERMINATION OF 4-AMINOBUTYRIC ACID, ASPARTATE, GLUTAMATE AND GLUTAMINE AND THEIR ^{13}C STABLE-ISOTOPIC ENRICHMENT IN BRAIN TISSUE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A selected-ion monitoring method was developed for measuring 4-aminobutyric acid, aspartate, glutamate, and glutamine in brain tissue. Natural isotopes of these amino acids and their stable-isotopic enrichment following intravenous infusion of a precursor, [^{13}C]glucose, were quantitated. Frozen mouse brain tissue was homogenized in cold 80% ethanol, and the supernatant, equivalent to 1 mg of wet weight brain tissue, was extracted using solid-phase bonded silica ion-exchange columns. Aspartate and glutamate (dicarboxylic acids) were isolated from strong anion-exchange columns, whereas 4-aminobutyric acid and glutamine (neutral amino acids) were isolated from strong-cation exchange columns. *n*-Butyl ester pentafluoropropionyl amide derivatives of these amino acids were analyzed by gas chromatography–mass spectrometry using a methane positive chemical ionization mode after gas chromatographic separation on a wide-bore, fused-silica capillary column. The method is applicable to determination of brain concentrations of these amino acids as well as their fluxes following administration of a stable-isotopic tracer.

INTRODUCTION

Several amino acids are considered to be neurotransmitters in the mammalian central nervous system [1–3]. Aspartate (ASP) and glutamate (GLU), dicarboxylic acids, exhibit excitatory activity [4] and 4-aminobutyric acid (GABA), a neutral amino acid, exhibits inhibitory activity [5]. All three of these neurotransmitter amino acids have been implicated in the pathogenesis or potential treatment of epilepsy [6–9]. These three, and glutamine (GLN), another neutral amino acid, are also closely related metabolically [10,11]. Amino acid concentrations by themselves represent a static situation, and compartmentation of

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amino acids may mask the actual dynamic changes taking place. Therefore, fluxes and turnovers of amino acids may be more representative of the on-going active processes. Neurotransmitter turnover appears to be a better index of neuronal activity than neurotransmitter concentrations [12–14]. Fluxes and turnovers can be determined following incorporation of a label from a labeled precursor [12,14,15]. Administration of [^{13}C]glucose results in incorporation of $^{13}C_2$ into neurotransmitter amino acids in the brain, probably through acetate via the tricarboxylic acid cycle [16].

Both amino acid analyzer [17,18] and high-performance liquid chromatographic (HPLC) [19–21] methods are commonly used for quantitation of these amino acids. Neither of these methods, however, has the sensitivity or inherent specificity of selected-ion monitoring or the ability to simultaneously quantify naturally occurring and isotopically enriched substances.

Bertilsson and Costa [22] reported one-step derivatization of glutamate, GABA, and their stable-isotopically enriched counterparts by forming the hexafluoroisopropyl ester pentafluoropropionyl amide products for gas chromatographic–mass spectrometric (GC–MS) analysis. However, this procedure has the drawback of yielding multiple and unstable derivatives [23,24]. Acid-catalyzed esterification of the carboxylic moiety of amino acids is commonly employed for GC–MS analysis, but the derivatization causes deamidation of glutamine to glutamate [23,25,26], therefore yielding only a sum concentration of these two amino acids rather than individual concentrations. Frank et al. [27] reported a GC method for glutamate and glutamine analysis in cerebrospinal fluid in which glutamine quantitation was based on a cyclic intermediate generated during the conversion of glutamine into glutamate.

MacKenzie and Tenaschuk [25], in a qualitative GC method, avoided the deamidation problem by forming N(O)-*tert.*-butyl dimethylsilyl derivatives of aspartate, glutamate, and glutamine from plants. Another approach to circumventing the deamidation problem is separation of glutamine from glutamate prior to derivatization by using ion-exchange chromatography [28]. Darmann et al. [26] used anion-exchange chromatography to separate glutamine from glutamate in plasma or blood prior to forming N-acetyl-*n*-propyl derivatives for GC–MS analysis. Corradetti et al. [23] described a method for analyzing GABA, aspartate, glutamate, and glutamine and their stable-isotopic enrichment in brain slice superfusates using ion-exchange chromatography to separate dicarboxylic from neutral amino acids followed by formation of methyl ester, pentafluoropropionyl derivatives and selected-ion monitoring.

