Journal of Chromatography, 337 (1985) 21—27
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2356

RAPID GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC QUANTITATION OF γ-AMINOBUTYRIC ACID IN BIOLOGICAL SPECIMENS

ERIC J. NORMAN*,*, ELIZABETH L. WEE**, HELEN K. BERRY*** and ERNEST F. ZIMMERMAN**

Departments of Medicine (Hematology-Oncology) and Pediatrics, Children's Hospital Research Foundation (Cell Biology** and Inborn Errors of Metabolism***), University of Cincinnati College of Medicine, Cincinnati, OH 45267 (U.S.A.)*

(First received June 13th, 1984; revised manuscript received August 28th, 1984)

SUMMARY

A mass fragmentographic method for γ-aminobutyric acid (GABA) quantitation using the heptafluorobutyryl-cyclohexyl-GABA derivative is described. Both capillary and packed column gas chromatography were used. This procedure employs 2,2[$^2H_2$]GABA as an internal standard and allows the rapid, sensitive, and specific measurement of GABA with a minimum of sample clean-up. Application of the method is demonstrated in mouse embryonic brain, body, and palate and human platelets, plasma, cerebrospinal fluid, and urine.

INTRODUCTION

γ-Aminobutyric acid (GABA) is present in high concentration in the mammalian brain and is believed to be a major inhibitory neurotransmitter [1]. Levels in cerebrospinal fluid (CSF) may reflect altered GABA metabolism in various neurologic and psychiatric diseases [2—6]. High levels are also present in non-neuronal tissue of rat pancreatic islets [7], human insuloma [7], rat ovary [8], and rat Fallopian tube [9], whereas lower GABA concentrations occur in human blood [10] and peripheral tissues [11, 12]. GABA is involved in respiratory regulation [13], cardiovascular control [14], temperature regulation [15], palate morphogenesis [16], and polyamine interrelations during neuronal development [17]. Measurements of GABA in body fluids and tissues will help elucidate its metabolic role and diagnostic value.

Gas chromatography—mass spectrometry—selected ion monitoring

0378-4347/85/$03.30

(GC—MS -SIM) with deuterated GABA as an internal standard provides a sensitive, rapid, and accurate method for GABA quantitation. The heptafluorobutyryl (HFB)-cyclohexyl-GABA derivative described in this paper with its properties of a moderatively high GC retention temperature and abundant high mass ions gives it selected advantages for GABA analysis over derivatives previously described [18—21]. This work reports a rapid method for GABA measurement and its application to a wide variety of biological specimens.

EXPERIMENTAL

Materials

2,2[$^2H_2$]GABA (99%) was purchased from Merck Sharp & Dohme Canada, (Pointe Claire-Dorval, Quebec, Canada); GABA from Sigma (St. Louis, MO, U.S.A.), heptafluorobutyric anhydride from Regis Chemical (Morton Grove, IL, U.S.A.) and hydrogen chloride lecture bottle from Matheson Gas Products (Morrow, GA, U.S.A.). GABA and GABA-d_2 stock solutions of 10 mg/ml of deionized, distilled water were prepared and diluted to 20 ng/ml solutions. A stock solution of 1.5 *M* hydrochloric acid in cyclohexanol was prepared by bubbling hydrogen chloride gas from the lecture bottle into the cyclohexanol [22]. Human CSF specimens were obtained from University and Children's Hospitals (Cincinnati, OH, U.S.A.). Human platelets and plasma were received from the Hoxworth Blood Center (Cincinnati, OH, U.S.A.).

Sample preparation

Palates of day 14.5 embryos of AJ mice were dissected as previously described [16]. Brains and bodies from the same developmental age embryo were also used. Five brains or bodies were homogenized in 1 ml and 50 palate pairs were homogenized in 0.1 ml of deionized, distilled water using a Thomas tissue hand homogenizer (A.H. Thomas, Philadelphia, PA, U.S.A.). Aliquots were taken for protein determination by the method of Lowry et al. [23]. The remaining homogenates were diluted with ethanol to a final concentration of 80%. The internal standard, GABA-d_2 (100 ng), was added to the palate homogenate or aliquots (50—500 μl) of brain or body homogenates. Human plasma (1 ml), platelets (1 ml), and pooled child CSF (1 ml) were lyophilized with 100 ng GABA-d_2 and 0.5 ml of 80% ethanol was added. Samples were centrifuged at 10,000 *g* for 30 min. The supernatant was transferred to glass vials and evaporated to dryness by a stream of nitrogen at 80°C [22]. Other CSF specimens (200 μl) and all urine samples (200 μl), after addition of 100 ng of GABA-d_2, were directly evaporated to dryness at 80°C with nitrogen without lyophilization.

