ELSEVIER

Journal of Chromatography B, 708 (1998) 49–54

JOURNAL OF CHROMATOGRAPHY B

Determination of γ-aminobutyric acid in microdialysis samples by microbore column liquid chromatography and fluorescence detection[1]

Jan Kehr

Department of Neuroscience, Karolinska Institute, Doktorsringen 12, plan 6, 171 77 Stockholm, Sweden

Received 25 September 1997; received in revised form 19 December 1997; accepted 19 December 1997

Abstract

A method for the determination of γ-aminobutyric acid (GABA) at concentrations as low as 1.5 n*M* in microdialysis samples is described. A high-performance liquid chromatography (HPLC) autosampler was used for precolumn derivatizations of samples with phthalaldehyde–2-mercaptoethanol reagent, as well as for automated injections of acetonitrile to wash the microbore column between injections. This permitted the use of only one isocratic pump and reduced the run-to-run analysis time to below 20 min, without any risk for late-eluting peaks. Three different microbore columns were evaluated. The optimal column was a 150×1 mm I.D., C_{18} reversed-phase silica column with a particle size of 3 μm, operated at a flow-rate of 50 μl/min. The mobile phase was 0.1 *M* sodium acetate buffer, pH 5.4, containing 20% acetonitrile. Under these conditions, the column exhibits a low back-pressure of about 11.1 MPa and is usable for at least 1000 injections. Good correlation was found between fluorescence and electrochemical detector responses for *o*-phthaldialdehyde–GABA derivatives, although the latter detector type generally required more skill and maintenance for routine use. Microdialysis samples of 10–20 μl, recovered from various rat brain nuclei, were assayed using the described method.

Keywords: γ-Aminobutyric acid

1. Introduction

γ-Aminobutyric acid (GABA) is one of the most thoroughly documented neurotransmitters in the central nervous system and is used by as many as 40% of all neurones. It is located predominantly in the cortex, basal ganglia, hippocampus, hypothalamus, amygdala, cerebellum, medulla and spinal cord [1]. Microdialysis permits the monitoring of extracellular GABA levels as a reflection of GABA release and uptake under various pharmacological, physiological or pathological stimuli. It has been reported previously that at least 50% of GABA overflow sampled by microdialysis originates from synaptic terminals [2], thus representing the neuronal pool involved in signal transduction.

Determination of GABA in microdialysis samples requires an ultrasensitive analytical method, since the basal GABA levels in a typical microdialysis sample are often in the range of 0.1–0.5 pmol (5–50 n*M*). An isocratic high-performance liquid chromatog-

[1]Part of this work was done at CMA/Microdialysis AB, Stockholm, Sweden.

0378-4347/98/$19.00
PII S0378-4347(97)00657-9

raphy (HPLC) method, based on electrochemical detection of GABA derivatized with *o*-phthaldialdehyde (OPA)–2-methyl-2-propanethiol and conventional reversed-phase C_{18} columns has been described [3]. The limit of detection was 50 fmol for GABA, with a retention time of 3.5–4 min. However, several other peaks, corresponding to an excess of thiol from the derivatization reagent, ammonia, ethanolamines and other unidentified compounds, eluted after the GABA derivative. The main drawback of this method was the shortened life-time of the glassy carbon working electrode, reflected by a gradually increasing background signal, from about 5 to 100 nA in a few months. This was most probably due to a high concentration of acetonitrile (50%) or its electroactive impurities in the resulting mobile phase. The whole system was particularly susceptible to contamination and often gave rise to unexplainable artefacts. In addition, the considerable odour of the 2-methyl-2-propanethiol reagent could cause some practical problems with the location of the HPLC apparatus, and with storage and handling of the reagent, etc.