Unfortunately, none of the above methods was specifically designed to quantify or study isotopic incorporation of GABA, aspartate, glutamate, and glutamine in mammalian tissue. The present study, therefore, was designed to develop a relatively simple and reliable method to quantitate all four amino acids in mammalian brain tissue using GC–MS for isotopic incorporation studies.

EXPERIMENTAL

Chemicals

Natural isotopes of L-amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). The following deuterated amino acids were obtained from MSD Isotopes

(St. Louis, MO, U.S.A.): [2,2-$^2H_2$]4-aminobutyric acid (98 atom % ^2H), [2,2,3,3,4,4-$^2H_6$]4-aminobutyric acid (99 atom % ^2H), [2,3,3-$^2H_3$]L-aspartic acid (98.3 atom % ^2H), [2,3,3,4,4-$^2H_5$]L-glutamic acid (99.2 atom % ^2H), [2,4,4-$^2H_3$]DL-glutamic acid (98 atom % ^2H), and [2,3,3,4,4-$^2H_5$]L-glutamine (98.7 atom % ^2H). [$^{13}C_6$]D-Glucose (98.6 atom % ^{13}C) was also from MSD Isotopes. Pentafluoropropionic anhydride and ethyl alcohol were purchased from Pierce (Rockford, IL, U.S.A.) and 3.0 *M* hydrochloric acid in *n*-butanol was obtained from Regis (Morton Grove, IL, U.S.A.). Deionized water was obtained using the Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Organic solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Other chemicals were of the best commercially available grades.

Animals

Male NIH general purpose mice (25–30 g) were used. [$^{13}C_6$]D-Glucose (22.4 μmol in 0.1 ml saline) or saline (0.1 ml) was administered intravenously via the tail vein by constant infusion over 5.5 min. The mice were decapitated and the heads were cooled rapidly by swirling in physiological saline at $-0.5°C$ for 2 min to minimize postmortem changes [29]. The brain was then excised and placed on a chilled aluminum plate kept on ice. A cortical slice (25–50 mg) was quickly dissected from each hemisphere, weighed and frozen immediately in a dry ice–acetone bath. Tissues were stored at $-20°C$.

Extraction

Tissue samples were homogenized in cold 80% ethyl alcohol (100 μl per mg wet tissue weight) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.). After centrifugation, the supernatant was transferred to clean tubes and stored in the refrigerator. Solid-phase, bonded silica ion-exchange columns (Bond-Elut, Analytichem International, Harbor City, CA, U.S.A.) were used for separation of the dicarboxylic amino acids from the neutral amino acids. The 1-ml cartridges contained 100 mg of sorbent in the form of a strong anion-exchange packing (SAX; trimethylaminopropyl) or a strong cation-exchange packing (SCX; benzenesulfonylpropyl). The SAX and SCX columns were conditioned by sequential washing with 1 ml of each of pentane, methanol, and deionized water. In addition, SAX columns in the chloride form (higher selectivity) were converted into the acetate form (lower selectivity) by washing twice with 1 ml of 1 *M* sodium hydroxide, four times with 1 ml of deionized water, twice with 1 ml of 2 *M* acetic acid, and finally four times with 1 ml of deionized water. The conditioning of the ion-exchange columns was performed using a vacuum manifold (Vac-Elut, Analytichem International). The following manipulations (sample applications, washes, and elutions), however, were performed with a slow, constant positive air pressure applied manually using a 35-ml syringe.

For each sample, 200 μl of 80% ethyl alcohol were combined with 100 μl of 0.1 *M* acetic acid. To this were added 100 μl of supernatant, and the mixture was mixed briefly by vortexing. Then 25 μl of aqueous solution containing the internal standards (250 ng each of [$^2H_6$]GABA and [$^2H_5$]GLN and 750 ng each of [$^2H_3$]ASP and [$^2H_5$]GLU) were added and the samples were mixed again. The

mixture was applied to SAX columns and the unretained analyte was collected in tubes containing 150 μl of 0.1 *M* hydrochloric acid. The SAX columns were washed with 1 ml of deionized water and the wash was collected in the same tubes as the unretained solution.