Sample derivatization

Cyclohexyl-GABA was prepared by adding 200 μl of 1.5 *M* hydrochloric acid in cyclohexanol and heating for 15 min at 115°C as previously described [22]. The mixture was evaporated to near dryness at 115°C with a stream of nitrogen and the residue was heated with 100 μl of heptafluorobutyl anhydride in 200 μl of ethyl acetate for 15 min at 80°C. The sample was then evaporated to dryness at 80°C and dissolved in 100 μl of methanol. The HFB-cyclohexyl-

GABA was stable in methanol at room temperature for over three years.

Gas chromatography—mass spectrometry

Initially, GABA quantitation was accomplished using a Finnigan 3200 electron-impact mass spectrometer interfaced to a Finnigan 9500 gas chromatograph. The system was equipped with a Teknivent 29K data system (Teknivent, St. Louis, MO, U.S.A.). A 3% OV-17 column was used for isothermal injections at 185°C [22]. In later experiments quantitation was accomplished using a Hewlett-Packard (HP) 5970A mass selective detector equipped with an HP 5790GC having a 12.5 m × 0.2 mm I.D., cross-linked dimethyl silicone capillary column. The system was interfaced to an HP 2671G printer, an HP 9825B computer and an HP 9134A Winchester disc drive for data storage. After injection of 1–4 μl of sample the column was programmed from 150°C to 275°C at 15°C/min, with GABA eluting at 188°C. Improved sensitivity and specificity were obtained using the capillary column. Quantitation was accomplished by monitoring the ions at *m/z* 282 (GABA) and *m/z* 284 (GABA-d_2) with *m/z* 254, 299 (GABA) and *m/z* 256, 301 (GABA-d_2) as confirming ions. For each day's analysis, levels of GABA were calculated from a standard curve employing at least five points containing 100 ng of GABA-d_2 and increasing amounts of GABA from 10 to 1000 ng.

RESULTS AND DISCUSSION

The mass spectra of derivatized GABA and 2,2[$^2H_2$]GABA are shown in Fig. 1a and b, respectively. The peaks at *m/z* 299 (GABA) and *m/z* 301 (2,2[$^2H_2$]-GABA), representing the $(M-82)^+$ ion, result from the loss of the cyclohexyl group with a hydrogen transfer [22]. A standard curve was prepared by plotting the peak height ratio of *m/z* 282 (GABA)/*m/z* 284 (GABA-d_2) versus increasing amounts of GABA from 10 to 1000 ng. Each sample contained 100 ng of GABA-d_2. A linear regression analysis of the data is described by the equation $y = a + bx$ where the intercept $a = 0.1241$, the slope $b = 0.0098$, and the correlation coefficient $r^2 = 0.9999$. Fig. 2 illustrates the selected ion recordings for GABA quantitation in the mouse embryonic brain, body, and palate obtained on the Finnigan system. HFB-cyclohexyl-GABA gave increased GC retention temperatures compared with other GABA derivatives, such as pentafluoropropionyl-methyl-GABA, HFB-heptafluoro-1-butanyl-GABA [19] and pentafluoropropionyl-hexafluoroisopropanyl-GABA [18]. The increased retention temperature of HFB-cyclohexyl-GABA has the advantage of effecting better separation from other substances which might interfere with the analysis. Pentafluoropropionyl-hexafluoroisopropanyl-GABA was not recommended for analysis of picomole quantities due to variable yields [21]. McCaman et al. [20] prepared the trimethylsilyl derivative of GABA and his procedure, like the work of Faull et al. [19], required a time-consuming ion-exchange column purification step prior to derivatization. Colby and McCaman [21], using the N-dinitrophenyl-ethyl-GABA, with a high GC retention temperature, analyzed GABA without prior ion-exchange column purification although eight extraction steps were employed prior to GC—MS injection.

Improved resolution and sensitivity were obtained using the capillary column.

