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Note

Simultaneous determination of γ-aminobutyric acid and polyamines by high-performance liquid chromatography

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Several lines of evidence indicate the occurrence and the physiological importance of γ-aminobutyric acid (GABA) both in the nervous system as a neurotransmitter and in peripheral tissues of mammals [1–4]. The main biosynthetic route of GABA production is via glutamate decarboxylation. However, GABA may be also formed in the oxidative degradation of the diamine putrescine [5]. How far this may represent an alternative pathway with physiological importance is the object of active studies.

Putrescine, its metabolic derivatives spermidine and spermine, and cadaverine are aliphatic amines ubiquitously present both in prokaryotic and eukaryotic cells, and appear to be involved in crucial metabolic events [6–8].

Numerous analytical methods have been devised for the separation and/or quantitation of GABA: radioreceptor assay [9], the enzymic "GABA-ase" method [10], thin-layer chromatography with fluorescence detection [11], and high-performance liquid chromatography (HPLC), followed by electrochemical [12] or spectrofluorimetric detection [13].

The di- and polyamines are most often assayed by very sensitive and specific HPLC methods with fluorimetric detection, based on their separation as ion pairs with post-column derivatization [14] or as dansyl derivatives [15–23].

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In this paper we describe a fast, reproducible and specific procedure for the simultaneous determination of dansyl derivatives of GABA, diamines (putrescine, cadaverine) and polyamines (spermidine, spermine) in rat brain samples, and preliminary data on some peripheral tissues are reported.

EXPERIMENTAL

Chemicals

GABA, diamine and polyamine hydrochloride salts and 1,7-diaminoheptane were obtained from Sigma (St. Louis, MO, U.S.A.). Spectrophotometric-grade acetonitrile was from Merck (Darmstadt, F.R.G.). All other chemicals were analytical-reagent grade.

Apparatus

HPLC was performed on a Perkin-Elmer (Norwalk, CT, U.S.A.) chromatograph composed of two Series 10 pumps, equipped with a loop injector valve with a 50-μl loop and a solvent programmer Model Series 10 LC controller. The analyses were performed on a LiChrosorb RP-18 column (25 cm$\times$0.4 cm I.D.) filled with a 5-μm C_{18} reversed-phase packing (Merck). Detection was accomplished using a 204-A fluorescence spectrophotometer (Perkin-Elmer) with a continuous-flow cell (excitation wavelength 340 nm, emission 545 nm). The signal of the fluorescence detector was recorded by an LCI-100 computing integrator equipped with a time-event module (Perkin-Elmer).

Animals and sample preparation

Male Sprague–Dawley rats weighing ca. 200 g were used. The tissues were rapidly removed from decapitated animals, frozen in liquid nitrogen and processed as previously reported [23]. After homogenization with 0.2 *M* perchloric acid (10%, w/v) in the presence of 25 nmol of 1,7-diaminoheptane as internal standard, the samples were centrifuged at 8000 *g* for 15 min. Aliquots of supernatants were adjusted to pH 9.5–10.5 with 1.5 *M* sodium carbonate buffer and added to 1.5 volumes of dansyl chloride (15 mg/ml acetone). Derivatization was carried out overnight at room temperature, and the dansyl amides were extracted with an equal volume of benzene. The benzene phase was then removed and evaporated, and the residue dissolved in methanol. Before the injection, the samples were filtered through 0.45-μm membrane filters for organic solvents (Millex HV, Millipore, Molsheim, France). The same procedure was followed for the external standard, which was prepared from a stock solution in which GABA and each amine (putrescine, cadaverine, spermidine, spermine) were $4 \cdot 10^{-4}$ *M* in 0.2 *M* perchloric acid. GABA was separated in the form of N-dansyl-2-pyrrolidinone, that is the reaction product of GABA with the excess of dansyl chloride [16,24].