The combined unretained analyte and washes from SAX columns were applied to SCX columns, which subsequently were also washed with 1 ml of deionized water. The SAX and SCX colums were eluted with 500 μl of 2 *M* hydrochloric acid in methanol.

Derivatization

Eluates were evaporated in vacuo using a Savant Speed Vac (Savant Instruments, Farmingdale, NY, U.S.A.). To the residue were added 100 μl of 3 *M* hydrochloric acid in *n*-butanol, and the samples were placed in a water-bath at 70°C for 90 min. The samples were then evaporated, 100 μl of pentafluoropropionic anhydride were added to the residue, and the samples were placed in a water-bath at 60°C for 30 min. The samples were evaporated again, the residue was dissolved in 100 μl of ethyl acetate, and 1–2 μl of the derivatives were used for each injection.

Gas chromatography–mass spectrometry

A Model 5987A gas chromatograph–mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) was equipped with a Model 5880A gas chromatograph and 1000E data system. A 15 m×0.52 mm I.D. cross-linked and bonded 5% phenylmethyl (1.5-μm film) DB-5 fused-silica capillary Megabore column (J&W Scientific, Rancho Cordova, CA, U.S.A.) was used for chromatographic separation. It was installed in a packed column inlet fitted with a direct flash vaporization liner. The flow from the column to the source of the spectrometer was via a direct transfer line. The carrier and reagent gas was methane adjusted to a flow-rate of 7 ml/min. The source pressure was ca. 700 mTorr, and the source temperature was 200°C. The filament current was 300 μA and the electron energy was 200 eV. The injection port and transfer line temperature was 260°C. The oven temperature was held at 130°C for 2 min and then programmed at 25°C/min to 170°C.

Quasi-molecular ions were monitored for the natural abundance isotopes of ASP (*m/z* 392), GLU (*m/z* 406), GABA (*m/z* 306), and GLN (*m/z* 406). In addition, appropriate ions were monitored for deuterated compounds and isotopes of interest. A dwell-time of 50 ms was used for each mass, and ions were monitored in three isotope groups: GABA (1.20–2.50 min), ASP (2.50–4.20 min), and GLU or GLN (4.20–5.00 min). Amino acid concentrations were determined based on the peak-height ratio (standard to internal standard) for a known amount of standard determined concurrently.

Extraction efficiency

The amino acid extraction efficiency was determined for corresponding deuterium-labeled analogues in the presence of brain supernatant. This allowed determination of extraction efficiency from the actual biological matrix. It was desirable to ascertain the percentage recovery of each amino acid on both the SAX and SCX columns in order to determine if there was any cross-contamina-

TABLE I

EXTRACTION EFFICIENCY FOR AMINO ACIDS

Percentage recovery was determined for deuterated amino acids added to supernatant from mouse cortex. Each value represents the mean of duplicate experiments. See text for details.

Amino acid	Recovery (%)	
	SAX column	SCX column
GABA	<0.5	81.6
ASP	97.2	<0.5
GLU	94.0	<0.5
GLN	<0.5	87.0

tion. The deuterated analyte was extracted and its peak-height ratio was normalized to the non-extracted internal standard, which was added to the column eluate. This ratio was compared with that obtained for an eluate spiked with non-extracted deuterated analyte and internal standard. For example, for determination of GABA recovery, $[^2H_2]$GABA was used as deuterated analyte in the presence of endogenous GABA and $[^2H_6]$GABA was used as the internal standard. A similar approach was used for the other amino acids.

RESULTS AND DISCUSSION

A relatively simple procedure was developed for quantitation of ASP, GLU, GABA, and GLN in the brain. After homogenization of brain tissue in 80% ethyl alcohol, the supernatant was extracted in series, first by the SAX and then by the SCX columns. The dicarboxylic acids, ASP and GLU, were eluted from the SAX columns and the neutral amino acids, GABA and GLN, from the SCX columns. The extraction efficiency was good for all four amino acids and there was no detectable cross-contamination between the SAX and SCX columns (Table I). The dicarboxylic acids were found exclusively on the SAX column and the neutral amino acids on the SCX columns.