Using fluorescence detection of OPA–2-mercaptoethanol (MCE)-derivatized amino acids, the detector can be left unattended for at least 1500–2000 h (half-life of a lamp). However, the limit of detection for GABA is only 0.5–1 pmol/sample for a typical chromatographic separation on a normal bore reversed-phase column with gradient elution [4]. To achieve GABA detection levels below 100 fmol, it is thus necessary to ''scale down'' the chromatographic system by using a microbore column. The system can be further simplified by using isocratic separation with an automated wash-out step between injections.

2. Experimental

2.1. Instruments and chemicals

A microbore LC system comprises a CMA/250 LC pump, a CMA/260 degasser, a CMA/200 refrigerated microsampler, a CMA/280 fluorescence detector equipped with a 6-μl cell (all from CMA/Microdialysis, Stockholm, Sweden). Originally, a low pressure switching valve was installed directly at the pump inlet, allowing one-step gradient elutions, as described elsewhere [5]. Later, a modified version of the autosampler program was used for in-between injections of acetonitrile as a fast eluent for column wash.

The CMA/280 fixed wavelength detector operated at a maximal excitation of 330–365 nm and emission of 440–530 nm. Data were recorded by an EZChromdata acquisition system (Scientific Software, San Ramon, CA, USA). For microbore separations, the following columns were used: (A) 100×1 mm I.D., C_{18} silica, 3 μm particle size (Bioanalytical Systems, West Lafayette, IN, USA), (B) 100×1 mm I.D., C_{18} silica, 5 μm particle size (BAS, Tokyo, Japan), (C) 150×1 mm I.D., C_{18} silica, 3 μm particle size (BAS). The mobile phases for all three columns consisted of 0.1 *M* sodium acetate buffer, pH 5.4 containing 12% acetonitrile (ACN) and 2.5% 1-propanol (columns A and B) or 20% ACN (column C). Flow-rates were 70 μl/min for column A, 90 μl/min for column B and 50 μl/min for column C.

Amino acid standard solutions (protein hydrolysate, AA-S-18; physiological basics, B; acidics and neutrals, AN; acidics, neutrals and basics, ANB), OPA solution (OPA incomplete) and 2-mercaptoethanol (MCE) were purchased from Sigma (St. Louis, MO, USA), sodium acetate, acetonitrile and methanol were from Merck (Darmstadt, Germany). The MCE stock solution was prepared by diluting 100 μl of concentrated MCE with 900 μl of methanol. The working OPA–MCE reagent was prepared fresh daily by pipetting 14 μl of MCE stock solution into 1 ml of OPA incomplete in a 1.5-ml glass vial. The vial was sealed with a silicone rubber septum and placed in the first reagent position in the autosampler. Another vial was filled with 1.5 ml of ACN and placed in the second reagent position. Usually, 10 (or 20) μl of a microdialysis sample were mixed with 1 (or 3) μl of OPA–MCE reagent, and 9 (or 20) μl were injected onto the column using either a 10-μl or a 20-μl injection loop. The reaction time was 60 s at +6°C. Following an analysis time of 2 min 30 s, 10 (or 20) μl of the second reagent (ACN) were pipetted into the loop and allowed to remain there for an additional 13 min 30 s. This ''wait in loop'' time was required to delay the injection of ACN until the GABA peak was eluted out of the column (about 14 min). Usually, 10 μl

injections of ACN were sufficient for the fast elution of remaining amino acids out of the column and for its reequilibration within the 2 min and 30 s period.

3. Results and discussion

The separation of GABA in a mixture of physiological amino acids using column A is shown in Fig. 1a. The chromatogram in Fig. 1b illustrates a typical microdialysis sample from rat brain (striatum) under basal conditions. The identity of the GABA peak was verified by varying the composition and concentrations of the amino acid standards, spiking microdialysis samples with GABA standard and analyzing samples taken during various physiological stimuli [5].

