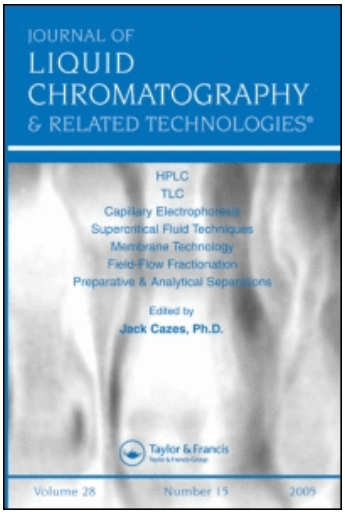

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DETERMINATION OF BETA-AMINO-ISOBUTYRIC ACID IN URINE AND SERUM USING PRE-COLUMN DERIVATIZATION TECHNIQUE

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ABSTRACT

Beta-amino-isobutyric acid (BAIBA) in urine and serum has recently been used as a biological marker for exposure to carcinogens, lead and several toxic substances. This paper describes an automated gradient high-performance liquid chromatographic method (HPLC) for the determination of BAIBA with fluorescence detection in combination with pre-column O-phthalaldehyde (OPA) derivatization. The method is able to separate and detect 25 fmol of BAIBA within 30 minutes. The mean value for free BAIBA obtained from 20 normal subjects was 40.7 nmol/umol creatinine and 2.4 nmol/ml for urine and serum, respectively. The procedures described here are relatively simple and are useful for screening of low level exposure to potential carcinogens.

INTRODUCTION

Beta-aminoisobutyric acid (BAIBA) is a non-essential amino acid. Increased BAIBA urinary excretion has been observed in subjects with cancer, pulmonary tuberculosis, after surgery and exposure to toxic substances (1,2). Recently, Farkas et al (3) also suggested that the increase of urinary excretion of BAIBA was due to the damage of DNA on exposure to lead. The normal level of BAIBA in serum was found to be low in the range of 1-5 nmol/ml (4,5). Elevation of serum levels of BAIBA in uremic patients were also reported by Gejyo et al (6).

Numerous HPLC methods have been developed recently to replace dedicated and expensive amino acid analyzers for the determination of amino acids. The growing interest in BAIBA as a probe for DNA and RNA metabolism and as a biological marker for cancer, lead poisoning and exposure to carcinogen have necessitated the development of a more rapid, sensitive and reliable method for its determination.

In this paper, we describe an automated gradient HPLC system using pre-column derivatization with fluorescence detection for very low levels of BAIBA. It is capable of separating BAIBA from other amino acids as well as interferences of gama-amino-n-butyric acid (GABA) and alpha-amino-n-butyric acid (AABA) in a single analysis within 30 min. The specificity and sensitivity of BAIBA determination by the present method is better than other methods described in the literature.

MATERIALS AND METHODS

Apparatus

A completely automated gradient system equipped with a master pump (Model 305) controlling a slave pump (Model 302) and an autosampling injector (Model 231-401) was used (Gilson, Villiers-le-Bel, France). The analytical column used was a replaceable cartridge (Partisphere 5 C_{18}, 110 mm X 4.7 mm I.D.) protected by a guard cartridge system (Whatman, Clifton, NJ, U.S.A.). For detection and quantitation, a Shimadzu Model RF-535 fluorescence detector (excitation at 330 nm, emission at 450 nm) and a Shimadzu CR-5A integrator was used (Kyoto, Japan). Two disposable Whatman filters for on-line filter and degasser devices were connected to the gradient system.

Reagents and Chemicals

DL-beta-amino-isobutyric acid (BAIBA), L-alpha-amino-n-butyric acid (AABA), gamma-amino-n-butyric acid (GABA), physiological amino acid standard solution containing acidic and neutral amino acid (2.5 umol/ml), ortho-phthalaldehyde (OPA) and the other 25 types of crystalline amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Sulfosalicylic acid, sodium acetate acid, sodium hydroxide and methanol (HPLC grade) were obtained from E. Merck (Darmstadt, F.R.G.). Sodium

tetraborate and 2-mercaptoethanol were from BDH (Poole, U.K.). Distilled and deionized water was used in the preparation of all solutions.

Collection of Samples

Random urine samples were collected and stored frozen below -20°C without preservative.