Solid-phase, bonded silica ion-exchange columns were used in lieu of more traditional polystyrene resin ion-exchange columns (Dowex AG 1 and AG 50). The former offer several advantages over the latter, requiring smaller solution volumes and shorter equilibration times, avoiding the swelling phenomenon and permitting dry column storage.

n-Butyl ester pentafluoropropionyl derivatives were chosen over their shorter methyl ester pentafluoropropionyl congeners, because of anticipated lower volatility and, therefore, less likely loss on evaporation. These derivatives with higher mass ions and elution at higher GC temperatures can also improve specificity. Using chemical ionization instead of electron impact MS allowed monitoring of higher masses, which can also improve specificity and sensitivity. Since acid-catalyzed esterification causes deamidation, it was necessary first to separate GLN from GLU, and although GLU and GLN were analyzed as GLU, they were quantitated separately.

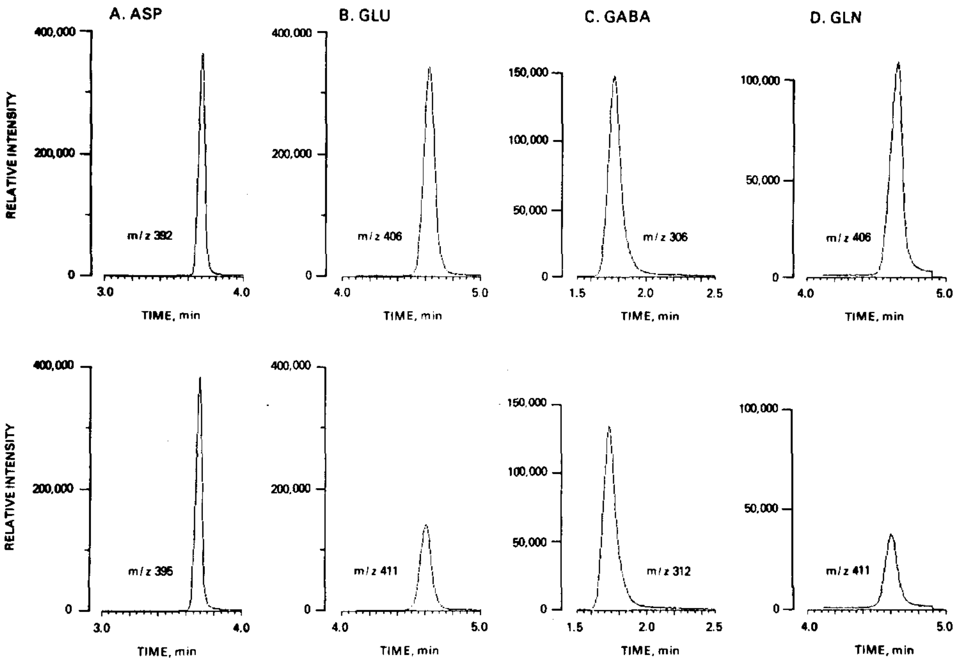

Fig. 1. Selected-ion chromatograms of (A) ASP, (B) GLU, (C) GABA, and (D) GLN representing endogenous amino acids (upper tracings) and their corresponding deuterated internal standards (lower tracings). The equivalent of 1 mg of wet tissue weight was extracted after the addition of internal standards (750 ng each of [$^2H_3$]ASP and [$^2H_5$]GLU and 250 ng each of [$^2H_6$]GABA and [$^2H_5$]GLN). Chromatograms A and B represent eluates from SAX columns and chromatograms C and D represent eluates from SCX columns. The mouse cortex sample was shown to contain 770 ng of ASP, 1690 ng of GLU, 215 ng of GABA, and 666 ng of GLN. See text for details.

