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Note

High-performance liquid chromatographic determination of β-aminoisobutyric acid in the picomole range

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The non-protein amino acid β-aminoisobutyric acid (β-AIBA), a normal degradation product of thymine in DNA and thymine, the minor constituent of transfer RNA [1], is excreted in low amounts in human urine. Normal individuals, particularly those of oriental origin and American Indians occasionally (5—10%) excrete elevated levels of β-AIBA [2]. This increase in β-AIBA excretion is genetically controlled [3]. Increased β-AIBA urinary excretion has been observed in subjects with cancer [4], pulmonary tuberculosis, children with Down's syndrome, after surgery, accidental exposure to radiation (see ref. 4 for other cited references) and lead poisoning [5, 6]. It is not clear whether serum levels of β-AIBA are also elevated under pathological conditions.

The serum level of β-AIBA in normal subjects is quite low. β-AIBA in serum is determined by ion-exchange chromatography [7—9]. Heretofore, the most sensitive method, described by Kuo et al. [9], for its determination detects β-AIBA at nanomolar concentrations and requires the use of a dedicated expensive Beckman Model 121 amino acid analyzer. We describe a rapid and highly sensitive analysis of β-AIBA by high-performance liquid chromatography (HPLC) using fluorescence detection of eluates [10, 11] with *o*-phthalaldehyde (OPA). β-AIBA analysis from serum or urine (before or after hydrolysis) is completed in less than 20 min and sensitivity for its detection is increased over 100-fold to 5—10 pmol.

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EXPERIMENTAL

Apparatus

The HPLC system (Waters Assoc., Milford, MA, U.S.A.) consisted of two Model 6000A pumps, a Model 720 systems controller, a Model 710 WISP automated sample injector, a reversed-phase Radial-Pak C_{18} cartridge, 5 μm particle size (10 cm × 8 mm) housed in an RCM-100 radial compression module. An ion-exchange steel column (30 cm × 3.9 mm) was packed with W-3H cation-exchange resin (Beckman Instruments, Palo Alto, CA, U.S.A.). The eluate reaction system consisted of an Eldex Model A30-S pump with a monitor. OPA reagent was pumped into a mixing chamber where derivatization with OPA takes place in stainless-steel tubing (1 m × 0.5 mm I.D.). Fluorescence detection (excitation at 365 nm and emission at 410 nm) was measured with a Kratos Model FS-950 fluorescence detector equipped with a flow cell (20 μl illuminated volume, 28 μl total volume). A six-port high-pressure switching valve (Valco Instruments, Houston, TX, U.S.A.) was used to inject eluent from the reversed-phase column to the ion-exchange column. A Model 119CL amino acid analyzer (Beckman Instruments) was also used for some studies. Peaks on the chromatograms were quantitated by a Perkin-Elmer Sigma 10 data station.

Reagents

DL-β-Aminoisobutyric acid, sodium tetraborate, 2-mercaptoethanol, Brij-35 (30%, w/v) and *o*-phthalaldehyde (Sigma, St. Louis, MO, U.S.A.), monobasic sodium phosphate (Mallinckrodt, St. Louis, MO, U.S.A.), concentrated sodium citrate solution, reagent-grade ninhydrin and sequanal-grade Piersolve (ethylene glycol monomethylether) (Pierce, Rockford, IL, U.S.A.), sulfosalicylic acid, 50% sodium hydroxide solution and ethylenediaminetetraacetic acid tetrasodium salt dihydrate (J.T. Baker, Phillipsburg, NJ, U.S.A.) and hydrochloric acid (Fisher Scientific, Fairlawn, NJ, U.S.A.) were used. Creatinine Analyzer-2, reagents picric acid solution and alkaline buffer were from Beckman Instruments, HPLC-grade ultrapure water (conductivity > 16 mΩ) generated by a water purification system (Millipore, Milford, MA, U.S.A.), consisting of a 1-μm particulate filter, one organic filter and two ion-exchange filters connected in series, was used in the preparation of all solutions.

Buffers

Two buffers were used for the chromatography; 0.10 *M* sodium dihydrogen phosphate, pH 2.5 (buffer A) and 0.4 *M* sodium citrate, pH 4.20 (buffer B). The ion-exchange column was regenerated with 0.3 *M* sodium hydroxide, 1% ethylenediamine tetraacetic acid (EDTA). Buffers were filtered through a 0.22-μm Magna Nylon 66 filter (Fisher Scientific).

OPA reagent

A solution containing 0.8 g OPA in 1 l of 1.0 *M* potassium borate was adjusted to pH 10.4, then filtered through a 0.22-μm filter (Millipore). Then 2 ml of 2-mercaptoethanol and 1 ml of Brij-35 were added.