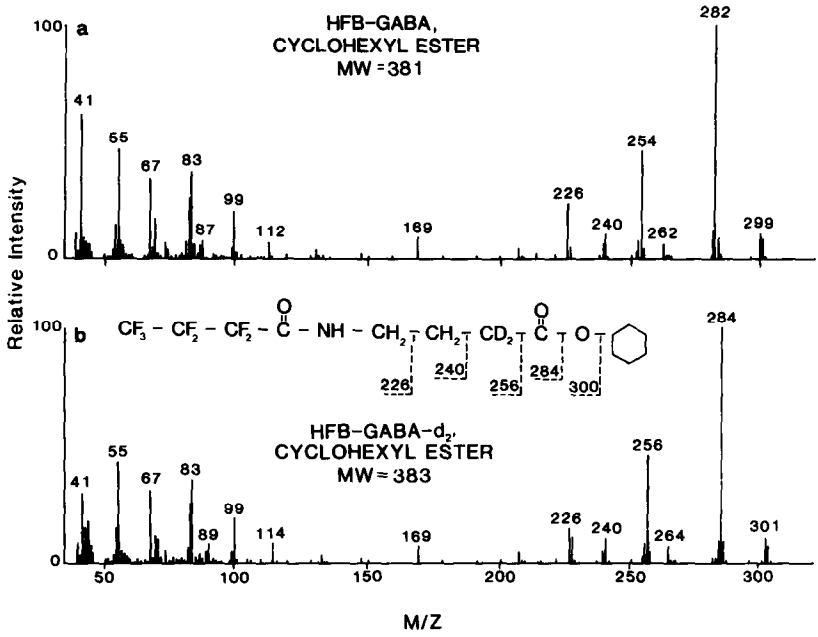

Fig. 1. Electron-impact mass spectra of (a) GABA and (b) GABA-d_2 heptafluorobutyryl cyclohexyl-GABA derivatives.

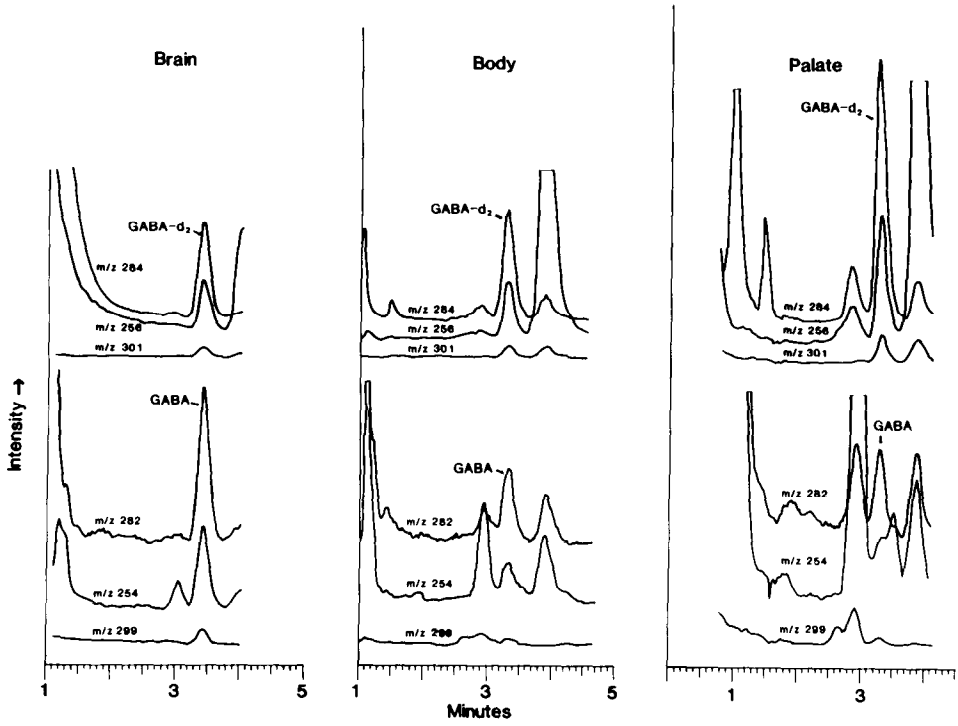

Fig. 2. Selected ion recordings for GABA quantitation in mouse embryonic brain, body, and palate obtained on the Finnigan 3200 GC—MS system.

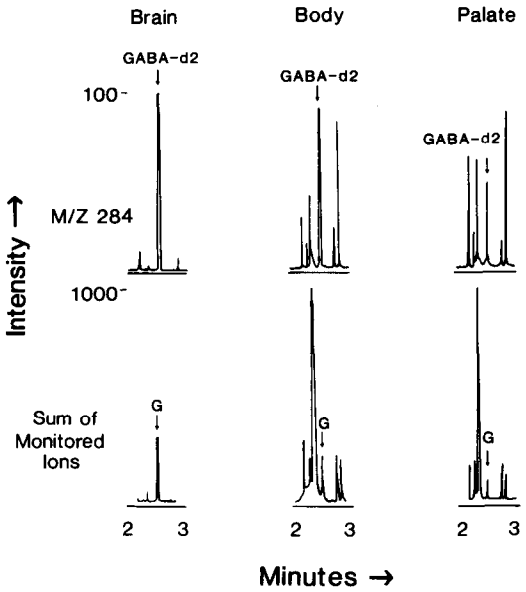

Fig. 3. Selected ion recordings for GABA quantitation in mouse embryo brain, body, and palate obtained on the HP 5970A GC—MS system. The top trace represents the recording for *m/z* 284 (GABA-d_1) and the bottom trace is the sum of the monitored ions. G = GABA (*m/z* 282, 254, 299) and GABA-d_2 (*m/z* 284, 256, 301).