About 3 ml of blood was collected without anticoagulant by venipuncture. It was allowed to clot at room temperature for about 30 min followed by centrifugation (4°C) at 1000 g for 10 min. The serum was separated and stored frozen at -20°C.

Standards Preparation

A stock standard solution containing 2.0 umol/ml of BAIBA, GABA and AABA was prepared by dissolving 10.31 mg of each crystalline amino acid in 50 ml of 0.1 M hydrochloric acid. Working standards were prepared weekly by diluting the stock solution 2000-, 400-, 200-, 50- and 10-fold in order to obtain concentrations of 1, 5, 10, 40 and 200 nmol/ml respectively. Other individual amino acid standard solutions used for interference studies were prepared in the same manner.

A 25 nmol/ml of physiological amino acids standard solution was prepared by diluting the 2.5 umol/ml of acidic and neutral amino acid standard solution (Sigma) with a 100-fold of 0.1 M hydrochloric acid.

Sample Preparation

For the determination of free BAIBA, 150 ul of 4 % (v/v) sulfosalicylic acid was added to a 50 ul aliquot of urine or serum contained in a 1.5 ml microfuge tube. The sample was mixed thoroughly on a Vortex mixer. 300 ul of methanol was added to the deproteinised sample and allowed to stand at below 4°C for 20 min for complete deproteinization. The sample was then centrifuged at 10,000 x g for 2 min. The supernatant was carefully transferred into a Costar centrifuge filter unit and centrifuged for another 30 sec at 10,000 x g. The filtrate was then transferred into an amber sampling vial for automatic pre-column derivatization and HPLC analysis.

For the determination of total urinary BAIBA, 1 ml of urine was hydrolyzed with 1 ml of 6 M hydrochloric acid in a sealed Pyrex glass tube for 24 hours at 110°C. After the sample was cooled, 1 ml of 6 M sodium hydroxide was added. The neutralized

hydrolysate was centrifuged at 1,000 x g at 4°C for 5 min. 150 ul of the supernatant was added with 350 ul of methanol into a Costar centrifuge filter unit and allowed to stand below 4°C for 20 min. After micro-centrifugation, we proceeded to HPLC analysis as mentioned above.

HPLC Conditions

Two buffers with pH adjusted to 6.2 with 10 % (v/v) acetic acid were used for the gradient separation chromatography: buffer A, 15 mM sodium acetate contained in 25 % (v/v) methanol and buffer B, 10 mM sodium acetate contained in 50 % (v/v) methanol. The gradient separation profiles for the separation of BAIBA from other amino acids of urine and serum samples are shown in Figure 1. The analytical column was equilibriated with two cycles of gradient separation before analysis.

OPA Reagent

The OPA reagent was prepared at least 24 hours before use. 54 mg of OPA was dissolved in 1 ml of methanol followed by 10 ml of 0.1 M sodium tetraborate (pH 9.4) and 100 ul of mercaptoethanol. A 20 ul of mercaptoethanol was added once every fourteen days to the reagent in order to maintain maximal yield of OPA-amino acid (6,7).

Pre-column Derivatization

The pre-column derivatization was carried out automatically by using a Gilson auto-injector equipped with a programmable microcomputer. The OPA derivatization and sample injection were programmed as follow: 20 ul of OPA reagent and 10 ul of treated sample together with 0.5 ml of buffer A were introduced into an amber sampling vial. This was followed by drawing and dispensing the mixture twice at lower speed to reach a reaction time of 2 min. 5 ul (for urine sample) or 20 ul (for serum sample) of the OPA derivatized amino acids was injected into the HPLC system.

RESULTS

Fig.2a shows that BAIBA was well separated from GABA, AABA and other amino acids using the above described method. The retention time was comparable with that for physiological amino acids standard provided by Sigma (25 nmol/ml), Fig. 2b. Over 25 types of other amino acids standard had also been tested individually. It was confirmed

