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# High-performance liquid chromatographic determination of β-alanine, β-aminoisobutyric acid and γ-aminobutyric acid in tissue extracts and urine of normal and (aminooxy)acetate-treated rats

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**Abstract**

A method is described for the simultaneous determination of β-alanine, β-aminoisobutyric acid and γ-aminobutyric acid in biological materials. Amino acids including these β- and γ-amino acids were derivatized with 4-dimethylaminoazobenzene-4′-sulfonyl (dabsyl) chloride and dabsyl amino acids formed were separated by reversed-phase high-performance liquid chromatography. Dabsyl derivatives of these β- and γ-amino acids were well separated from other dabsyl-amino acids. The method was applied to the determination of these β- and γ-amino acids in trichloroacetic acid extracts of various tissues and to the urine of normal rats and those injected with (aminooxy)acetate (AOA). AOA injection (15 mg per kg of body mass) produced remarkable increase in β-alanine contents in liver, kidney and urine (10.2, 4.6 and 25.7 times, respectively).


*Keywords:* Alanine; Aminoisobutyric acid; Aminobutyric acid

## 1. Introduction

(Aminooxy)acetate (AOA) is a potent inhibitor of aminotransferases [1]. Simultaneous administration of AOA and L-cysteine to rats resulted in the increase in excretions of taurine and hypotaurine [2], which are β-amino acid containing sulfonic and sulfinic group, respectively. In order to study the metabolism of these amino acids, we described a method for the determination of hypotaurine and taurine by reversed-phase high-performance liquid chromatography (RP-HPLC) [3]. During these studies, we noticed that excretion of other β-amino acids such as β-alanine (BALA) and β-aminoisobutyric acid (BAIBA), and γ-aminobutyric acid (GABA) increased in the urine of AOA-treated rats.

There are many reports describing HPLC analysis of GABA in the brain [4–9], but only a few HPLC methods have been reported for the determination of BALA and BAIBA [10]. Thus, we developed a simple method of tissue extraction and simultaneous determination of BALA, BAIBA and GABA by RP-HPLC.

## 2. Experimental

### 2.1. Materials

BAIBA and AOA were obtained from Sigma (St.

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Louis, MO, USA). BALA, GABA, DL-ethionine (Eth), other amino acids, acetonitrile (HPLC grade) and acetone (analytical reagent grade) were purchased from Wako (Osaka, Japan). 4-Dimethylaminoazobenzene-4′-sulfonyl (dabsyl) chloride was obtained from Dojin Laboratories (Kumamoto, Japan). Male Wistar rats weighing 250–300 g were used and fed MF diet of Oriental Yeast (Tokyo, Japan).

### *2.2. Preparation of standard dabsyl amino acids and calibration graphs*

Eth was used as the internal standard in the present method. A solution of 0.4 m*M* Eth was mixed at various ratios shown below with a solution containing 0.4 m*M* BALA, 0.4 m*M* BAIBA and 0.4 m*M* GABA. To 200 μl of the mixture, 1.0 ml of 0.1 *M* sodium hydrogen carbonate buffer (pH 9.0) (buffer A) and 1.0 ml of 2 m*M* dabsyl chloride solution in acetone were added. The dabsylation reaction was performed at 70°C and solutions of dabsyl-amino acids in 70% ethanol was prepared according to Chang et al. [11]. RP-HPLC was performed as described below and peak area ratios of both β- and γ-amino acids to that of Eth were plotted against the molar ratio of the corresponding amino acid and Eth.

