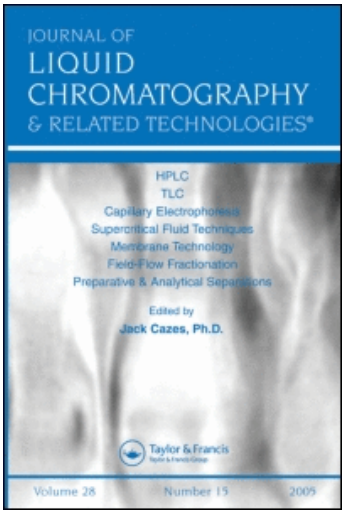

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DETERMINATION OF γ-AMINOBUTYRIC ACID IN THE MOUSE HYPOTHALAMUS AND HIPPOCAMPUS USING LIQUID CHROMATOGRAPHY/ELECTROCHEMISTRY

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ABSTRACT

A method is described for the rapid detection and measurement of γ-aminobutyric acid (GABA) in crude extracts of mouse brain. This study includes optimisation of the pre-column derivatization of GABA by o-phthalaldehyde in the presence of ethylmercaptan as well as of the chromatographic and electrochemical conditions.The GABA levels have been measured in the hypothalamus and hippocampus of control mice and mice treated with three compounds known for their activity on brain GABA levels.

INTRODUCTION

There is now considerable evidence that the γ-aminobutyric acid (GABA) system : GABA, glutamate decarboxylase (GAD) and GABA

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oxoglutarate transaminase (GABA-T) is involved in the neuroendocrine regulation of the hypothalamo-pituitary axis. Administration of GABA to rats has been shown to cause release of growth hormone (GH) (1) while GABA inhibitors decrease GH secretion (2). There is an indication that TRH is similarly regulated, as basal plasma TSH levels in rats dropped following GABA injection (1). A number of other studies suggest that the GABA system may be involved in the release of other pituitary hormones including Luteinizing Hormone (LH) (3), Prolactin (PRL) (4)and Melanocyte Stimulating Hormone (MSH) (5).
Earlier studies in this laboratory have examined the role of the dopaminergic system in TRH regulation (6) and it is planned to extend this work to include the effect of GABA on TRH, since it has been shown that GABA may be involved in the release of GH and PRL (8). However, GABA and other amino acids cannot be directly measured by the method of HPLC and electrochemical detection developed in the laboratory (6).

Numerous methods have been reported for the determination of brain amino-acids but they all require derivatization of the amino group. The methods available include : gas chromatography and electron capture detection of acylated derivatives (9); spectrophotometric detection of ninhydrin derivatives (10) or dansyl derivatives (11); liquid scintillation spectrometry of dinitrophenyl derivatives (12); enzymatic conversion of the aminoacid to fluorescent by-products (13-14) ; fluorescence detection of fluorescamine (15); dansyl (16), or o-phthalaldehyde (OPA) derivatives (17-26).

Roth (18) first reported the formation of a fluorescent S-substituted isoindole by the reaction of amines with OPA and β-mercaptoethanol (β -MCE) or ethylmercaptan (EMC). More recently, it has been shown that such compounds may undergo anodic oxydation (27). The present study was undertaken to determine the optimum conditions for the measurement of GABA by high performance liquid

chromatography and electrochemical detection (LCEC) of the S-substituted isoindole derivatives.This particular combination of derivation, separation and detection was chosen as derivative preparation is simple and the LCEC rapid and sensitive. The methods have been applied to the determination of GABA in two regions of the mouse brain (hypothalamus and hippocampus) in normal animals and mice treated with valproic acid (VPA), vinyl GABA (V-GABA) and aminooxyacetic acid (AOAA).

MATERIALS AND METHODS

1. Chromatographic system

The HPLC system comprised a Waters 6000 A pump with a U6K injector, a Waters RCM Module radial compression system, a Waters Radial Pak A column (C8, 10 cm, 8 mm ID., 10 µm particle size). The effluent was analysed with a Metrohm 641 electrochemical detector fitted with two vitreous carbon electrodes EA 286-1 and a Ag/AgCl reference electrode (EA 442). The potential used was 0.75 V and the sensitivity 10 nA. The detector output was recorded on a Kipp and Zonen recorder with a 1 V input.

