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Rapid determination of β-aminoisobutyric acid by reversed-phase high-performance liquid chromatography

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SUMMARY

For the determination of β-aminoisobutyric acid (BAIBA) in urine samples in which the β-alanine concentrations are higher than those of BAIBA, the resolution between these two amino acids, separated by reversed-phase liquid chromatography on an octadecylsilane column, was optimized. The chromatographic analysis included precolumn derivatization of amino acids with *o*-phthalaldehyde, followed by a 15-min isocratic elution and detection at 340 nm. Because of its simplicity, this method should be useful for monitoring urinary excretion of BAIBA.

INTRODUCTION

2-Aminoisobutyric acid (β-aminoisobutyric acid; BAIBA), a non-protein amino acid, is a product of thymine metabolism from both DNA and transfer RNA [1–3] and has been identified in human urine by Crumpler et al. [4] and

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Fink et al. [5]. BAIBA is excreted in small amounts in the urine of normal human subjects. Increased urinary excretion of BAIBA has been observed in subjects with cancer [6] or exposed to radiation [7] or lead [8,9].

In northern Mexico, in the area La Region Lagunera, the inhabitants are chronically exposed to high concentrations of arsenic (0.24–1.0 mg/l in the drinking water) [10], which has increased the incidence of skin cancer [11]. In a pilot study we are monitoring the urinary excretion of BAIBA in a group of these subjects, in order to determine if an increase in BAIBA levels in urine constitutes a biochemical indicator of the effects of arsenic on health. The results in this study will be published elsewhere.

In previous work [9,12–15] ion-exchange chromatography was used to determine BAIBA. We found some inconveniences with these methods: if the urine samples contained concentrations of β-alanine (β-Ala) higher than that of BAIBA, it was not possible to separate these two compounds with the dual-column method described by Kuo et al. [12], as the resolution R_s was only 1.0 [16]. Buschman et al. [13] overcame this interference by using two columns. A Radial-Pak column was used first in order to prefractionate the sample, then the fraction containing BAIBA (with relatively small amounts of β-Ala) was injected onto a second cation-exchange column where it was completely separated. Using an automated cation-exchange column chromatographic procedure, Van Gennip et al. [14] reported elution times of 67 min for β-Ala and 70 min for BAIBA at 47°C.

We describe in this paper a reversed-phase high-performance liquid chromatographic (HPLC) method using an octadecylsilane column. With this system we were able to obtain a clean separation between the amino acids present in human urine, particularly between β-Ala and BAIBA, which was optimized to give a resolution $R_s = 6.8$. The total analysis time is 18 min.

EXPERIMENTAL

Apparatus

A System Gold liquid chromatographic system from Beckman (San Ramon, CA, U.S.A.) was combined with (a) a Model 126 programmable solvent module with an Altex 210A injection valve, (b) a Model 166 programmable UV–VIS detector module, (c) a NEC PC 8300 controller system and (d) a Model 427 integrator. An Ultrasphere ODS (5 μm) column (250 mm × 4.6 mm I.D.) protected by an Ultrasphere ODS (5 μm) guard column (45 mm × 4.6 mm I.D.), both from Beckman, was used for separation. Samples and solvents were filtered through 0.22-μm porous membranes (XX3001200 and GVWP 04700; Millipore, Bedford, MA, U.S.A.).

Reagents

HPLC-grade water was obtained from deionized water with a Norganic filter apparatus (Millipore XX1504-710). Methanol (LiChrosolv grade) was obtained from Merck (Darmstadt, F.R.G.), sodium acetate from Allied Chemical (Morristown, NJ, U.S.A.) and *o*-phthalaldehyde (OPA) from Beckman. HPLC-grade glacial acetic acid was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Individual crystalline samples of L-amino acids were obtained from Pierce (Rockford, IL, U.S.A.; AMAC Standard Kit No. 20065), β-alanine (Cat. No. 23, 972-0) from Aldrich (Milwaukee, WI, U.S.A.) and DL-β-aminoisobutyric acid (Cat. No. A-8504), L-α-amino-*n*-butyric acid (α-ABA) (Cat. No. A-6407) and γ-amino-*n*-butyric acid (γ-ABA) (Cat. No. A-6282) from Sigma (St. Louis, MO, U.S.A.).