Amino acid standards

Physiological amino acid standard solution containing acidic and neutral amino acids (2.5 μmol/ml) was purchased from Sigma. A six amino acids standard solution, containing 100 nmol/ml each of tyrosine, phenylalanine, β-alanine, α-aminoisobutyric acid, β-AIBA and γ-aminoisobutyric acid, was prepared by dissolving crystalline amino acids (Sigma) in water. Similarly a standard solution of β-AIBA was also prepared.

Collection of serum and urine

From healthy donors ranging in age from 18 to 74 years old, blood (10—15 ml) was drawn without anticoagulant and allowed to clot at room temperature for 30 min. The serum was separated by centrifugation at 0—4°C (600 *g* for 15 min) and stored frozen in capped plastic tubes at −80°C. Random samples (10—15 ml) of urine were collected in plastic specimen containers and stored frozen at −80°C prior to analysis. Serum from children with leukemia was provided by Dr. Lorrie Odom of Children's Hospital, Denver, CO, U.S.A.

Sample preparation

For the determination of total β-AIBA (free and conjugated) a 0.5-ml aliquot of serum or urine was mixed with 0.5 ml of concentrated hydrochloric acid and hydrolysed in a sealed Pyrex glass tube (10 × 1.2 cm) for 24 h at 110°C. After hydrolysis, the hydrolysate was dried under vacuo over sodium hydroxide pellets. The dried material was taken up in 1.0 ml water and filtered through a 0.22-μm filter. For the determination of free β-AIBA, 0.5 ml of 4% sulfosalicylic acid was added to a 0.5-ml aliquot of urine or serum contained in a 1.5-ml microfuge tube. The sample was mixed thoroughly on a Vortex mixer and clarified by centrifugation at 8800 *g* for 5 min in an Eppendorf Model 5412 centrifuge.

Separation of β-AIBA and quantitation by fluorescence detection

Method 1. The analytical system used for the determination of free and total β-AIBA in urine and for free β-AIBA in serum is illustrated in Fig. 1A. Sample was injected onto the ion-exchange column equilibrated with buffer B at 65°C, the effluent was reacted with OPA reagent at room temperature and amino acids were quantitated by fluorescence detection. After each run, the ion-exchange column was regenerated by passing 3 ml of 0.3 *M* sodium hydroxide—1% EDTA and equilibrated with 15 ml of buffer B. In some experiments, the β-AIBA content was also determined by the amino acid analyzer (Model 119CL) as described by Kuo et al. [9] except that the analytical buffer consisted of 0.35 *M* sodium citrate (pH 4.18)—0.5% isopropanol, the flow-rate of ninhydrin and elution buffer was 20 ml/h and a standard reaction coil, 15.6 m × 0.3 mm, was used. By increasing the flow-rate of ninhydrin, we have increased the sensitivity of detection of β-AIBA to 0.5 nmol. β-AIBA was eluted in 40 min.

Method 2. Release of large amounts of phenylalanine and tyrosine on hydrolysis of serum proteins interfered with the separation of small amounts of β-AIBA on an ion-exchange column (method 1). Therefore, the hydrolysate was fractionated on a reversed-phase column equilibrated with buffer A and the