2. Mobile Phase

The mobile phase was a mixture (2/3,v/v) of 0.1 M KH_2PO_4 and methanol (HPLC grade). The solution was adjusted to an apparent pH of 5.5 with 1M H_3PO_4 and was filtered through a 0.45 µm Millipore filter before use.

3. Reagents

The derivatizing solution consisted of 27 mg o-phthalaldehyde (OPA)(Aldrich), 1 ml of absolute ethanol and 40 µl of EMC (Aldrich) in 10 ml of 0.1 M sodium tetraborate. The 0.1 M sodium tetraborate was prepared by dissolving 0.78 g of boric acid in 100

ml of water and adjusting the pH to 9.17 with 5N NaOH.The working OPA-EMC solution, prepared fresh each week, was stored at 0°C. The GABA stock solution, 20.6 mg in methanol-water 50/50 was stored at 0°C for up to one month.

4. Biological Sample Preparation

Male mice (26-30 g, 6 weeks old) were sacrificed by decapitation and the brains quickly removed. The hypothalamus and hippocampus regions were dissected out on ice and stored at -80°C until extraction. The samples were homogenized in an Ultra-Turrax with 1 ml of cold methanol and centrifuged at 8000 g and 4°C for 10 minutes. The protein content of the tissue pellets were measured by Lowry method (28).

5. Derivatization

The initial studies to optimize GABA measurement were made on 150 µl aliquots of GABA stock solution diluted to 10 ml in water (3.09 ng/ µl). 100 µl of the diluted solution were incubated for exactly 2 min at 25° with 100 µl of the OPA-EMC solution. At the end of the 2 min., 10 µl of the derivatization mixture were injected onto the HPLC.

Tissue samples and associated GABA standards were derivatized by incubating 50 µl of supernatants or diluted GABA with 200 µl OPA-EMC solution for 2 minutes as for the standard samples. At the end of the two minutes 10µl of the derivatization mixture were injected onto the HPLC column.

6. Internal Standard

Standard samples included 2-amino butyric acid (2-ABA) (Janssen) as internal standard (20.6 mg/100 ml).However, 2-ABA was not enough resolved from contaminating peaks in the tissue

samples, so GABA itself was added to these samples and used as internal standard . Two 400µl aliquots of tissue supernatant were prepared. 100 µl of methanol:water (50/50) was added to one aliquot and 100 µl of 1/10 dilution of stock GABA solution was added to the other.

Derivatization was carried out by incubating (2 min.) 50 µl GABA standard,50 µl supernatant without standard and 50 µl GABA-supernatant with 200 µl of OPA/EMC. 20 µl aliquots of GABA standard, and supernatant preparations were analysed to give peaks H_1 and H_2 respectively . 10 µl of GABA-supernatant preparation were analysed to give H_3.Relative recoveries were calculated from the expression $(2H_3-H_2)/H_1$.

7. Drug Treatment

Mice were given the three drugs :

a- aminoxyacetic acid (AOAA) (hemihydrochloride, Sigma) dissolved in 0.9 % NaCl adjust to pH 6.5 with 2 N NaOH, was injected (i.p.) at 50 mg/kg ,3 hrs before sample removal.

b- γ-vinyl GABA (VGABA) (Merrel), dissolved as above, was injected at 800 mg/kg i.p. 4 h before sacrifice

c- sodium valproate (VPA) (Labaz), dissolved as above, was injected at 300 mg/kg i.p. 1 h before sacrifice.

Control mice were given saline 1ml/100 g. All manipulations were performed between 8 h and 13 h.

RESULTS AND DISCUSSION

1. Optimization of GABA Measurement

It has been reported that breakdown of some OPA-aminoacid derivatives occurs rapidly even in the presence of a thiol

stabilizing agent (21-23,29-30). Changing the thiol brought no appreciable improvement in stability unless the bulky tert-butyl group was incorporated (31). Ethylmercaptan C_2H_5SH (EMC), which gives the same result as mercapto ethanol $SHCH_2-CH_2OH$ (MCE) (32), was used in this study.