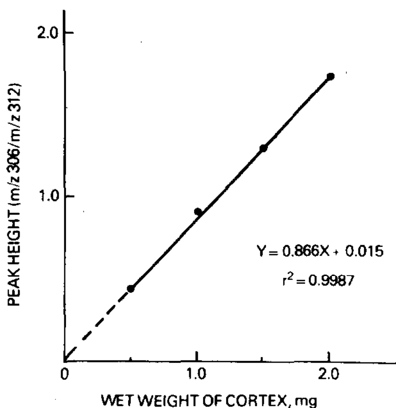

Fig. 2. Relationship between the amount of GABA and the sample size. Different volumes of supernatant were extracted and analyzed for endogenous GABA (*m/z* 306) after the addition of the internal standard, [$^2H_6$]GABA (*m/z* 312). The line was drawn and the equation of the line was determined using a linear least-squares method. See text for details.

TABLE II

AMINO ACID CONCENTRATIONS IN MOUSE BRAIN CORTEX

Amino acid	Concentration (mean ± S.D.) (μmol/g wet tissue weight)	
	GC-MS*	HPLC**
GABA	2.15±0.27	2.41±1.27
ASP	5.80±0.51	3.52±0.72
GLU	11.23±0.92	10.73±2.12
GLN	5.44±1.79	5.28±1.70

*SIM method with male NIH general purpose mice (see text); $n=4$.
**Data from Westerberg et al. [19], who used an HPLC method with male Swiss mice; $n=7$.

GC separation was achieved using a wide-bore, fused-silica capillary column. This type of column offers a compromise between the inherent advantages of packed and narrow capillary columns in terms of capacity, resolution, inertness, and speed of analysis.

Representative selected-ion chromatograms of mouse cortical extracts containing ASP, GLU, GABA, and GLN, and the corresponding internal standards, are shown in Fig. 1. The identity of the peaks was also confirmed by obtaining electron-impact, methane positive chemical ionization and methane negative chemical ionization mass spectra for the peaks. The spectra (not shown) were consistent with the mass spectra reported for the same and analogous derivatives [23,30].

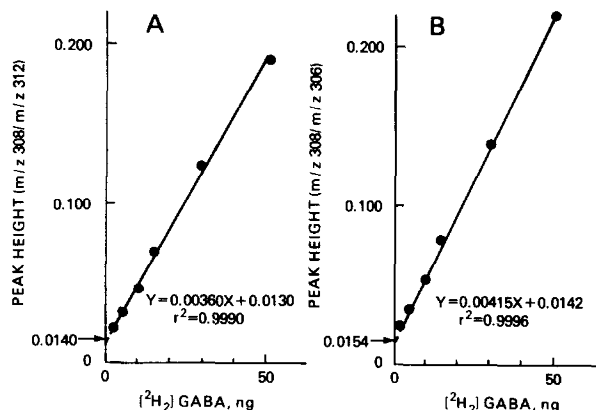

Fig. 3. Relationship between the amount detected and the amount added of [$^{2}H_2$]GABA. Supernatant from mouse cortex containing 240 ng of endogenous GABA was spiked with various amounts of [$^{2}H_2$]GABA (2.5-50.0 ng). A constant amount (250 ng) of the internal standard, [$^{2}H_6$]GABA, was also added to all samples. The data were plotted after normalizing the response of [$^{2}H_2$]GABA (*m/z* 308) to either (A) the internal standard (*m/z* 312) or (B) the endogenous GABA (*m/z* 306). The arrows show the corresponding ratios determined for the same sample in the absence of exogenous [$^{2}H_2$]GABA. The line was drawn and the equation of the line was determined using a linear least-squares method. See text for details.

TABLE III

ISOTOPIC ENRICHMENT OF AMINO ACIDS IN BRAIN CORTEX FOLLOWING ADMINISTRATION OF LABELED GLUCOSE TO MICE

Saline (0.1 ml) with and without labeled glucose (24.2 µmol) was administered via the tail vein by constant infusion over 5.5 min. Each value represents the mean ± S.D. of four male NIH general purpose mice. The values for the [$^{13}C_6$]glucose group are different from the saline-infused control group, $p < 0.01$.