Fig. 3 illustrates the selected ion recordings for GABA analysis of mouse embryonic brain, body, and palate specimens using the HP 5970A GC—MS system. Similar GABA quantitation was accomplished for various human body fluids as shown in Fig. 4. Table I lists the GABA concentration in the various biological specimens. The mean concentration of GABA in mouse embryonic brain of 6.54 is lower than the 14.1 nmol/mg of protein reported for mature mouse brain [24] as might be expected. Concentrations of GABA in mouse embryonic body or palate have not been previously reported. The greater concentration in the body than the palate is not surprising since recent reports have indicated high GABA levels in selected tissues such as pancreatic islets [7], ovary [8], and Fallopian tube [9]. The successful measurement of GABA in the palate illustrates the applicability of the method to the trace levels found in complex biological tissues.

GABA content in blood platelets has not previously been reported to our knowledge; however, GABA-α-oxoglutarate transaminase activity has been demonstrated [25]. GABA uptake in platelets from Down's syndrome patients was shown to be less than control [26]. Ferkany et al. [27] reported GABA concentration in pooled human plasma of 326 ± 28 (n = 7) pmol/ml which is comparable to our value. Stability studies with blood GABA have shown no significant changes in GABA content after 24 h at room temperature [27].

CSF GABA levels shown in Table I are higher than previously reported [4]. CSF GABA exists loosely bound and conjugated in forms such as homocarnosine [28, 29]. The increased GABA content of CSF specimens may reflect the enzymatic hydrolysis of GABA conjugates since CSF GABA increases rapidly at room temperature [30, 31]. More likely, the higher CSF

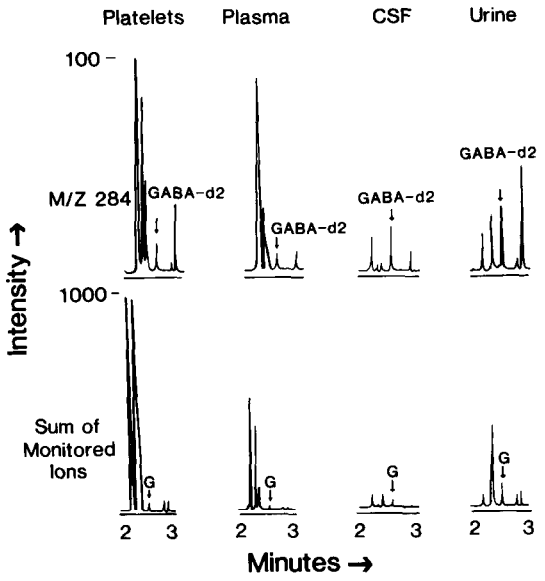

Fig. 4. Selected ion recordings for GABA quantitation in human platelets, plasma, CSF, and urine obtained on the HP 5970A GC—MS system.

TABLE I

LEVELS OF GABA IN VARIOUS BIOLOGICAL SPECIMENS

Specimen	*n*	GABA (nmol ± S.E.)
Mouse embryonic brain (nmol/mg of protein)	14	6.54 ± 0.29
Mouse embryonic body (nmol/mg of protein)	9	0.59 ± 0.02
Mouse embryonic palate (nmol/mg of protein)	9	0.19 ± 0.10
Human platelets (nmol/ml)	2	0.55 ± 0.05
Human plasma (nmol/ml)	4	0.41 ± 0.02
Human CSF adult (nmol/ml)	4	1.08 ± 0.13
Human CSF children pooled (nmol/ml)	1	1.19
Human urine (nmol/mg of creatinine)	2	3.41 ± 0.40

GABA levels shown in Table I result from the derivatization procedure; the acid conditions used for deproteinization liberate loosely bound GABA [28]. Some conjugated GABA may also be hydrolyzed during derivatization. To avoid pitfalls from artifactual increases in free CSF GABA levels, Grove and co-workers [31, 32] and Böhlen et al. [33] suggested that total GABA released, after hydrolysis, representing conjugated plus free, would be a more reliable clinical indicator of brain GABA concentration. In a recent review, Grove et al. [34] concluded that there are no advantages and many disadvantages to measuring free GABA instead of total GABA concentration. The GABA concentration obtained for human urine was 3.41 nmol/mg of creatinine. The clinical significance of GABA excretion is not known.