### *2.3. Preparation of dabsyl amino acids from tissue extracts and urine*

Blood was collected by heart puncture under ether anesthesia using EDTA as an anticoagulant. Liver, kidney and brain were taken out and washed with ice-cold saline. When AOA was injected, an AOA solution, neutralized with sodium hydroxide, containing 2.5 mg $ml^{-1}$ of water was injected to rats subcutaneously on the back at a dose of 15 mg per kg of body mass. Two hours later, tissue samples were collected as above. Further procedure was performed at 0–4°C. Blood plasma, obtained by centrifugation, liver, kidney and brain were homogenized with 4 volumes of 7% trichloroacetic acid (TCA). Homogenates were centrifuged at 10 000 *g* for 10 min and resulting supernatants (TCA extracts) were separated. One half ml of the TCA extract of blood plasma was evaporated to dryness under reduced pressure at 50°C. To the dried material, 10 μl of 0.4 m*M* Eth, 1.0 ml of buffer A and 1.0 ml of 2 m*M* dabsyl chloride solution in acetone were added and the resulting mixture was heated at 70°C for 10 min. After evaporation at 50°C under reduced pressure, dabsyl-amino acids were dissolved in 0.5 ml of 70% ethanol.

To 50 μl of TCA extracts of liver, kidney and brain, 50, 40 and 20 μl, respectively, of 0.4 m*M* Eth, 1.0 ml of buffer A and 1.0 ml of dabsyl chloride solution were added. The mixtures were heated and evaporated as above. The dabsylated materials were dissolved in 2.0 ml of 70% ethanol.

To 50 μl of urine, 25 or 50 μl of 0.4 m*M* Eth (depending on the contents of amino acids to be determined) was added as an internal standard and processed as above. The dabsylated materials were dissolved in 2 ml of 70% ethanol.

The 70% ethanol samples thus obtained were filtered through a Milex filter (0.2 μm) and routinely 10 μl was subjected to RP-HPLC analysis.

Recovery experiments were performed using liver extracts and urine. To 0.5 ml of a TCA-extract of the liver obtained as above, a mixture containing 20, 50, or 100 nmol each of BALA, BAIBA and GABA was added together with 20 nmol of Eth. After dabsylation and chromatography described as above, recoveries of these amino acids were calculated. In case of urine, a mixture containing 50, 100, or 200 nmol each of BALA, BAIBA and GABA was added together with 20 nmol of Eth to 2.0 ml of 1:10 diluted urine, and recoveries of these amino acids were obtained as above. Because of high contents of these amino acids in the urine, a diluted urine was used in the recovery experiments.

### *2.4. RP-HPLC*

The RP-HPLC system used was the same as that used for the analyses of taurine and hypotaurine [3] except that a TSKgel ODS-80Ts column (250×4.6 mm, I.D., particle size 5 μm) was used instead of a 150-mm column. Ten microliters of a sample solution obtained above was applied to the system. Routine chromatography was performed at 28±1°C instead of 16±1°C [3] using a solvent system consisted of 50 m*M* sodium acetate (pH 4.15)

(solvent A) and acetonitrile (solvent B). Elution was performed with the concentration of solvent B as follows: 0–40 min, linear gradient from 35 to 40%; 40–80 min, isocratic at 40%. Then the column was washed with 5% solvent A-95% solvent B for 20 min and reconditioned with 75% solvent A-35% solvent B.

Rarely, an irregular peak pattern of BAIBA was observed when its content in blood plasma was small (less than 2–3 nmol $ml^{-1}$). Under such circumstances, rechromatography was performed using a modified elution procedure as follows: 50 m*M* sodium acetate buffer, pH 3.65, was used as solvent A and isocratic elution at 40% solvent B was continued for 40–90 min after the linear gradient of solvent B from 35 to 40% from 0 to 40 min. Likewise, when an irregular peak pattern of GABA was observed rarely upon the analyses of blood plasma and kidney, rechromatography was performed using a modified elution procedure as follows: 50 m*M* sodium acetate, pH 3.90, was used as solvent A and elution was performed with the concentration of solvent B as follows: 0 to 10 min, a linear gradient of 35 to 36%; 10 to 100 min, isocratic at 36%.

## 3. Results and discussion

Fig. 1 shows a chromatogram of standard dabsyl amino acids including dabsyl derivatives of BALA, Eth, GABA, and BAIBA. These amino acid derivatives were eluted at about 45, 54, 57 and 62 min, respectively, and they were well separated from other amino acid derivatives. Dabsyl derivatives of hydrophilic amino acids were eluted before dabsyl-glycine, and those of hydrophobic amino acids such as phenylalanine, tryptophan, leucine and isoleucine were eluted after dabsyl-BAIBA.