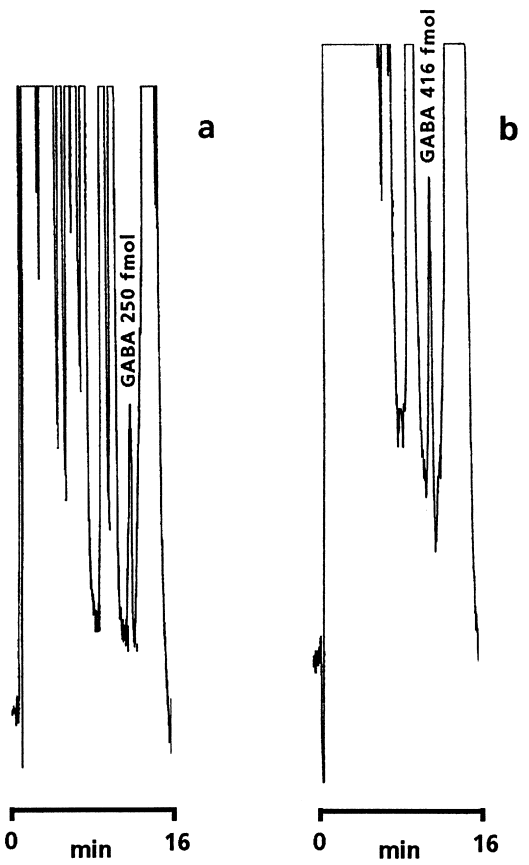

Fig. 1. Chromatograms of: (a) a 10-μl standard mixture containing more than 30 different physiological amino acids at concentrations of 500 n*M*, spiked with 25 n*M* GABA. An elution mode based on a one-step gradient on microbore column A was used. After sample derivatization and injection, the low-pressure valve was switched after 11 min 30 s to a second mobile phase (95% ACN, 2.5% propanol, 2.5% water) for 60 s. (b) a 10-μl volume of a typical microdialysis sample from rat striatum. A CMA/12 probe with a membrane length of 2 mm was perfused with Ringer's solution at 2 μl/min.

In addition, an excellent correlation ($r=0.999$, $n=10$) was found between GABA levels detected by the method described and those obtained using an electrochemical detection method [3]. However, the microbore column (A) has a relatively short life-time, which lead to a gradual increase in the column back-pressure. This was in spite of all the efforts made to carefully prepare and filter the mobile phases and the very clean nature of microdialysis samples. In fact, a new column has a back-pressure of about 15.2 MPa at a flow-rate of 70 μl/min. After some 500–700 injections, the back-pressure reached 25.3–30.4 MPa and, in several cases, was above the highest operating pressure of the non-metallic HPLC pump (35.5 MPa). After testing several batches of A-type columns, it was concluded that the change in the microbore column was caused by the poor mechanical stability of the packing material, which was unable to withstand repeated one-step gradient flushes, rather than by operational conditions, such as dust particles or crystals clogging the inlet of the column.

Therefore, a column of type B, which had with higher permeability and a more rigid (5 μm particle size) packing material, was tested. This column operates at back-pressures of only 6.1 MPa under the same chromatographic conditions as used with column A. Representative chromatograms for the separation of GABA in a standard mixture and in rat striatal dialysates are shown in Fig. 2a,b. Although a baseline separation of GABA can be obtained from a standard mixture, when the microdialysis sample is run, the column capacity is clearly overloaded by high concentrations of amino acids that elute before and after the GABA peak (Fig. 2b).