Chromatography

The gradient programme is reported in Table I. The pumps, controlled by a solvent programmer, delivered the mobile phase through the analytical column at a flow-rate of 1.8 ml/min. The samples were eluted with a gradient of 40–99%

TABLE I

COMPOSITION OF THE GRADIENT

Solvent A consisted of 1.2 m*M* disodium hydrogenphosphate; solvent B consisted of 80% acetonitrile and 20% 1.2 m*M* disodium hydrogenphosphate.

	Elution time (min)	Percentage of solvent	
		A	B
	0.0	60	40
	5.5	27	73
	11.5	27	73
	16.0	1	99
End time	24.0		
Equilibration time	8.0		

solvent B in A, which was interrupted and followed by two isocratic periods of 6 and 8 min, respectively.

RESULTS

Fig. 1 shows typical chromatograms of standard (A), rat brain (B), rat liver (C) and rat kidney (D) samples, obtained under the conditions indicated in Table I.

The fluorescence intensity was measured at the same sensitivity for dansyl derivatives of GABA, diamines (putrescine, cadaverine) and polyamines (spermidine, spermine). An integrator coupled to the fluorimeter quantified the peaks and was calibrated every day with a standard mixture of 250 pmol of each compound to obtain the values of the response factors, in order to be able to establish the relationship between peak areas and concentrations. The quantification was based on the ratio between the peak areas of the compounds to be determined and that of the internal standard, taking into account the response factors. The recovery was measured by adding known amounts (10–50 nmol) of GABA and each diamine and polyamine to a fraction of tissue homogenate and subtracting the values of the control (a fraction of tissue homogenate with no addition). Using this procedure, the recovery was 92–98% for all the tissues studied (results not shown).

The chromatograms showed satisfactory resolution and symmetrical peak shapes, both with the standard and tissue extracts. The retention times (Fig. 1A) were 4.60 min for GABA (as N-dansyl-2-pyrrolidinone), 10.69 min for putrescine, 11.62 min for cadaverine, 14.74 min for 1,7-diaminoheptane, 17.80 min for spermidine and 22.40 min for spermine. They were constant in repeated analyses and exactly the same for the standard and tissue samples. The standard amount applied to the column corresponded to 125 pmol for each compound examined. Under the chromatographic conditions used, spermidine and spermine were strongly retained on the column during the initial gradient portion of the pro-

