*Journal of Chromatography*, 309 (1984)139—144
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2111

## Note

# Determination of γ-aminobutyric acid in physiological samples by a simple, rapid high-performance liquid chromatographic method

A.S. HERRANZ, J. LERMA and R. MARTIN DEL RIO*

*Departamento de Investigacion, Centro "Ramon y Cajal", Ctra. Colmenar Viejo Km 9, Madrid 34 (Spain)*

(First received December 1st, 1983; revised manuscript received February 13th, 1984)

Since the discovery of γ-aminobutyric acid (GABA) in the nervous system, various methods for its quantitation have been developed [1—11]. Most of them, however, display low sensitivity [2—4], require expensive equipment [5—9] or are difficult to handle [10, 11].

The introduction of high-performance liquid chromatography (HPLC) represented a considerable improvement in analytical techniques and it has been used for the determination of a great number of compounds. HPLC has also been applied to the measurement of amino acids [12, 13], and some HPLC methods for GABA determination have recently been reported [14—16]. The purpose of the present work is to describe a new rapid method for GABA analysis in physiological samples using a simple reversed-phase HPLC procedure with *o*-phthalaldehyde (OPA) pre-column derivatization.

## MATERIALS AND METHODS

### *Chromatographic equipment*

A Beckman HPLC apparatus Model 344 was used. The chromatographic system included a controller Model 421 CRT, a pump Model 112, an injection valve Model 340 with a 20-μl loop, and a data processor/recorder Chromatopac C-R1B (Shimadzu) for peak integration. Analyses were performed on a 150 × 4.6 mm I.D. column, pre-packed with 5-μm Ultrasphere-ODS (Beckman). The detector consisted of a Gilson Spectra/Glo fluorometer with a 15-μl micro-flow cell, a quartz-halogen lamp, and the combination of 360-nm excitation with 455-nm emission filters. The sensitivity dial of the fluorometer was usually set at 100 range units.

0378-4347/84/$03.00 

A Beckman 121/MB automatic amino acid analyser was used for free GABA determination.

*Reagents*

The following chemicals were purchased: amino acid standard solution from Beckman; GABA from Sigma (St. Louis, MO, U.S.A.); methanol (HPLC grade), concentrated hydrochloric acid and 2-ethanethiol from Scharlau (Barcelona, Spain); acetic and boric acids from E. Merck (Darmstadt, F.R.G.); and OPA from Fluka (Buchs, Switzerland). Distilled, deionized water was used, further purified by passage through a filter for adsorption of organic substances (Norganic, Millipore) and a 0.4-$\mu$m Millipore filter.

*Chromatographic conditions*

Optimum chromatographic conditions were as follows: isocratic solvent of water—methanol (20:80) with a flow-rate of 1.0 ml/min. All analyses were performed at room temperature (22—25° C).

*Esterification of GABA and amino acid standard mixture*

To obtain the corresponding methyl ester derivatives, an amino acid standard mixture solution containing eighteen $\alpha$-amino acids (2.5 m*M*) and the GABA solution (1.7 m*M*), were diluted (× 100, by vol.) with absolute methanol containing hydrochloric acid at a final concentration of 0.1 *M*. The esterification reaction was maintained overnight at room temperature.

*Saponification of the GABA methyl ester derivative*

The saponification reaction of GABA methyl ester was obtained by diluting an aliquot of the esterified sample five times with 0.2 *M* sodium hydroxide and heating the resultant solution at 60° C overnight in a sealed tube. For quantitation of free GABA, the sample was acidified by adding concentrated hydrochloric acid immediately before analysis.

*Tissue GABA extraction and esterification*

Sprague—Dawley female rats were killed by decapitation and their brains and oviducts were rapidly dissected and frozen in liquid nitrogen. Tissues were then weighed and resuspended in 25 vols. (w/v) of a solution of absolute methanol containing 0.1 *M* hydrochloric acid, and ground with a glass homogenizer. The tissue GABA extraction and the esterification reaction were left overnight at room temperature. Samples were centrifuged at 5000 *g* for 10 min and the clear supernatant was used for analysis.

*Derivatization procedure*

The method used is essentially that of Hill et al. [17] with minor modifications. Two hundred microlitres of sample were mixed with 50 $\mu$l of 0.5 *M* potassium borate buffer (pH 10.5); 50 $\mu$l of methanol and 100 $\mu$l of 2-ethanethiol solution (10 $\mu$l/ml in absolute methanol) were then added to this mixture. The amino acid derivatization was initiated by adding 100 $\mu$l of OPA solution (10 mg/ml in methanol) and the reaction was acidified 1 min later with 100 $\mu$l of acetic acid (5% in methanol) in order to lower the pH to 5—6. Then 20 $\mu$l of

the final solution were injected into the chromatographic system. The 2-ethanethiol and OPA solutions were kept in light-protected tubes and prepared weekly.