We have presented a rapid, reliable method for GABA measurement in a wide variety of biological specimens through the GC—MS—SIM analysis of HFB-cyclohexyl-GABA. This methodology provides researchers and clinicians

with the means to study the metabolic role of GABA and to determine the diagnostic importance of GABA in detecting disease states.

REFERENCES

1 E. Roberts, T.M. Chase and D.B. Tower (Editors), GABA in Nervous System Function, Raven Press, New York, 1976, p. 1.
2 W. Löscher, D. Rating and H. Siemes, Epilepsia, 22 (1981) 697.
3 H. Kuroda, N. Ogawa, Y. Yamawaki, I. Nukina, T. Ofuji, M. Yamamoto and S. Otsuki, J. Neurol., Neurosurg. Psychiatry, 45 (1982) 257.
4 T.L. Perry, S. Hansen, R.A. Wall and S.G. Gauthier, J. Neurochem., 38 (1982) 766.
5 R.J. Abbott, I.F. Pye and S.R. Nahorski, J. Neurol., Neurosurg. Psychiatry, 45 (1982) 253.
6 K. Kasa, S. Otsuki, M. Yamamoto, M. Sato, H. Kuroda and N. Ogawa, Biol. Psychiatry, 17 (1982) 877.
7 Y. Okada, H. Taniguchi and C. Shimada, Science, 194 (1976) 620.
8 S.L. Erdö, J. Neurochem., 40 (1983) 582.
9 S.L. Erdö, B. Rosdy and L. Szporny, J. Neurochem., 38 (1982) 1174.
10 D.F. Schafer, J.M. Fowler and E.A. Jones, Proc. Soc. Exp. Biol. Med., 167 (1981) 301.
11 B. Haber, K. Kuriyama and E. Roberts, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 28 (1969) 577.
12 M. Zachmann, P. Tocci and W.L. Nyhan, J. Biol. Chem., 241 (1966) 1355.
13 J. Hedner, T. Hedner, B. Bergman and D. Lundberg, J. Develop. Physiol., 2 (1980) 401.
14 F.V. DeFeudis, Pharmacol. Res. Commun., 14 (1982) 567.
15 G. Sparagli and F. Pavan, Neuropharmacology, 11 (1972) 45.
16 E.L. Wee and E.F. Zimmerman, Teratology, 28 (1983) 15.
17 N. Seiler, S. Sarhan and B.F. Roth-Schechter, Develop. Neurosci., 4 (1981) 181.
18 L. Bertilsson and E. Costa, J. Chromatogr., 118 (1976) 395.
19 K.F. Faull, J.R. DoAmaral, P.A. Berger and J.D. Barchas, J. Neurochem., 31 (1978) 1119.
20 M.W. McCaman, B.N. Colby and R.E. McCaman, J. Neurochem., 33 (1979) 967.
21 B.N. Colby and M.W. McCaman, Biomed. Mass Spectrom., 5 (1978) 215.
22 E.J. Norman, H.K. Berry and M.D. Denton, Biomed. Mass Spectrom., 6 (1979) 546.
23 O.H. Lowry, N.J. Rosebrough, A.F. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
24 R. Bernasconi, H. Bittiger, J. Heid and P. Martin, J. Neurochem., 34 (1980) 614.
25 H.L. White, Science, 205 (1979) 696.
26 L. Enns and E.E. McCoy, Brit. J. Pharmacol., 71 (1980) 553.
27 J.W. Ferkany, L.A. Smith, W.E. Seifert, Jr., R.M. Caprioli and S.J. Enna, Life Sci., 22 (1978) 2121.
28 T.N. Ferraro, B.V. Manyam and T.A. Hare, J. Neurochem., 41 (1983) 1057.
29 J.J. Ohisalo, K. Murros, B.B. Fredholm and T.A. Hare, Arch. Neurol., 40 (1983) 623.
30 W. Löscher and I. Ahnfelt-Ronne, J. Neurochem., 39 (1982) 251.
31 J. Grove, P.J. Schechter, G. Tell, L. Rumbach, C. Marescaux, J.M. Warter and J. Koch-Weser, J. Neurochem., 39 (1982) 1061.
32 M.G. Palfreyman, S. Huot and J. Grove, Neurosci. Lett., 35 (1983) 161.
33 P. Böhlen, S. Huot, M. Mellet and M.G. Palfreyman, Brain Res. Bull., 5 (1980) 905.
34 J. Grove, M.G. Palfreyman and P.J. Schechter, Clin. Neuropharmacol., 6 (1983) 223.