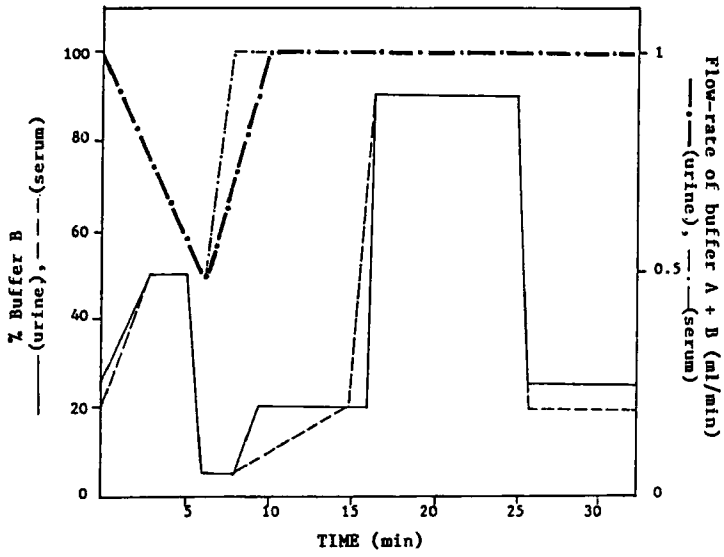

Figure 1. Gradient elution profiles for BAIBA analysis in urine and serum.

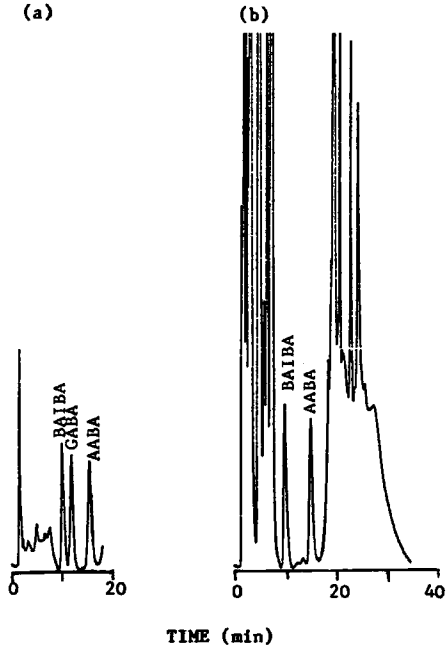

Figure 2. Chromatograms of (a) an aqueous standard mixture of BAIBA, GABA and AABA (each 20 nmol/ml) and (b) Physiological amino acid standards (Sigma, each 25 nmol/ml).

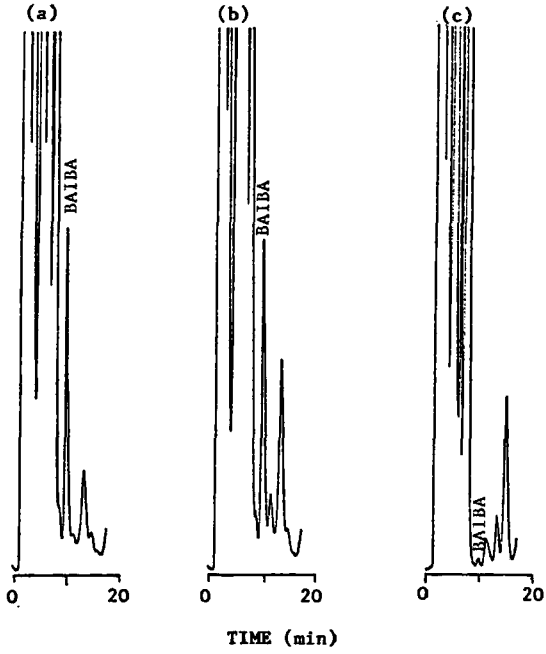

Figure 3. Chromatograms of (a) free urinary BAIBA (242 nmol/ml), (b) total urinary BAIBA (240 nmol/ml) and (c) serum specimen of a normal subject (1.6 nmol/ml).

that none of these amino acids have the same retention time as the BAIBA eluted by the above conditions described.

Fig.3 a and 3b show the chromatograms of urine specimen collected from a male subject. The chromatogram for serum sample from the same subject is shown in Fig.3c, it was noted that the concentration of BAIBA was much lower when compared with that of the urinary BAIBA (Fig.3b).