## RESULTS

The carboxyl group of GABA was esterified as described in Materials and methods and the progress of the reaction was monitored at different times by measuring the remaining unmethylated GABA. Determinations were performed with an automatic amino acid analyser until the non-reacting GABA was below the detection limit ($\simeq$ 100 pmol.). Fig. 1 shows the gradual decline in the reaction mixture of free (unmethylated) GABA with time. Conversion to the methyl derivative is complete after 4 h. Recovery of the free form of GABA following saponification with sodium hydroxide ranged between 98 and 100%, demonstrating that the amino acid had previously been totally converted to the methyl ester form.

When the GABA methyl ester was combined with OPA, it produced a fluorescent compound which, in our optimum chromatographic conditions, eluted in a well defined peak within a short time (4.9 $\pm$ 0.12 min), as shown in Fig. 2A.

In order to ascertain the resolution of GABA from $\alpha$-amino acids, a mixture of eighteen $\alpha$-amino acid methyl esters was added to the GABA standard. Fig. 2A depicts a typical chromatogram obtained. It can be seen that the $\alpha$-amino acid mixture eluted in a broad peak well ahead of GABA. Moreover, the methyl ester of two $\beta$-amino acids ($\beta$-alanine and $\beta$-aminoisobutyric acid), which might be present in physiological samples, also eluted far away from the $\alpha$-amino acids and were well separated from the GABA peak (Fig. 2B).

Flow-rates higher than 1.0 ml/min and a methanol content greater than 80% shortened the analysis time but diminished the resolution of the GABA peak. The converse conditions improved the resolution, but a longer analysis time was required. The optimum derivatization reaction time was 1 min. A shorter reaction time diminished the fluorescence response but longer periods, up to 10 min, did not improve it.

The fluorescence response of GABA was examined in the 1—100 pmol range.

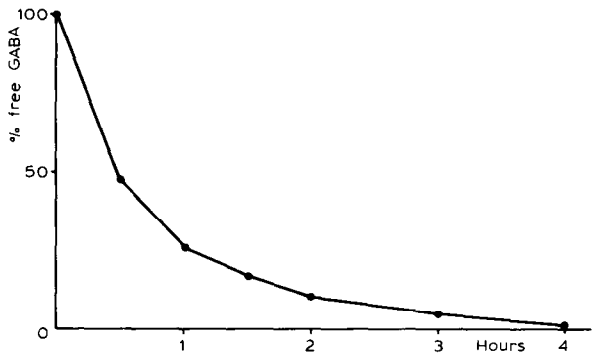


**Fig. 1. Declining concentration of free GABA with increasing esterification time. Each point, represented as a percentage of the initial amount (100%), is the mean of two determinations.**