The stability of the OPA/EMC derivative was investigated by preparing a derivatization mixture and periodically monitoring the LCEC response.The detector response is a function of derivative formation and decreases at a rate of 7 %/min for reaction times between 1 to 4 min (Fig. 1). A reaction time of exactly 2 min at 25°C was adopted as a compromise between sufficient time to prepare samples for injection and loss of detector response due to degradation.

The composition of the mobile phase was studied to obtain the best compromise between resolution and analysis time. The C8 Radialpack compressed column was used as C18 retention times were excessive. Increasing methanol content decreased the retention times of the GABA/OPA derivatives. 50 % MeOH in 0.1 M KH_2PO_4 (apparent pH 5.5) give the best separation of GABA from other aminoacids (AA) (Fig. 2). GABA had the longest retention time (29 min) and was eluted last.60 % MeOH was used as the organic modifier when GABA was determined alone (standards) and the retention time was reduced to 11 min. There were no derivatives following the GABA peak in mouse brain samples and then the total analysis time was approximately 12 min. A chromatogram of the GABA standard is shown in Figure 3.

A good linear relationship existed between GABA concentration and the peak height over the range 0.2 to 25 ng when the reaction time 2 min was carefully controlled. The detection limit was 0.2 ng (signal: noise = 2.5/1). This limit is far below the quantity of GABA present in the regions of mouse brain examined.

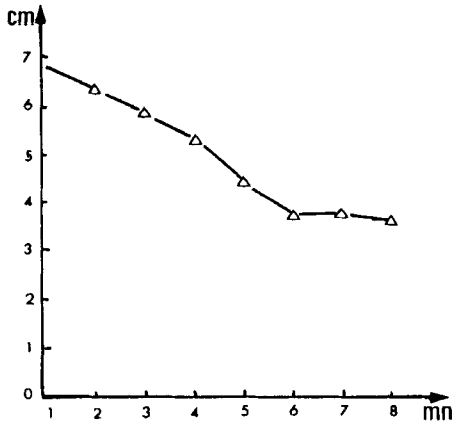

FIGURE 1 : Effect of derivatizing time on GABA peak heigh
Column:Microbondapack C8 10 µ ; Mobile phase : 0.1 M KH_2PO_4, 60 % methanol , pH 5.5 ; flow rate 1.5 ml/min ; sensitivity 10 nA ; detector potential 0.7 V.

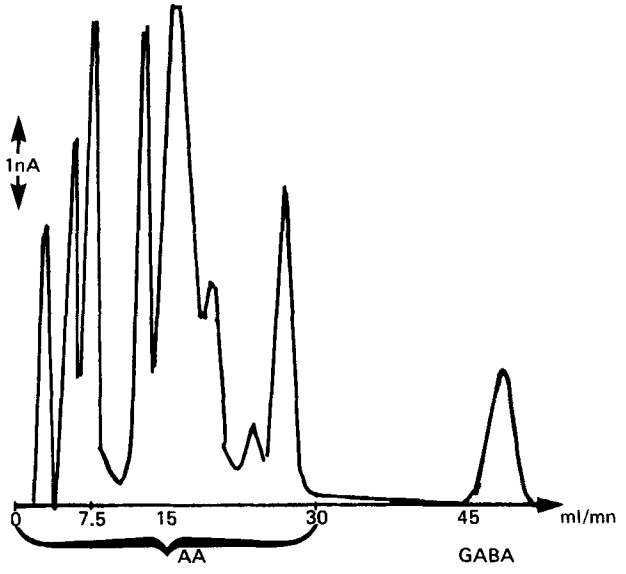

FIGURE 2 : Chromatogram of mouse brain tissue extracts.
Column Microbondapack C8 10 µ ; Mobile phase : 0.1 M KH_2PO_4 (pH 5.5), 50 % methanol ; flow rate 1.5 ml/min ; sensitivity 10 nA ; detector potential 0.7 V.