Chromatographic procedure

Eluent A (50 m*M* sodium acetate buffer, pH 6.8) and eluent B (methanol) were degassed by vacuum filtration through a porous membrane (0.22 μm). Stock standard solutions of β-Ala, γ-ABA, BAIBA and α-ABA were prepared by dissolving each compound in water to a final concentration of 100 μmol/ml. Working standard solutions containing 2.5 μmol/ml of the amino acids were prepared from the stock solutions by dilution with water and 30 μl of each solution were mixed with 300 μl of OPA at room temperature. After 120 s, a 20-μl aliquot was injected, the OPA derivative being eluted from the column by isocratic elution [eluent A–B (62:38, v/v)]. The eluent was pumped at a flow-rate of 1.5 ml/min. The absorbance of the column eluate was monitored at 340 nm (sensitivity 0.01 a.u.f.s.).

In order to determine retention times (Table I), the OPA derivatives of β-Ala, γ-ABA, BAIBA and α-ABA were chromatographed individually. From the separation of mixtures, the resolution R_s and the separation factor α (Table II) were calculated. The molar responses are given in Table I. Linearity of the

TABLE I

MOLAR RESPONSES AND RETENTION TIMES ON THE CHROMATOGRAM OF THE COMPOUNDS UNDER STUDY

See Fig. 1.

Amino acid	Concentration (pmol/20μl)	Detector response	Molar response (*M*)	Retention time (min)
β-Ala	194	101 228	522	9.9
γ-ABA	194	77 112	398	13.0
BAIBA	194	76 613	395	14.0
α-ABA	194	80 063	413	25.4

TABLE II

RESOLUTION (R_s) AND SEPARATION FACTORS (α)

See Fig. 1.

Amino acids	R_s	α
β-Ala-γ-ABA	6.0	1.4
β-Ala-BAIBA	6.8	1.5
γ-ABA-BAIBA	1.2	1.1
BAIBA-α-ABA	13.4	1.9

TABLE III

LINEARITY OF DETECTOR RESPONSE FOR BAIBA

Sample	Concentration (pmol/20μl)	Regression equation	Correlation coefficient (r)
BAIBA standard	48.5-291	$y=331.1x-2403.5$	0.999
Control urine spiked with BAIBA	48.5-291	$y=334.1x-88.1$	0.998

detector response for BAIBA was confirmed in the range 48.5–291 pmol (Table III).

In order to determine BAIBA in urine, an external standard procedure was employed. An OPA blank was run before each BAIBA standard.

Sample preparation

Urine samples were obtained from arsenic-exposed and unexposed subjects. Immediately after collection, the urine samples were sterilized by filtration through a porous membrane (0.45 μm) and cooled to 0–4°C for transport to the laboratory. Prior to analysis, 500 μl of the urine samples were deproteinized with 750 μl of methanol [17,18]. In order to prove that the BAIBA concentration was not altered by treatment with methanol, tests were made with known concentrations of BAIBA standard in control urine. The urine–methanol mixtures were shaken for 2 min and centrifuged at 7520 *g* for 5 min; the supernatant solutions were filtered through porous membranes (0.22 μm). A 30-μl volume of each solution was derivatized with 300 μl of OPA at room temperature. After 120 s, 20 μl were injected onto the column under the chromatographic conditions mentioned above, except that after elution of BAIBA the proportion of eluent B was increased from 38 to 100% within 3 min in order to wash the column for 5 min. Before each run, the column was equilibrated with eluent A–B (62:38, v/v) for 10 min.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of BAIBA from a mixture of seventeen amino acids. None of the amino acids interfered with BAIBA. Statistical analyses