Infusate	(M+2)/M			
	GABA	ASP	GLU	GLN
Saline	0.0142±0.0008	0.0259±0.0006	0.0269±0.0005	0.0274±0.0003
[$^{13}C_6$] Glucose	0.0394±0.0087	0.0347±0.0022	0.0594±0.0046	0.0368±0.0020

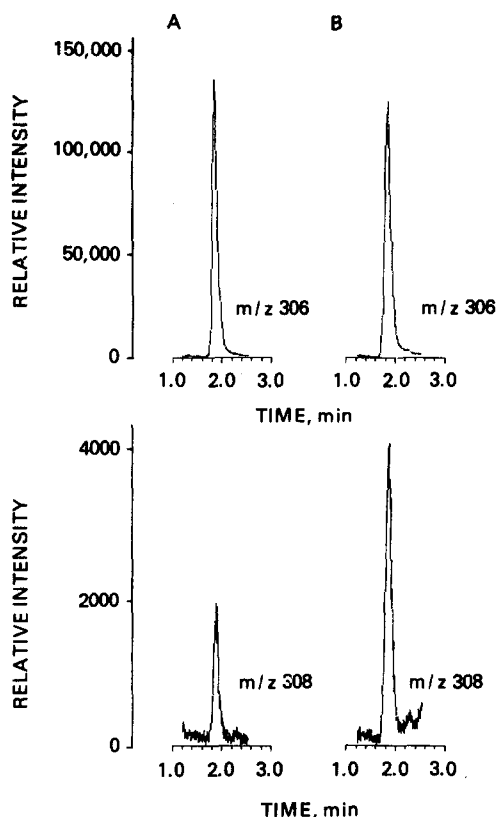

Fig. 4. Selected-ion chromatograms for GABA extracted from (A) saline-infused and (B) [$^{13}C_6$]glucose-infused (22.4 µmol per 5.5 min) mice representing natural isotopic abundance of GABA (upper tracings) and the M+2 isotopes (lower tracings). See text for details.

The relationship between the amount of tissue extracted and the amount of amino acid detected was examined by extracting different volumes of supernatant in the presence of the same amount of internal standard. The equivalent of 0.5–2.0 mg of wet tissue weight was analyzed. Good linearity over this range was shown for all four amino acids, as illustrated for GABA (Fig. 2).

An apparent loss of deuterium from deuterated amino acids, most notably ASP, in the presence of biological matrix was avoided under the above extraction conditions.

The amino acid levels determined in mouse cortex by the above method are summarized in Table II. They were found comparable with values reported by Westerberg et al. [19], who used an entirely different method.

Commercially available [$^{2}H_{2}$]GABA was used to examine the linearity between the amount of M+2 isotopic enrichment, where M represents the nominal mass of the quasi-molecular ion and the peak-height ratio response. Various amounts of [$^{2}H_{2}$]GABA were used to spike mouse cortex supernatant to which the internal standard was also added. The peak height of the M+2 isotope was normalized either to M due to endogenous [$^{2}H_{0}$]GABA or to M+6 due to [$^{2}H_{6}$]GABA, the internal standard. The resultant data plotted against the amount of [$^{2}H_{2}$]GABA added (Fig. 3) show good linearity for either method of normalization. In addition, extrapolation of the data to zero concentration of exogenous [$^{2}H_{2}$]GABA yields values similar to those determined experimentally in the absence of [$^{2}H_{2}$]GABA (Fig. 3).

In vivo incorporation of $^{13}C_{2}$ was determined after intravenous infusion of [$^{13}C_{6}$]glucose in mice. Isotopic enrichment was observed for all four amino acids (Table III) and was more pronounced for GABA and GLU than for ASP and GLN. Selected-ion chromatograms for M and M+2 ions of GABA were obtained for animals infused with labeled glucose and for the saline-infused controls (Fig. 4).

CONCLUSION

This report describes a selected-ion monitoring method for determination of ASP, GLU, GABA, and GLN concentrations and their isotopic enrichment in brain tissue. The method is also applicable to measuring fluxes of these amino acids in brain. The usefulness of this method in studying basic mechanisms of epilepsies will be evaluated in future studies.