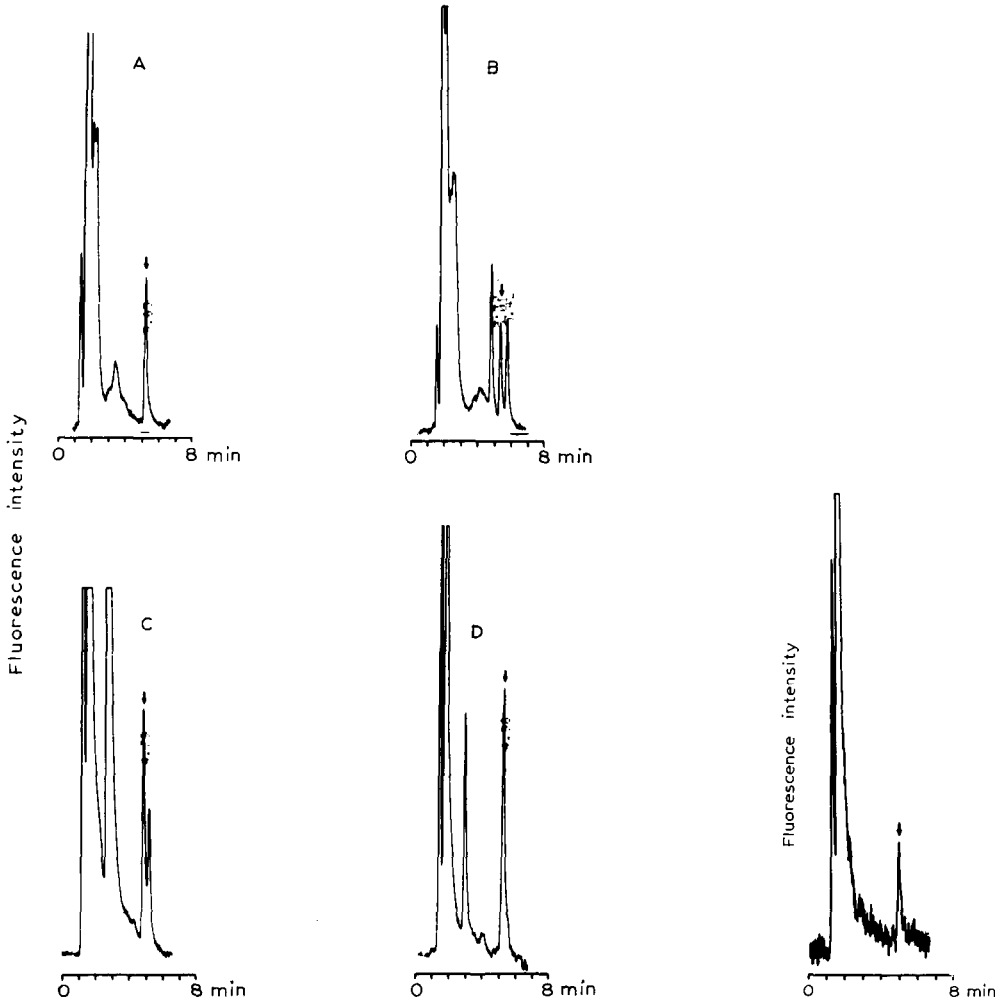


Fig. 2. Arrows indicate the GABA methyl ester peaks in four different situations: (A) in a mixture of eighteen $\alpha$-amino acids; (B) as in A with $\beta$-alanine (peak ahead of GABA) and $\beta$-aminoisobutyric acid (peak slower than GABA) included; (C) in a chromatogram of brain extract representing 13.3 $\mu$g of tissue; (D) in a chromatogram of oviduct extract representing 6.6 $\mu$g of tissue. In all cases the recorder attenuation was set at $2^4$.

Fig. 3. Typical chromatogram showing the analysis of 1 pmol of GABA (arrow). Recorder attenuation was set at $2^1$.

The analysis of 1 pmol gave peak heights with a signal-to-noise ratio higher than 2 (Fig. 3), demonstrating the high sensitivity of this method. The regression line between GABA amount and fluorescence response was calculated with data obtained from assays performed on three different days, and was linear over the concentration range studied ($r = 0.99, P < 0.05$).

*Tissue GABA determination*

Brains and oviducts of five female rats were processed as described in Materials and methods and the free GABA content in pellets (non-extracted),

supernatants (non-esterified GABA), and in saponified supernatants (extracted and esterified GABA) was measured with the aid of an amino acid analyser. In addition, GABA from five other animals was extracted and measured as previously described [4]. Brain and oviduct GABA values were considered to be the total GABA content in these tissues.

TABLE I

FREE GABA CONTENT IN TOTAL, PELLET, SUPERNATANT AND SAPONIFIED SUPERNATANT OF RAT BRAIN AND OVIDUCT

Data are expressed as mean ± S.D. and percentage of the total amount.

| | Total | | Pellet | | Supernatant | | Saponified supernatant | |
|---|---|---|---|---|---|---|---|---|
| | μmol/g | % | μmol/g | % | μmol/g | % | μmol/g | % |
| Brain | 2.01 ± 0.19 | 100 | <0.032 | <1.6 | <0.095 | <4.7 | 1.96 ± 0.15 | 97.5 |
| Oviduct | 4.65 ± 0.31 | 100 | <0.087 | <1.8 | <0.024 | <4.4 | 4.45 ± 0.32 | 95.7 |

Table I shows the free GABA content found in pellet, supernatant, and saponified supernatant, expressed in μmol/g original tissue and also as a percentage of total GABA. It can be seen that the non-extracted and non-esterified GABA are less than 2% and 5%, respectively, and that the GABA ester obtained in the supernatant, measured as free GABA after saponification, is 95—98% of the total GABA.

The GABA derivative was further analysed with the HPLC method. Fig. 2C and D shows typical chromatograms obtained from samples of both structures, brain and oviduct. The GABA peak was well resolved and the coefficients of variation obtained from the five samples analysed were 2.2% and 4.0%, respectively, for brain and oviduct.