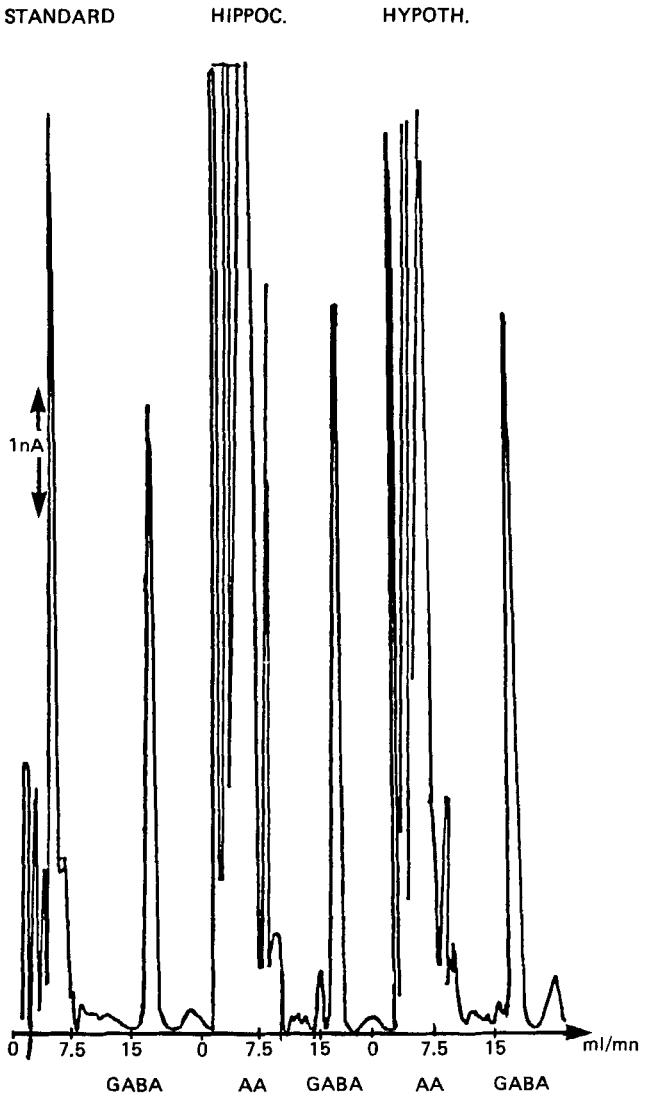

FIGURE 3 : Chromatogram of a standard and of mouse hippocampus and hypothalamus.
Column Microbondapack C8 10 µ; Mobile phase : 0.1 M KH_2PO_4; 60 % methanol ; pH 5.5 ; flow rate 1.5 ml/min; sensitivity 10 nA ; detector potential 0.7 V ; Injection volume 10 µl.

2. Recovery and Internal Standard

The 2-ABA peak is eluted with a short retention time in the chromatographic system used. This peak is buried in the mass of other amino acid derivatives when it is included in tissue samples and, thus,is unsuitable for use as an internal standard. The system employing exogenous GABA was therefore adopted for internal standardization (see methods).The relative recoveries of GABA were as follows :

100.18 ± 5.9 % for hypothalamus (n = 10)
100.10 ± 2.79 % for hippocampus (n = 10).

3. GABA Content of Mouse Hypothalamus and Hippocampus

The measurement of tissue GABA levels is complicated by the fact that GABA rapidly increases when an animal dies .The GABA levels of microwave treated brains are equivalent to those determined after decapitation into liquid nitrogen, but the GABA levels of brains obtained by decapitation and dissection at 20°C are 18 per cent higher (31). In the present study the mice were decapitated and hypothalami and hippocampi rapidly dissected out on ice, and immediately frozen on dry ice.

Lasley et al. (32) used 10 volumes of cold methanol to extract GABA from rat substantia nigra but no work has been done on GABA extraction from mouse hypothalamus and hippocampus.In this study, each hypothalamus or hippocampus was extracted with 1 ml cold MeOH.As 50 µl of this methanolic extract was reacted with 200 µl of OPA/EMC, 20 µl of the reaction mixture contained 15-25 ng of GABA, which is within the range of standards used.