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REFERENCES

1 F.V. DeFeudis and P. Mandel (Editors), Amino Acid Neurotransmitters, Raven Press, New York, 1981.

2 F. Fonnum (Editor), Amino Acids as Chemical Transmitters, Plenum Press, New York, 1977.

3 L. Hertz, E. Kvamme, E.G. McGeer and A. Schousboe (Editors), Glutamine, Glutamate, and GABA in the Central Nervous System, Alan R. Liss, New York, 1983.
4 J.C. Watkins and R.H. Evans, Ann. Rev. Pharmacol. Toxicol., 21 (1981) 165.
5 E. Roberts, T.N. Chase and D.B. Tower (Editors), GABA in Nervous System Function, Raven Press, New York, 1976.
6 B.S. Meldrum, Int. Rev. Neurobiol., 17 (1975) 1.
7 D.B. Tower, in E. Roberts, T.N. Chase and D.B. Tower (Editors), GABA in Nervous System Function, Raven Press, New York, 1976, p. 461.
8 B. Meldrum, Epilepsia, 25 (Suppl. 2) (1984) S140.
9 M.J. Croucher and B.S. Meldrum, in R.G. Fariello, P.L. Morselli, K.G. Lloyd, L.F. Quesney and J. Engel (Editors), Neurotransmitters, Seizures, and Epilepsy II, Raven Press, New York, 1984, p. 227.
10 F. Fonnum, in C.J.Pycock and P.V. Taberner (Editors), Central Neurotransmitter Turnover, University Park Press, Baltimore, 1981, p. 105.
11 J.L. Johnson and E. Roberts, Brain Res., 323 (1984) 247.
12 N.H. Neff, P.F. Spano, A. Gropetti, C.T. Wang and E. Costa, J. Pharmacol. Exp. Ther., 176 (1971) 701.
13 F. Moroni, D. Malthe-Sorensson, D.L. Cheney and E. Costa, Brain Res., 150 (1978) 333.
14 E. Costa, D.L. Cheney, C.C. Mao and F. Moroni, Fed. Proc., Fed. Am. Soc. Exp. Biol., 37 (1978) 2408.
15 F. Moroni, in P. Mandel and F.V. DeFeudis (Editors), GABA: Biochemistry and CNS Function, Plenum Press, New York, 1979, p. 189.
16 L. Bertilsson, C.C. Mao and E. Costa, J. Pharmacol. Exp. Ther., 200 (1977) 277.
17 F. Nicoletti, V. Perciavalle, S. Beretta, C. Speciale, M.C. Polizz, F. Patti, D. de Simone, M.A. Sortino, R.M. di Giorgio and U. Scapagnini, Eur. J. Pharmacol., 103 (1984) 133.
18 D.K. Norris, R.A. Murphy and S.H. Chung, J. Neurochem., 44 (1985) 1403.
19 E. Westerberg, A.G. Chapman and B.S. Meldrum, J. Neurochem., 41 (1983) 1755.
20 I.C. Allen, A. Grieve and R. Griffiths, J. Neurochem., 46 (1986) 1582.
21 J.W. Geddes and J.D. Wood, J. Neurochem., 42 (1984) 1624.
22 L. Bertilsson and E. Costa, J. Chromatogr., 118 (1976) 395.
23 R. Corradetti, G. Monetti, F. Moroni, G. Pepeu and A. Wieraszko, J. Neurochem., 41 (1983) 1518.
24 J.M. Schaaf, A.W. Teelken, F.A.J. Muskiet, G. Nagel and B.G. Wolthers, J. Neurosci. Methods, 13 (1985) 257.
25 S.L. MacKenzie and D. Tenaschuk, J. Chromatogr., 322 (1985) 228.
26 D. Darmann, M.J. Manary and D.E. Matthews, Anal. Biochem., 147 (1985) 92.
27 H. Frank, A. Eimiller, H.H. Kornhuber and E. Bayer, J. Chromatogr., 224 (1981) 177.
28 M.K. Gaitonde, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 2, Plenum Press, New York, 1973, p. 321.
29 J.D. Wood, Int. J. Biochem., 13 (1981) 543.
30 G.K.-C. Low and A.M. Duffield, Biomed. Mass Spectrom., 11 (1984) 223.