Fig. 2 shows an example of the present HPLC

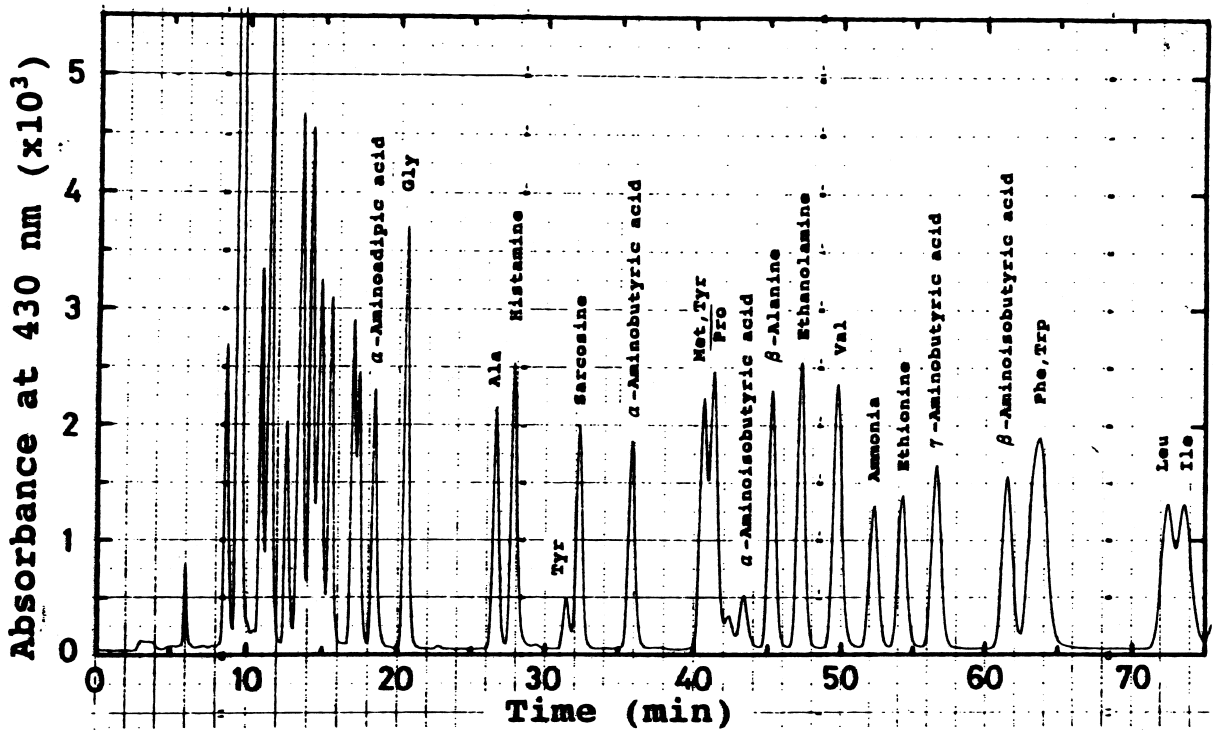


Fig. 1. Chromatogram of dabsyl derivatives of authentic amino acids including β-alanine, β-aminoisobutyric acid and γ-aminobutyric acid. A 10-μl volume of a mixture containing ca. 0.1 nmol each of dabsyl-amino acids was analyzed by RP-HPLC. For detailed chromatographic conditions, see Section 2.