The GABA content of the hypothalami and hippocampi of control mice is shown in Table 1 and fig.4. There are few values in the literature with which compare them. Van der Heyden and Korf

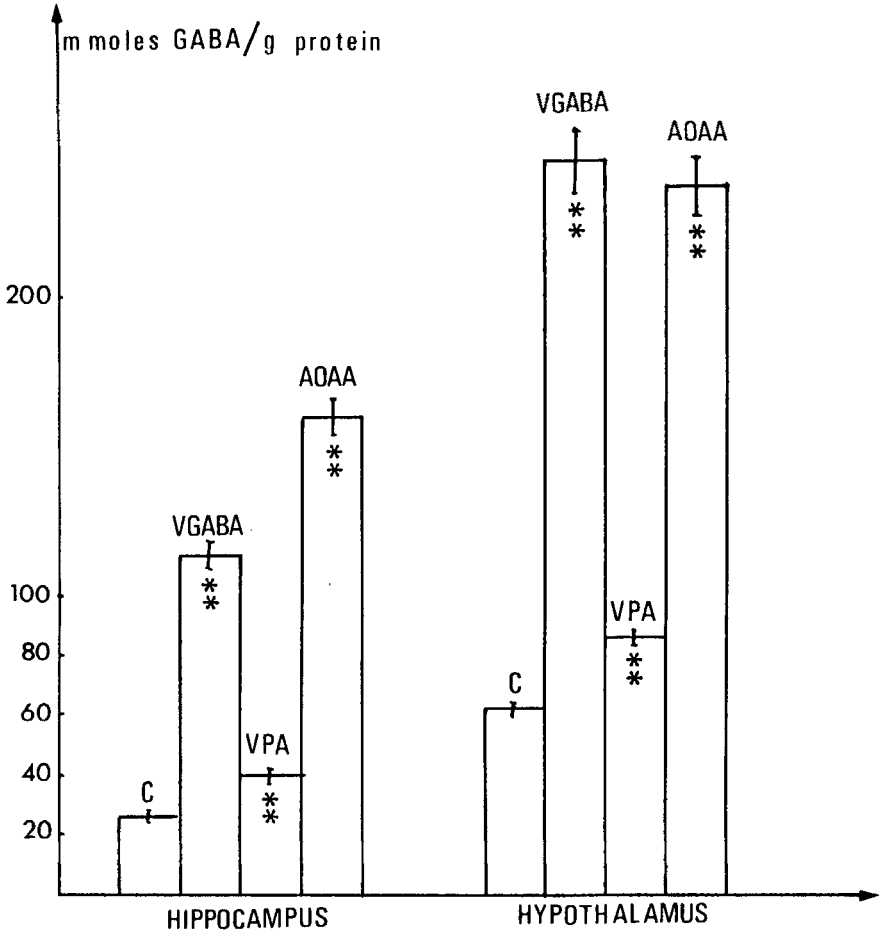

FIGURE 4 : Variation of GABA under the influence of a treatment.
C : Control.
VGABA : γ-vinyl GABA 800 mg/kg ip.
VPA : Sodium valproate 300 mg/kg ip.
AOAA : Aminoxyacetic acid hemihydrochloride 50 mg/kg ip.

TABLE 1

Mouse Brain GABA Content

		Hippocampus mmole/g protein			Hypothalamus mmole/g protein		
	n			Δ %			Δ %
Control	23	26.05	0.71		62.84	1.69	
Treatment							
VGABA	14	114.13	4.32	338 %	245.65	13.96	291 %
VPA	15	38.67	1.68	48 %	85.46	2.61	36 %
AOAA	15	158.56	7.5	509 %	237.8	9.7	278 %

(18) reported 2.05 mol/g wet tissue in the mouse hippocampus and we found 26.05 mol/g protein. These values are in the same range as there is around 1 g protein/10 g wet tissue. Smiler et al. (33) studied other regions of the brain and Gabellec et al. (34) worked on special hybrid mice.The adoption of the internal GABA standard system appears to be justified from the reproducibility obtained.The coefficient of variation for the mean GABA content are in the acceptable range of 3 % in this study , despite the instability of GABA derivatives.