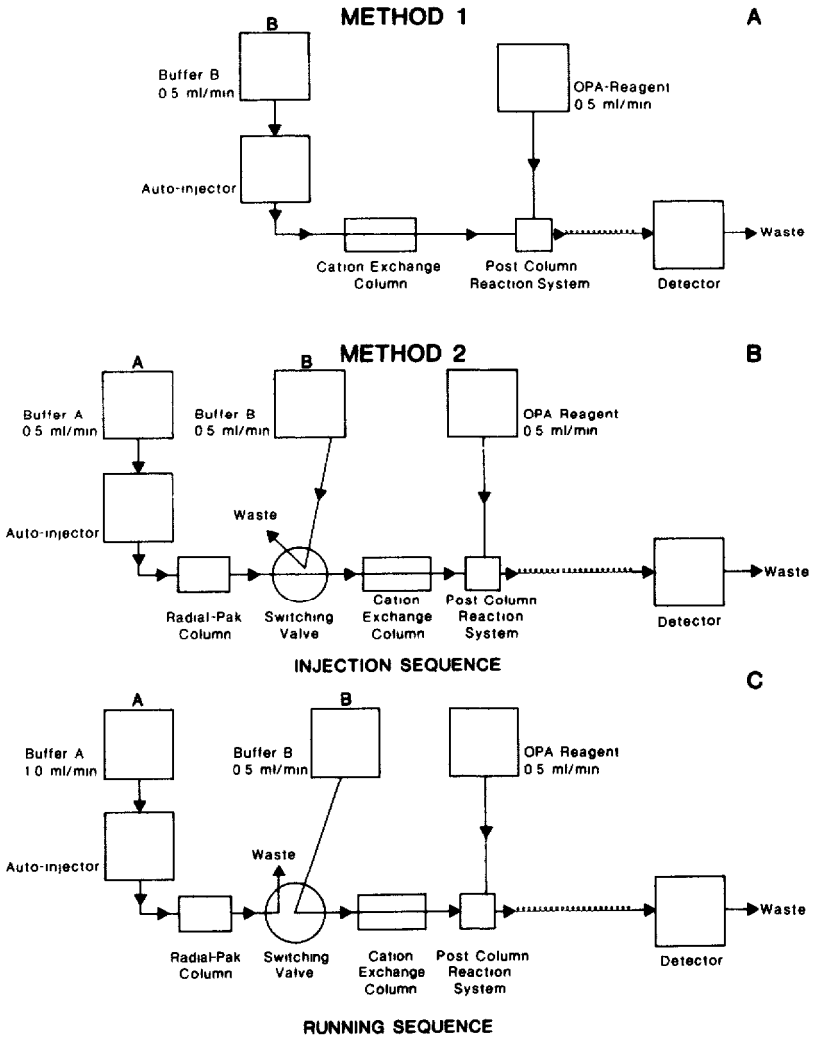

Fig. 1. Flow diagram for the separation of β-AIBA from serum and urine.

fraction containing β-AIBA, relatively free from phenylalanine and tyrosine (pre-determined using a physiological standard), was injected onto an ion-exchange column which was equilibrated with buffer B and processed as described for method 1. We have automated this procedure as shown in Fig. 1B and C. In the running sequence, both reversed-phase and ion-exchange columns were equilibrated with buffers A and B, respectively. The chromatography was initiated by injecting the sample onto the reversed-phase column; when the β-AIBA began to elute from the reversed-phase column, the six-port high-pressure switching valve was switched to position No. 2 (Fig. 1B) and the injection sequence for the ion-exchange column was initiated. The system remained in the injection mode until the eluent containing β-AIBA from the reversed-phase column was injected onto the cation-exchange column. After this, the six-port high-pressure switching valve was returned to the running

sequence (Fig. 1C) and β-AIBA, separated on the cation-exchange column, was detected by fluorescence. After the chromatography was completed, the cation-exchange column was regenerated by passing 3 ml of 0.3 *M* sodium hydroxide—1% EDTA, followed by passing 15 ml of buffer B. Although the six-port high-pressure switching valve and buffer valve at pump B can be switched manually, we have automated the switching of valves using a Waters Model 720 systems controller.

RESULTS AND DISCUSSION

Fig. 2A shows the elution profile of β-AIBA from a mixture of six amino acids, separated on an ion-exchange column and detected by post-column eluate derivatization with OPA (method 1). β-Alanine, a constituent of human urine which has elution characteristics very similar to those of β-AIBA in other chromatographic systems, was clearly separated from β-AIBA. Furthermore, 5—10 pmol of β-AIBA were rapidly and reproducibly detected by this procedure. However, this procedure is not suitable for the determination of β-AIBA from hydrolysed serum due to release of large amounts of amino acids including phenylalanine and tyrosine which interfere with the separation and detection of relatively small amounts of β-AIBA. Therefore, chromatographic conditions were determined to elute β-AIBA relatively free from phenylalanine, tyrosine and β-alanine. The interfering amino acids were removed by passing the serum hydrolysate through a reversed-phase column. The fraction eluting after these amino acids (approximate retention time 2—3 min after sample injection) was