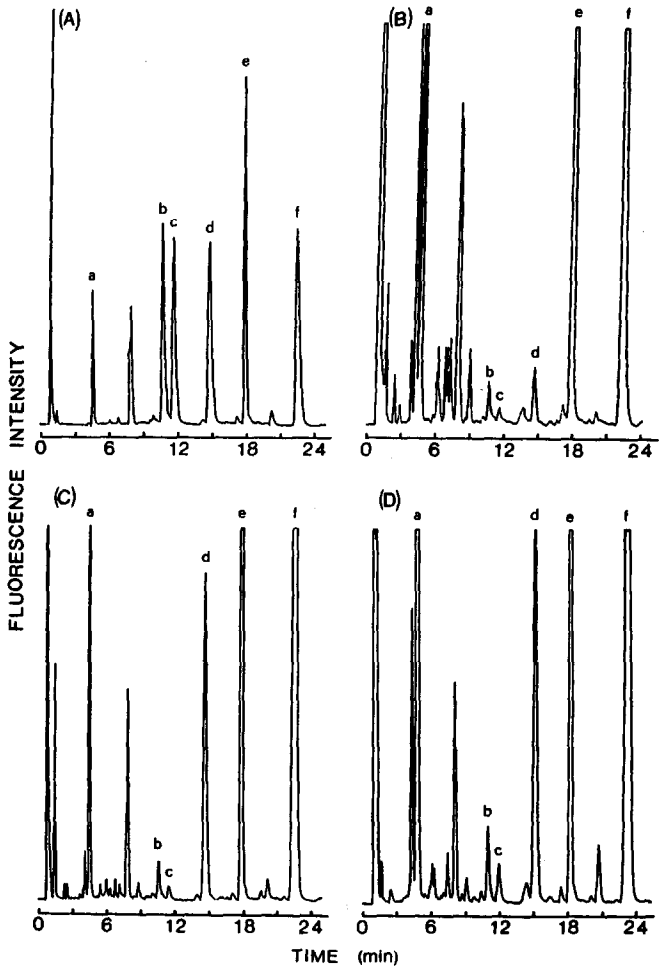

Fig. 1. Chromatograms obtained from dansylated (A) standard mixture (125 pmol of each compound), (B) rat brain, (C) rat liver and (D) rat kidney. Peaks: a = GABA (N-dansyl-2-pyrrolidinone); b = putrescine; c = cadaverine; d = 1,7-diaminoheptane; e = spermidine; f = spermine. For details of separation conditions see Experimental.

gramme, and eluted during the final isocratic period, when the concentration of the organic solvent was the highest.

The limit of detection, which is defined as the minimum amount of dansylated GABA, diamines and polyamines injected that gives a signal-to-noise ratio of 2, was 10 pmol for GABA, 3 pmol for putrescine and cadaverine and 1 pmol for spermidine and spermine. The sensitivity of this separation is comparable with that obtained with previously used methods, in which GABA and polyamines were assayed separately by reversed-phase HPLC with fluorescence detection [13,14,23].

When applied to rat tissues (Fig. 1B–D), our technique revealed no interference due to the possible presence of amino acids and endogenous GABA deriva-

TABLE II

GABA, DIAMINE AND POLYAMINE CONCENTRATIONS IN BRAIN, LIVER AND KIDNEY OF RAT

The sample preparation and the chromatographic conditions are described in the Experimental section. Each value represents the mean ± S.D. of four samples.

Tissue	GABA (μmol/g of tissue)	Polyamines (nmol/g tissue)			
		Putrescine	Cadaverine	Spermidine	Spermine
Brain	1.19 ± 0.12	9.69 ± 0.79	1.62 ± 0.14	225.23 ± 15.77	118.63 ± 7.98
Liver	0.88 ± 0.09	39.27 ± 3.01	16.07 ± 1.48	894.85 ± 63.53	844.50 ± 50.63
Kidney	2.23 ± 0.25	52.53 ± 4.15	14.05 ± 1.17	492.82 ± 34.00	672.52 ± 44.39

tives. The chromatograms correspond to 2.4 mg of brain, 1.0 mg of liver and 1.1 mg of kidney.

Table II reports the values of cerebral, hepatic and renal GABA, putrescine, cadaverine, spermidine and spermine. The concentrations of GABA in the brain and of diamines and polyamines in peripheral tissues were in good agreement with published data [22,23,25,26]. The values of GABA found in the liver and the kidney were higher than those reported in the literature [25,26], and thus it was possible that some tissutal component interfered in the separation of the cyclic dansyl derivative of GABA in these tissues. In the brain this interference is not substantial owing to the very high cerebral GABA concentrations.

DISCUSSION

We present here a method that allows the rapid (24 min only) and simultaneous quantification from brain extracts of GABA and putrescine, cadaverine, spermidine and spermine. They were assayed as dansyl derivatives by reversed-phase HPLC using a simple mobile phase of a water solution and an organic solvent. The reproducibility and sensitivity of the method were excellent. The advantage was that in a single assay it was possible to separate compounds with different polarity, without time-consuming preparation, and to assay various tissue samples for each determination. This method could be suitable for studying the regulatory interactions between GABA and polyamines in the brain, since it is known that alterations of GABA levels cause changes in the activities of the principal enzymes involved in polyamine metabolism [27–30].

Experiments designed to exclude the possible interference of other compounds in the assay of GABA in the liver and the kidney are in progress to allow the practical application of this method to peripheral tissues.

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