4. Variations in cerebral GABA Content in Drug-treated mice.

The GABA accumulation produced by VPA was small compared to the 3 to 5-fold increases induced by the other inhibitors of GABA metabolism, AOAA and VGABA. For these two, the increase was much greater in the hippocampus than in the hypothalamus. The results are listed in table 1 and shown on Figure 4.

This study describes the optimization of methodology for LCEC determination of OPA-AA derivatives for the measurement of GABA in

mouse hypothalamus and hippocampus. Application of the method is illustrated by the determination of GABA levels after administration of three drugs which block GABA catabolism.

ADRESSES CHANGE

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REFERENCES

(1) Vijayan E. and S.M. Mac Cann, Effects of intraventricular injection of γ-aminobutyric acid (GABA) on plasma growth hormone and thyrotropin in conscious ovariectomized rats, Endocrinology, 103 , 1888, 1978.

(2) Fiok J., Z. Acs and E. Stark, Possible inhibitory influence of gamma-aminobutyric acid on growth hormone secretion in the rat, J. of Endocrinology, 91 , 391, 1981.

(3) Vijayan E. and S.M. Mac Cann, The effects of intraventricular injection of γ-aminobutyric acid (GABA) on prolactin and gonadotropin release in conscious female rats, Brain Research, 155 , 35, 1978.

(4) Ondo, J.G. and K.A. Pass, The effects of neurally active amino acids on prolactine secretion, Endocrinology, 98 , 1248, 1976.

(5) Taleisnik S., M.A. Celis and M.E. Tomatis, Release of melanocyte stimulating hormone by several stimuli through the activation of 5-hydroxytryptamine mediated inhibitory neuronal mechanism, Neuroendocrinology, 13 , 327, 1974.

(6) Rips R., M. Desiles and A.J. Puech, Is a dopaminergic system involved in TRH induced hyperthermia and in its potentiation by amphetamine, Prog. Neuropsychopharmacol., 3 , 337, 1979.

(7) Morier E. and R. Rips, A new technique for simultaneous determination of biogenic amines and their metabolites, J. Liquid Chromatogr., 5 , 151, 1982.

(8) Collu R. and Y. Tache, Hormonal effects exerted by TRH through the central nervous system, in : Central nervous system effects on hypothalamic hormones and other peptides, eds Collu et al. (Raven Press, New York) 1979, p. 97.

(9) Schmidt R. and M. Karobath, Specific and sensitive method for the determination of γ-aminobutyric acid using gas chromatography with electron capture or mass fragmentographic detection, J. Chromatogr., 139 ,101, 1977.

(10) Lee, P.L.Y. , Single-column system for accelerated aminoacid analysis of physiological fluids using five lithium buffers, Biochem. Med., 10 , 107, 1974.

(11) Zecca, Z., F. Zambotti, N. Zonta and P. Mantegazza, Determination of γ-aminobutyric acid in brain areas by high-performance liquid chromatography of dansyl derivatives with ultraviolet detection, J. Chromatogr., 233 , 307, 1982.

(12) Freeman, M.E., C. Co, T.R. Mote, J.D. Lane and J.E. Smith, Determination of content and specific activity of amino acids in central nervous system tissue utilizing tritium and carbon-14 dual labelling, Anal. Biochem., 106 , 191, 1980.

(13) Graham, L.T. and M.H. Aprison, Fluorometric determination of aspartate, glutamate and γ-aminobutyrate in nerve tissue using enzymic methods, Anal. Biochem., 15 , 487, 1966.

(14) Okada, Y, C. Nitsch-Hassler, J.S. Kim, I.J. Bak and R. Hassler, Role of γ-aminobutyric acid (GABA) in the extrapyramidal motor system. 1- Regional distribution of GABA in rabbit, rat, guinea pig and baboon CNS, Exp. Brain Res., 13 , 514, 1971.

(15) Rubinstein,M., S. Chen-Kiang, S. Stein and S. Udenfriend, Characterization of proteins and peptides by high performance liquid chromatography and fluorescence monitoring of their tryptic digests, Anal. Biochem., 95 , 117, 1979.