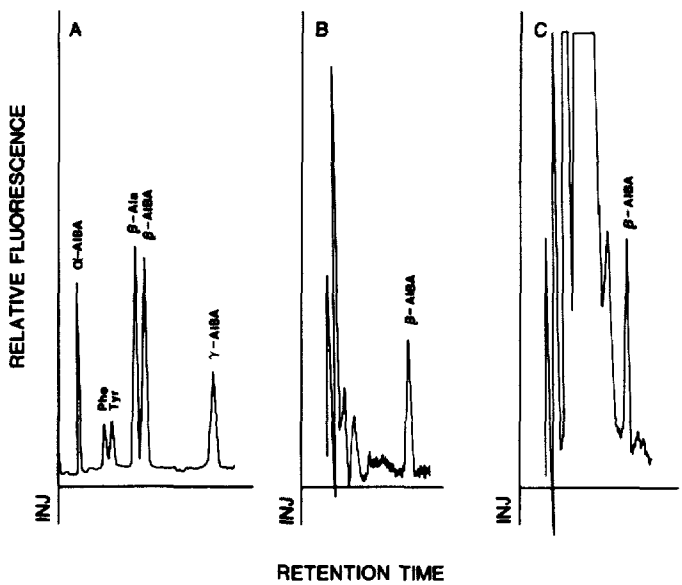

Fig. 2. Elution profile of β-AIBA. (A) Six amino acids standard mixture (100 pmol each) separated by method 1; (B) physiological amino acid standard (Sigma, 25 pmol each); (C) injection corresponding to 25 μl of normal adult male serum. Method 2 was used for B and C, and the sensitivity of fluorescence detection was four times greater than in A. Peaks: Phe = phenylalanine; Tyr = tyrosine; β-Ala = β-alanine.

further chromatographed on an ion-exchange column as described under method 2. Fig. 2B shows the separation of β-AIBA from a standard mixture containing 50 pmol of each of the 27 amino acids. Separation of β-AIBA from normal adult serum hydrolysate is shown in Fig. 2C; the sample injection corresponded to 25 μl of serum. In a separate experiment, nine hydrolysates of normal adult serum were spiked with 50 pmol of β-AIBA; the recovery of the spike ranged from 96 to 106%.

To determine linearity of response and sensitivity of method 1 and method 2, a mixture of six amino acids containing β-AIBA was used. There was a linear relationship between β-AIBA content (5 pmol to 2 nmol) and amount of fluorescence (data not shown).

The β-AIBA content of normal adult urine is considerably higher than in serum; furthermore, hydrolysis of urine compared to serum does not liberate large amounts of amino acids which might interfere in the separation and determination of β-AIBA. Therefore, method 1 can be used for rapid analyses of urinary β-AIBA (free and conjugated) and free β-AIBA from serum. Analysis of urinary β-AIBA from eight normal adult subjects by method 2 provided nearly identical values (Table I). The correlation coefficient (r) between method 1 and method 2 was 0.9935. Values for β-AIBA were similar (0.9948) to those obtained by the method of Kuo et al. [9] employing a dedicated

TABLE I

β-AIBA CONTENT OF HUMAN URINE DETERMINED BY DIFFERENT METHODS

Code	β-AIBA content (nmol/μmol of creatinine)		
	Amino acid analyzer	Method 1	Method 2
560	37	39	35
561	14	15	13
562	8	7	9
563	18	20	19
564	25	29	27
565	7	7	6
566	5	6	5
567	4	6	4

TABLE II

NORMAL VALUES FOR β-AIBA IN HUMAN URINE AND SERUM

Sample	Sex*	n	β-AIBA level** (mean ± S.D.)	R.S.D.*** (%)
Urine	M	40	6.0 ± 3.5	58
	F	43	24 ± 36	150
Serum	M	23	2.11 ± 0.93	42
	F	51	1.59 ± 0.60	38

*M = Male; F = female.
**Expressed as nmol/μmol of creatinine for urine and as nmol/ml for serum.
***R.S.D. = Relative standard deviation.

TABLE III

FREE AND TOTAL β-AIBA CONTENT OF SERUM OF CHILDREN WITH LEUKEMIA

Age (years)	Sex*	β-AIBA content (nmol/ml)		
		Free	Total	Total/free
5	F	1.6	4.4	2.75
5	F	0.9	3.2	3.6
5	F	2.0	4.7	2.35
6	M	1.7	1.6	0.94
7	F	2.8	4.2	1.5
8	M	2.1	3.2	1.5
10	M	0.9	3.0	3.33
13	F	2.0	4.0	2.0
14	F	1.9	6.4	3.5
16	M	3.4	6.3	1.85

*F = Female; M = male.

amino acid analyzer (Table I). The urinary β-AIBA content from normal adult male subjects (Table II) analyzed after acid hydrolysis (to measure free plus conjugated β-AIBA) is similar to that reported for free β-AIBA by Kuo et al. [9]. However, the β-AIBA content from normal adult females was much higher than that observed by Kuo et al. [9]. The reason for this difference is unclear (Table II). In normal adults, serum β-AIBA is present in free form [9]. The β-AIBA content of normal adult serum, free plus bound (Table II), was similar to that observed by others as free β-AIBA [7—9]. However, in children with leukemia, nine out of ten subjects, both free and conjugated forms of β-AIBA were detected (Table III). It remains to be ascertained whether under other pathological conditions β-AIBA in serum is present in conjugated form. In summary, the procedure described here provides a rapid and sensitive HPLC method for the determination of β-AIBA in biological fluids in the picomole range. *This HPLC method has an additional advantage in that it obviates* the use of an expensive amino acid analyzer.

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