The accuracy of the described method was assessed by comparing GABA values obtained from brain and oviduct with those measured by the amino acid analyser. The results obtained by these techniques differed by less than 5%.

## DISCUSSION

Various methods have been developed [1—11] for the quantification of GABA since its discovery in the nervous system more than 30 years ago [18, 19]. Recently, GABA—OPA [12, 13], dansyl-GABA [14, 15] and trinitrobenzene sulphonic acid—GABA derivatives [16] have been measured by HPLC. The present study describes a new HPLC method for GABA determination which quantifies its methyl ester after derivatization with OPA.

Although our method is based on obtaining the methyl ester of the amino acid, the derivative is actually formed during the tissue extraction step; hence there is no complicated handling procedure and this is one of several advantages over other OPA methods. The mobile phase needed is less harmful to the stationary phase than that of Lindroth and Mopper [12], Jones et al. [13] or Caudill et al. [16], thus increasing the life of the chromatographic system. The run time is quite short (less than 7 min) and the sensitivity (less than 1 pmol)

is satisfactory. The method has adequate precision (coefficient of variation = 4%), and, because of the isocratic procedure, the equipment needed is relatively inexpensive. Dansyl derivative methods [14, 15] also offer all of these advantages but the preparation of the sample is difficult and time-consuming, and the recovery of the derivative is relatively low, e.g. 87% [14]. Finally, the method described by Caudill et al. [16] is suitable, although the retention time of GABA is almost three times longer than in the present method.

Although it is not known why the $\beta$- and $\gamma$-amino acid methyl esters elute later than the $\alpha$-amino acids, it may be hypothesized that the absence of polar lateral chains in $\beta$- and $\gamma$-amino acids, the blocked carboxyl group and their capability to adopt a more extended conformation after derivatization than the $\alpha$-amino acids, make them more hydrophobic [12] thus allowing greater interaction with the stationary phase and subsequently increasing retention times.

Measurements of GABA levels in human cerebrospinal fluid may be helpful for diagnosis and therapy of brain disorders, thus the reported method provides with a rapid, sensitive, selective and relatively inexpensive assay for this purpose.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. J.M.R. Delgado for research facilities, Dr. E. Herrera for critical evaluation, C.S. Delgado and D. Muñoz for editorial help, and A. Latorre for technical assistance.

REFERENCES

1 N. Seiler, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 3, Plenum, New York, 1975, p. 409.
2 M.K. Gaitonde, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 2, Plenum, New York, 1974, p. 321.
3 F.W. Wagner and R.L. Liliedahl, J. Chromatogr., 71 (1972) 567.
4 R. Martin del Rio and A. Latorre Caballero, J. Neurochem., 34 (1980) 1584.
5 P. Bõlen, P.J. Schechter, W. Van Damme, G. Coquillat, J.C. Dosch and J. Koch-Weser, Clin. Chem., 24 (1978) 256.
6 T.A. Hare and N.V. Bala Manyam, Anal. Biochem., 101 (1980) 349.
7 J.A.M. Van der Heyden, K. Venema and J. Korf, J. Neurochem., 32 (1979) 469.
8 J.D. Huizinga, A.W. Teelken, F.A.J. Muskiet, J.V.D. Meulen and B.G. Wolthers, N. Engl. J. Med., 296 (1977) 692.
9 P.H. Wu, D.A. Durden and L. Hertz, J. Neurochem., 32 (1979) 379.
10 M. Otsuka, K. Obata, Y. Miyata and Y. Tanaka, J. Neurochem., 18 (1971) 287.
11 S.J. Enna, J.H. Wood and S.H. Snyder, J. Neurochem., 28 (1977) 1121.
12 P. Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667.
13 B.N. Jones, S. Pääbo and S. Stein, J. Liquid Chromatogr., 4 (1981) 565.
14 G.E. Griesmann, W. Chan and O.M. Rennert, J. Chromatogr., 230 (1982) 121.
15 L. Zecca, F. Zambotti, N. Zonta and P. Mantegazza, J. Chromatogr., 233 (1982) 307.
16 W.L. Caudill, G.P. Houck and R.M. Wightman, J. Chromatogr., 227 (1982) 331.
17 D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, Anal. Chem., 51 (1979) 1338.
18 E. Roberts and S. Frankel J. Biol. Chem., 187 (1950) 55.
19 J. Awapara, A.J. Landau, R. Fuerst and B. Seale, J. Biol. Chem., 187 (1950) 35.