DISCUSSION

Separation of BAIBA from GABA, AABA and other Amino Acids

Resolution is the most crucial parameter for amino acids analysis especially for biological samples. The determination of low level BAIBA in urine or serum was

known to be affected by large amounts of over 25 types of amino acids. Furthermore, GABA and AABA have relatively similar chromatographic characteristic as BAIBA and thus would affect its detection. Many studies on amino acids analysis were based on mobile phase with pH level at 4 to 5.5 (5-10). However, we found that BAIBA, GABA and AABA were retained in the C_{18} column when 20 mM sodium acetate buffer (pH 4.5 and 5.5) was used. Although addition of methanol as modifier would reduce the retention time, it does not improve their resolution of BAIBA, GABA and AABA.

In the present study, we looked into the separation of BAIBA, GABA and AABA with mobile phase contained 30 % v/v of methanol and pH varied from 4 to 6.4. The results showed that BAIBA was well separated from the two amino acids which appeared just after the BAIBA (Figure 2a).

When HPLC was used for separation of different amino acids in urine or serum samples, it usually takes at least 60 min for a single analysis (6-8). Since we are only interested in BAIBA determination, the analysis time could be shortened to 32 min. This can be achieved by eluting other amino acids with increasing methanol concentration of mobile phase before and after separation of BAIBA. However, changes of methanol concentration during analysis are bound to affect the fluorescence detection and result in unstable chromatographic baseline. In order to minimize this problem, the maximum difference of methanol concentration changes during the gradient separation was kept within 21 % (Figure 1). The precursors of BAIBA were eluted with 50 % each of buffer A and B, with a total methanol composition of 38 % for the first 6.5 min. The total methanol concentration was immediately reduced to 26 % and then maintained at 30 % until 16 min for the separation of the BAIBA, GABA and AABA. It was then increased to 47 % from 16.5 to 32 min to remove other amino acids from the column. Under these conditions , BAIBA was eluted at 10 min.

Sensitivity and Precision

The concentration of BAIBA in serum is known to be very low in the range of 1-5 nmol/ml (4,5). Therefore, detection sensitivity becomes another crucial problem for amino acid analysis using HPLC. The method described by Kuo et al (10), for detection at nanomolar concentrations required the use of dedicated and expensive amino acid

analyzer. On the other hand, the detection sensitivity can be increased to 5-10 pmol when using cation-exchange chromatography with post column derivatization, reported by Buschman et al (1986) (10). However, the sensitivity of the proposed method is even better. As shown in Figure 2a, the chromatogram of OPA-BAIBA standard was 1 pmol with an injection volume of 5 ul corresponding to 5 ul of 20 nmol/ml of standard used for pre-column derivatization as mentioned above. The lowest detection limit of BAIBA determination by present method (at a signal to noise of 3) was 25 fmole.

The calibration graphs were linear for concentrations of BAIBA in the range 0.025 - 10 pmole with typical regression equation $y = 0.016 + 0.000638 x$ (y is absolute weight, pmole; x is peak height, uV) and coefficient of correlation $r = 0.99$. The coefficients of variation of the linearity and slope of the calibration graphs for between-day analysis was less then 0.02 % and 4 % (n=3), respectively.

Automatic Pre-column Derivatization

Generally, pre- or post column derivatization for HPLC amino acids analysis is needed to enhance detectability and sensitivity. The used of pre-column derivatization technique for HPLC analysis is generally rapid, quantitative and cost effective. However, the reproducibility and accuracy of BAIBA determination were influenced by the reaction time with OPA, as OPA-BAIBA is only stable for 1-2 min. Therefore, a programmable auto-injector for automatic pre-column derivatization prior to HPLC separation is essential. The sampling procedures, dilution factor, mixing time and injection frequency could be programmed accordingly to address the requirements of the analysis. It also ensures accurate and precise sample volume, sampling speed and reaction time. The within- day coefficient of variation for of 12 runs by the present automatic system was less than 5 %.

Chromatographic Analysis of Urine and Serum Samples

The chromatograms of human urine for free BAIBA and total (free + conjugated) BAIBA are shown in Figure 3a and 3b, respectively. The levels of urinary total BAIBA of male subjects analyzed after acid hydrolysis were similar to the levels of free BAIBA analyzed after deproteinization. It was noted that for female subjects, the total urinary BAIBA content were slightly higher than free BAIBA. The equation of correlation

between free (x) and total (y) urinary BAIBA of 6 male subjects analyzed was y = -0.8 + 1.07 x with coefficient of correlation r = 0.99 and y = -8.90 + 1.19 x , r = 0.99 for 14 female subjects. This observation is in close agreement with earlier reports (10,11). The mean value of free urinary BAIBA of 20 normal subjects (female + male) analyzed was 217 nmol/ml (range 5-1025 nmol/ml) or 40.7 nmol/umol creatinine (range 2-215 nmol/umol creatinine).