Similar results were obtained on a 100×1 mm I.D. column that was packed with the same material but had a particle size of 3 μm. It was necessary to increase the efficiency of the separation by using a longer (150×1 mm I.D.) microbore column (C) and a flow-rate that was reduced to 50 μl/min. Under these conditions, the C-type column could last for at

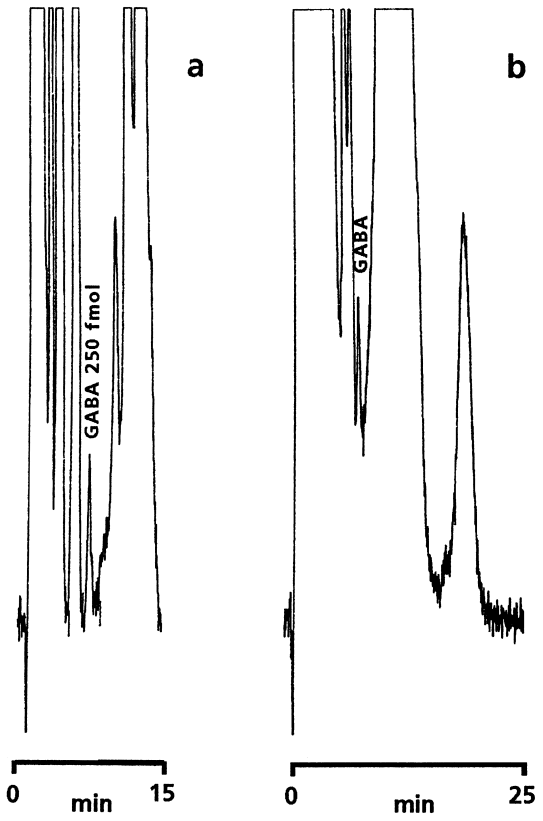

Fig. 2. Chromatograms of the same standard and microdialysis samples as in Fig. 1, separated on a 100×1 mm microbore column (B), packed with 5 μm C_{18} reversed-phase silica. In this case, switching of the low-pressure valve to ACN eluent occurred between 7 and 9 min.

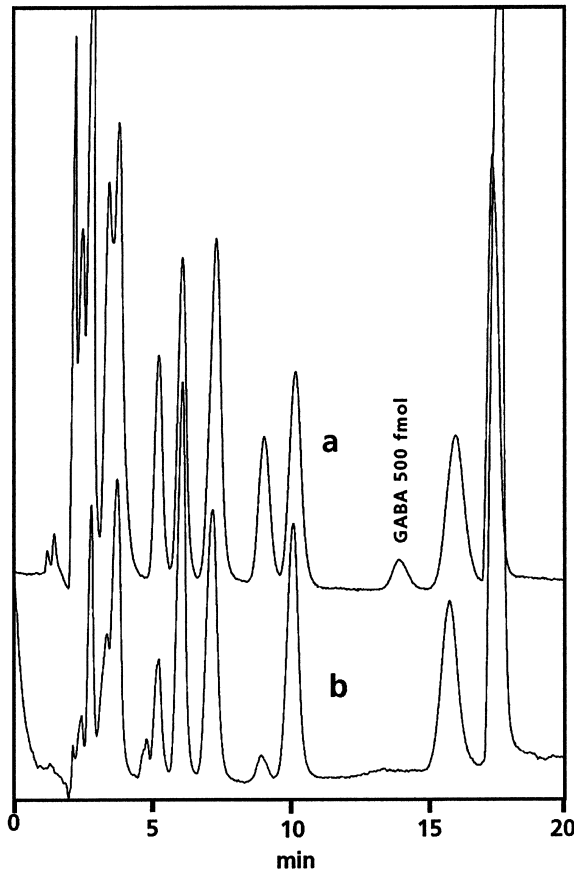

Fig. 3. Isocratic separations on microbore column C using automated wash-out steps between injections: (a) a 10-μl volume of an amino acid standard mixture (AN) at concentrations of 250 n*M* each, spiked with 500 fmol of GABA, and (b) a 10-μl volume of AN standard alone (not containing GABA).