(16) De Jong, C., G.J. Hughes, E. Van Wieringen and K.J. Wilson, Amino acid analyses by high performance liquid chromatography. An evaluation of the usefulness of pre-column Dns derivatization, J. Chromatogr., 241 , 345, 1982.

(17) Roth, M., Fluorescence reaction for amino acids, Anal. Chem., 43 , 880, 1971.

(18) Van Der Heyden J.A.M. and J. Korf, Regional levels of GABA in the brain : rapid semiautomated assay and prevention of posmortem increase by 3-mercaptopropionic acid, J. Neurochem., 31 , 197, 1978.

(19) Davis, T.P., C.W. Gehrke, C.W. Gehrke Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson, and C.H.J. Williams, High performance liquid chromatographic analysis of biogenic

amines in biological materials as o-phthalaldehyde derivatives, J. Chromatogr., 162 , 293, 1979.

(20) Hill, D.W., F.H. Walters, T.D. Wilson and J.D. Stuart, High performance liquid chromatograhy of amino acids in the picomole range, Anal. Chem., 51 , 1338, 1979.

(21) Lindroth P. and K. Mopper, High performance liquid chromatographic determinations of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phtalaldehyde, Anal. Chem., 51 , 1667, 1979.

(22) Lenda K. and G. Svenneby, Rapid high performance liquid chromatographic determination of amino-acids in synaptosomal extracts, J. Chromatogr., 198 , 516, 1980.

(23) Jones, B.N., S. Paabo and S. Stein, Aminoacid analysis and enzymatic sequence determination of peptides by an improved o-phtaldialdehyde precolumn labelling procedure, J. Liq. Chromatogr., 4 , 565, 1981.

(24) Caudill, W.L., G.P. Houck and R.M. Wightman, Determination of gamma-aminobutyric acid by liquid chromatography with electrochemical detection, J. Chromatogr., 227 , 331, 1982.

(25) Hill, D., L. Burnworth, W. Skea and R. Pfeifer, Quantitative HPLC analysis of plasma amino acids as orthophtaldialdehyde/ethanethiol derivatives, J. Liq. Chromatogr., 5 , 2369, 1982.

(26) Lindgren, S., N.E. Anden and M. Grabowska-Anden, A fluorimetric method for determination of GABA in tissues following cation exchange chromatography and condensation with o-phtalaldehyde, J. Neur. Trans., 55 , 243, 1982.

(27) Joseph M.H. and P.J. Davies, Electrochemical activity of o-phthalaldehyde mercapto ethanol derivatives of amino acids application to high performance liquid chromatographic determination of amino acids in plasma and other biological materials, J. Chromatogr., 277 , 125, 1983.

(28) Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem., 193 , 265, 1951.

(29) Hogan, D.L., K.L. Kraemer and J.I Isenberg, The use of high performance liquid chromatography for quantitation of plasma amino acids in man, Anal. Biochem., 127 , 17, 1982.

(30) Turnell D.C. and J.D.H. Cooper, Rapid assay for amino acids in serum or urine by pre-column derivatization and reversed phase liquid chromatography. Clin. Chem., (Winston- Salem, N.C.), 28 , 527, 1982.

(31) Allison, L.A., G.S. Mayer and R.E. Shoup, O-phthaldehyde derivatives of amines for high speed liquid chromatography electrochemistry, Anal. Chem., 56 , 1089, 1984.

(32) Lasley,S.M., R.D. Greenland and I. A. Michaelson, Determination of gamma-amino butyric and glutamic acids in rat brain by liquid chromatography with electrochemical detection, Life Sciences, 35 , 1921, 1984.

(33) Smiler, S., C. Gensburger, L. Ciesielski and P. Mandel, Time course of the increase in GABA level in different mice brain regions following dipropylate treatment, Psychopharmacol., 2 , 123, 1978.

(34) Gabellec, M.M., M. Recassens, R. Benezia and P. Mandel, Regional distribution of GABA, GAD and GABA-T in the central nervous brains of C 57/BR, C3H/He and F1 hybrid mice, Neurochem. Res., 5 , 309, 1980.