Figure 3c shows the chromatogram of a serum sample with relatively low concentration of free BAIBA. Unlike urine samples, release of large amounts of phenylalanine and tyrosine on hydrolysis of serum proteins interfered with the separation of small amounts of BAIBA. Therefore, the proposed method is not recommended for the determination of total BAIBA in serum. However, it is believed that the present method would be able to address the issue if it is combined with column switching technique. The mean value of the free BAIBA level in serum of 20 normal subjects was 2.4 nmol/ml (range 0.6 - 6.5 nmol/ml). Seven serum samples collected from leukemia patients were also analyzed. Among these samples, 2 were found to contain relatively high free serum BAIBA levels, with concentrations at 38 and 85 nmol/ml, respectively.

CONCLUSION

The present method provides a specific and sensitive HPLC technique for the determination of BAIBA in the fentomole range. It is considered as a more cost effective procedure for the analysis of BAIBA in urine and serum. It may be useful for clinical screening of cancer and other pathological conditions.

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REFERENCES

1. Waalkes, T. P., Gehrke, C. W., Lakings, D. B., Zumwalt, R. W., Kuo, K. C., Jacobs, S. A. and Borek, E., Beta-Aminoaciduria in Patients with Burkitt's Lymphoma. J Nat Cancer Inst. 57, 435, 1976.

2. Nielsen, H. R., Sjolin, K. E., Nyhdm, K., Baliga, B. S., Wong R., and Borek, E. Beta-Aminoisobutyric Acid, A New Probe for the Metabolism of DNA and RNA in Normal and Tumorous Tissue. Cancer Res, **34**, 1381, 1974.

3. Farkas, W. R., Fischbein, A., Soloman, S. and Buschman, F. Elevated Urinary Excretion of Beta-Aminoisobutyric Acid and Exposure to Inorganic Lead. Arch of Envn Hlth, **42**, 96, 1987

4. Solem, E., Agurwal, D. P., and Goedde, H. W. The Determination of Beta-Aminobutyric Acid in Human Serum by Ion-Exchange Chromatography. Clin Chem Acta, **59**, 203, 1975

5. Gejyo, F., Kinoshita, Y., and Ikenaka, T. Elevation of Serum Levels of Beta-Aminobutyric Acid in Uremic Patients and the Toxicity of the Amino Acid. Clin Nephro, **8**, 520, 1977.

6. Joseph, M. H. and Marsden, C. A. Amino Acids and Small Peptides. Lim, C K, HPLC of Small Molecules, IRL Press, 1986 - p 13.

7. Joseph, M. H. and Davies, P. Electrochemical Activity of O-Phthalaldehyde-Mercaptoethanol Derivatives of Amino Acid. Application to High-Performance Liquid Chromatographic Determination of Amino Acid in Plasma and other Biological Materials. J Chromatogr, **277**, 125, 1983.

8. Goldsmith, R. F., Earl, J. W. and Cunningham, A. M. Determination of Gama-Aminobutyric Acid and other Amino Acids in Cerebrospinal Fluid of Pediatric Patients by Reversed-Phase Liquid Chromatography. Clin Chem, **33**, 1736, 1987.

9. Kehr, J., and Ungerstedt, U. Fast HPLC Estimation of Gama-Aminobutyric Acid in Microdialysis Perfusates: Effect of Nipecotic and 3-Mercaptopropionic Acids. J Neurovhem, **51**, 1308, 1988.

10. Buschman, F. L., Pell, G. A., and Sharma, O. K. High-Performance Liquid Chromatographic Determination of Beta-Aminoisobutyric Acid in the Picomole range. J Chromatogr, **374**, 129, 1986.

11. Kuo, K. C., Cole, T. F., Gehrlce, C. W., Waalkes, T. P., and Borek, E. Dual-Column Cation-Exchange Chromatographic Method for Beta-Aminoisobutyric Acid and Beta-Alanine in Biological Samples. Clin Chem, **24**, 1373, 1978.

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