least 1000 injections. During this time, the back-pressure was stable and did not exceed 11.1 MPa. Fig. 3a,b shows chromatograms of (a) 10 μl of amino acid standard mixture (AN) at concentrations of 250 n*M* each, spiked with GABA at a final concentration of 50 n*M*, and (b) 10 μl of AN standard alone (not containing GABA). As seen in Fig. 3b, no peak occurred at the position of GABA elution at 14 min. Also, injections of other standards (GABA alone, AA-S-18, B, ANB) and blank samples confirmed the complete separation of GABA from other physiological amino acids. An excellent linear relationship was observed between the concentration of GABA and the peak area, in the range from 5 n*M* to at least 1 μ*M* GABA, for both 10 and 20 μl sample volumes ($r=0.998$). The coefficients of variation (C.V.) for five injections (20 μl of sample each) containing 5 or 100 n*M* GABA in 250 n*M* AN standard were 13.4 and 2.3%, respectively. Similarly, the mean peak area of 50 n*M* GABA standard alone or in 250 n*M* AN varied by 6.4% for thirteen calibrations performed during a continuous run over eight days. Thus, column C was chosen for all further studies.

Separation of a typical microdialysis sample from rat brain (striatum) under basal conditions is shown in Fig. 4a. A CMA/12 Microdialysis probe with a membrane length of 2 mm was perfused with Ringer's solution at 2 μl/min. The GABA peak corresponds to 46.2 n*M*, i.e. 462 fmol in a 10-μl

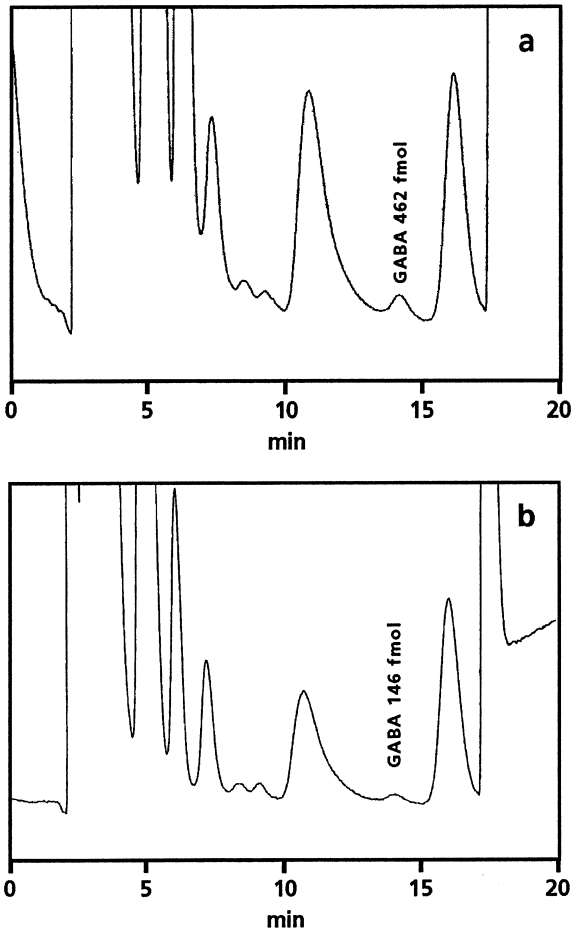

Fig. 4. GABA levels estimated in some typical microdialysis samples from rat brain: (a) striatum, 462 fmol of GABA, and (b) substantia nigra, 146 fmol of GABA.

perfusate. The chromatogram in Fig. 4b illustrates a similar case where a microdialysis sample was collected from a much smaller nucleus (substantia nigra). Here, GABA was recovered at a concentration of only 14.6 n*M*. However, GABA could still be clearly quantified, as illustrated in the window showing a zoomed GABA peak in Fig. 6.