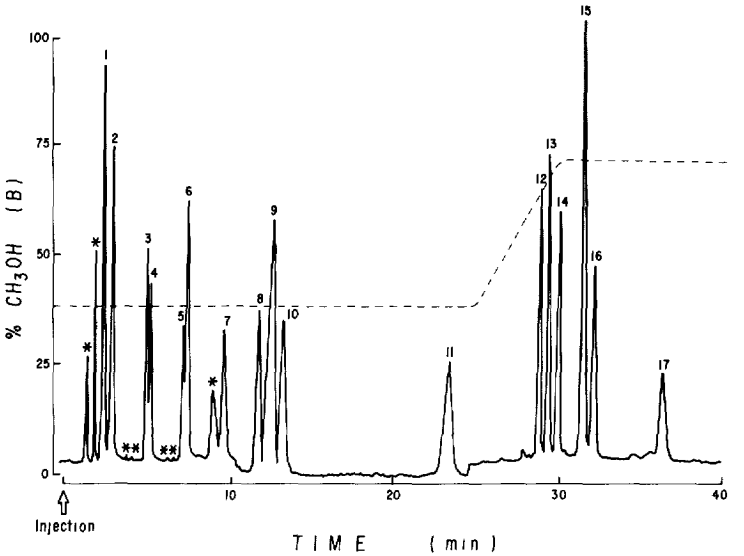

Fig. 1. Chromatogram showing the elution profile of a mixture of seventeen amino acids. Peaks: 1 = aspartic acid; 2 = glutamic acid; 3 = serine; 4 = histidine; 5 = glycine; 6 = threonine; 7 = β-Ala; 8 = tyrosine; 9 = γ-ABA; 10 = BAIBA; 11 = α-ABA; 12 = L-methionine; 13 = L-valine; 14 = phenylalanine; 15 = isoleucine; 16 = leucine; 17 = lysine. Separation was carried out at room temperature on an Ultrasphere ODS (5 μm) column (250 mm × 4.6 mm I.D) coupled to an Ultrasphere ODS (5 μm) guard column (45 mm × 4.6 mm I.D.). Mobile phase, initially 62% eluent A (50 m*M* sodium acetate buffer, pH 6.8)–38% eluent B (methanol); after 25 min, eluent B was increased linearly in 5 min from 38 to 70%; flow-rate, 1.5 ml/min; UV detection (340 nm, 0.01 a.u.f.s.). Peaks marked with asterisks also appear in the OPA blank runs.

TABLE IV

STATISTICAL ANALYSES

Parameter	Value
Standard deviation (s)	0 021
Variance (s^2)	$4.41 \cdot 10^{-4}$
Average value ($\bar{x}$)	0.292 nmol
Coefficient of variation	0.072
Confidence limit[a]	$\pm$0.008
Detection limit[b]	17.7 pmol

[a]95% based on 26 replicates.
[b]At a signal-to-noise ratio of 2.

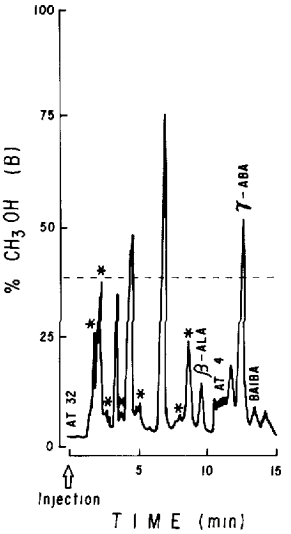

Fig. 2. Chromatogram of 30 μl of urine from an unexposed subject showing the β-Ala, γ-ABA and BAIBA peaks. Chromatographic conditions as in Fig. 1 and washing step as described under Experimental. Peaks marked with asterisks also appear in the OPA blank runs.

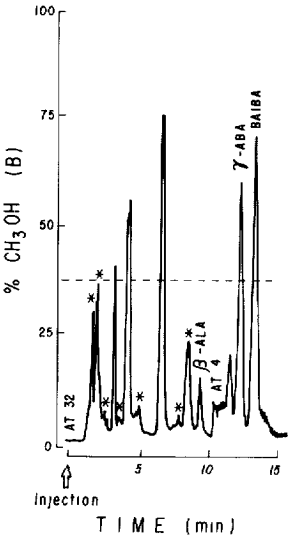

Fig. 3. Chromatogram of 30 μl of urine from an exposed subject, diluted 1 3 with eluent A, showing the β-Ala, γ-ABA and BAIBA peaks. Chromatographic conditions as in Fig. 2. Peaks marked with asterisks also appear in the OPA blank runs.

[19] (Table IV) were carried out using results from 26 control urines spiked with BAIBA in the range 48.5–291 pmol [20].

The resolution of β-Ala and BAIBA (Table II) demonstrated the efficiency of the technique. Even though the resolution between γ-ABA and BAIBA was only 1.2, it was sufficient for our purposes as the concentration of γ-ABA in the urine samples studied was low (Figs. 2 and 3).

The retention times (Table I) indicate that the proposed technique fulfils the necessary requirements for speed and the statistical analyses demonstrate its reliability. It can be concluded that the proposed technique is simple, rapid, efficient and reliable.

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