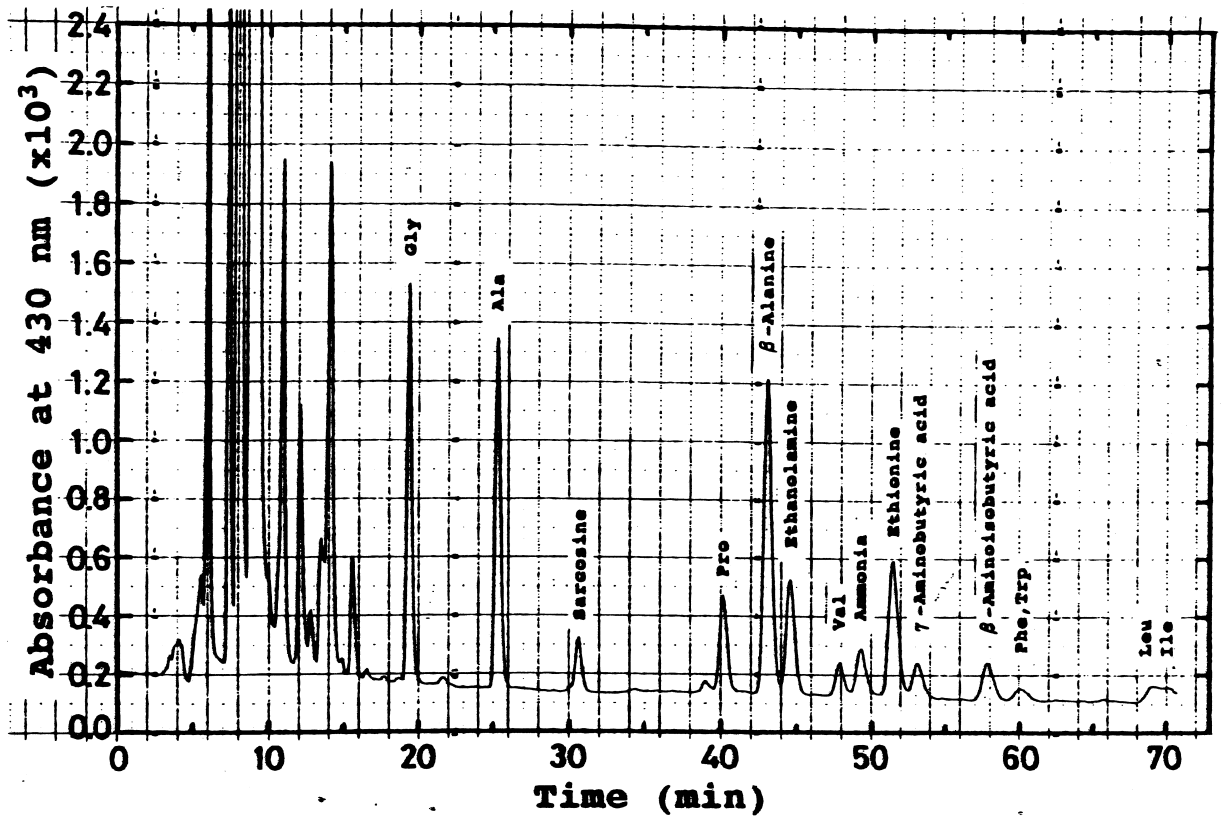


Fig. 2. Chromatogram of dabsyl derivatives of a trichloroacetic acid extract of rat liver obtained at 2 h after the administration of (aminooxy)acetate. A sample corresponding to 20 μg of fresh tissue was analyzed by RP-HPLC. For detailed procedures, see Section 2.

analysis showing a chromatogram of TCA extract of rat liver obtained at 2 h after the administration of AOA. The peak of dabsyl-BALA was detected between dabsyl-proline and dabsyl-ethanolamine, and dabsyl-GABA and dabsyl-BAIBA were after dabsyl-Eth.

As described under Materials and methods, irregular peak patterns (a broad peak or a peak with a shoulder) were observed rarely, probably due to the presence of peptides, when contents of BAIBA in blood plasma and GABA in blood plasma and kidney were small. Under such cases, the standard elution procedure was slightly modified as described above and peaks of dabsyl-amino acids were confirmed by rechromatography with standard amino acid mixture. Confirmation was performed also by rechromatography after hydrolysis of the TCA-extracts or urine. In this procedure with urine, a large peak of dabsyl-ammonia due to hydrolysis interfered with the analysis of these amino acids. Therefore, hydrolyzed sample solution was brought to pH 12 with sodium hydroxide solution and dried with a flash evaporator at 50°C before dabsylation in order to eliminate ammonia formed by hydrolysis.