Similarly, perfusing other brain areas, such as the hippocampus, provides microdialysis samples with GABA concentrations at levels of around 10 n*M*. For reproducible analysis of such low concentrations, it is useful to increase the sample volume up to 20 μl, although this means overloading the microbore column (C), thus causing a dramatic reduction in its separation efficiency. However, it is still possible to achieve baseline separations of GABA in both amino acid standards and microdialysis samples, as illustrated in Fig. 5a,b. Furthermore, routine determinations of GABA concentrations at levels of 2–5 n*M*/sample are easily achievable, as shown in an enlarged part of a previous chromatogram (Fig. 6). The limit of quantitation at a signal-to-noise ratio of two was 1.14 n*M*, or 23 fmol, in a 20-μl sample, which corresponds to 20 fmol of GABA injected onto the column. This should be completely satisfac-

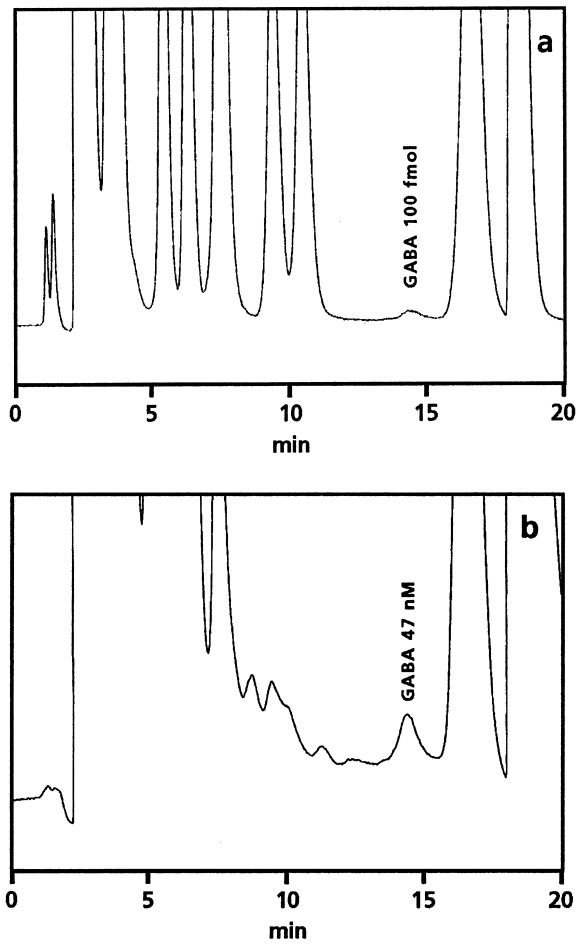

Fig. 5. Separations of GABA in 20 μl sample volumes: (a) the same standard mixture as in Fig. 1a, except that physiological amino acids at concentrations of 250 n*M* were spiked with 5 n*M* GABA, (b) microdialysis sample from rat striatum collected under the same conditions as in Fig. 1b but using the microbore column (C).

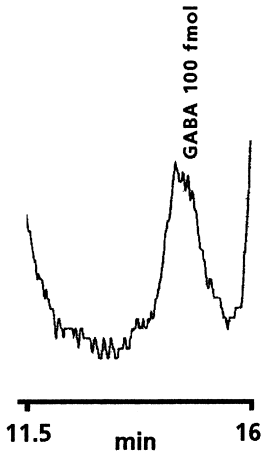

Fig. 6. An enlarged part of the chromatogram shown in Fig. 5a, showing a peak of 100 fmol of GABA and the corresponding noise level.

tory for most microdialysis experiments conducted on small laboratory animals.

References

[1] F. Fonnum, Z. Gottesfeld, I. Grofova, Brain Res., 143 (1978) 125.
[2] P.G. Osborne, W.T. O'Connor, J. Kehr, U. Ungerstedt, J. Neurosci. Methods, 37 (1991) 93.
[3] J. Kehr, U. Ungerstedt, J. Neurochem., 51 (1988) 1308.
[4] E. Westerberg, J. Kehr, U. Ungerstedt, T. Wieloch, Neurosci. Res. Commun., 3 (1988) 151.
[5] J. Kehr, Application Note no. 19, CMA/Microdialysis, Stockholm, 1994, p. 4.