Calibration curves of these three amino acid derivatives were constructed using liver extracts and urine samples and Eth as the internal standard. In these graphs, the peak-area ratio ($x$) of a dabsyl amino acid to that of dabsyl-Eth was plotted against the molar ratio ($y$) of this amino acid to Eth. The regression lines for dabsyl-BALA, -BAIBA and -GABA obtained in the range of 20 to 80 pmol (10 μl)$^{-1}$ of 70% ethanol solution injected to HPLC were: for liver extracts, $y=0.507x-0.004$, $y=0.574x-0.004$ and $y=0.535x-0.002$, and for urine samples, $y=0.590x-0.002$, $y=0.709x-0.004$ and $y=0.589x-0.000$. Correlation coefficients of these lines were all 1.000. The concentrations (μmol/

sample volume used) of BALA, BAIBA and GABA were calculated by these equations against the amount (μmol) of Eth added.

Table 1 shows an example of recovery experiments of BALA, BAIBA and GABA from liver extracts and urine samples. When 20, 50 or 100 nmol each of BALA, BAIBA and GABA were added to 0.5 ml of a liver extract, recoveries of these amino acids were 93.6±8.9 to 100.9±9.9%, and those of these amino acids were 98.3±1.2 to 106.0±4.1 when 50, 100 or 200 nmol each of these amino acids were added to 2.0 ml of a 1:10 diluted urine sample.

The detection limits of BALA, BAIBA and GABA were 0.5 pmol at a signal-to-noise ratio of 3 under the present derivatization and HPLC conditions. Therefore, approximately 10 nmol per g of fresh tissue and 1 nmol per ml of blood plasma or urine of these amino acids could be determined by the present method. The sensitivity of the present method is comparable to HPLC methods with precolumn *o*-phthalaldehyde (OPA)-mercaptoethanol derivatization and fluorometric [4] or electrochemical detection [5] and to HPLC method with precolumn dansylation and fluorometric detection [9], which were reported for the determination of GABA in the brain. Approximately 10-fold improvement of sensitivity was obtained by the use of sulfite [6,7] or *t*-butylthiol [8] in OPA derivatization. However, the present method uses a simple detector of visible range and gives stable chromatograms.

Intra-day precision of the present method was examined with a liver extract. Values (mean±S.D., $n=5$) obtained were 2.302±0.088, 0.319±0.024 and 0.448±0.028 μmol per g of fresh tissue for BALA, BAIBA and GABA, respectively. Thus, the coefficients of variation were all less than 10%. Likewise, the coefficients of variation of inter-day reproducibility for these amino acids were 8.0, 7.0 and 8.3% ($n=14$), respectively, when tissue samples were stored at −20°C for 6 months.

Table 2 is a summary of concentrations of BALA, BAIBA and GABA in rat liver, kidney, brain, blood plasma and urine and their increases at 2 h after the subcutaneous injection of 15 mg of AOA per kg of body mass. Contents of these amino acids in kidney, brain and blood plasma were calculated using regression lines obtained with liver extracts.

Similar levels of BALA content with those obtained in the present study were reported in postmortem control of human liver [12] and normal human serum [13]. In the present study, BALA contents in the liver and kidney increased significantly, namely 10.2- and 4.6-fold the normal levels, respectively, after AOA treatment. The contents of this amino acid in the urine increased 25.7-fold by AOA treatment and that in the blood plasma 2-fold, but the latter was statistically insignificant because the contents were variable.

BAIBA was not detected in the liver, kidney, brain and blood plasma by the present method, but it was detected in human serum [13,14]. After AOA treatment in the present study, small amounts of this amino acid were detected in the liver, kidney and blood plasma, but not in the brain. Seventeen-fold increase of BAIBA in the urine was observed by AOA treatment.

GABA contents in the brain and blood plasma determined by the present method were comparable

Table 1
Recovery of β-alanine (BALA), β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA)

| Sample | Amino acid added[a] | Recovery (%) | | |
|---|---|---|---|---|
| | | BALA | BAIBA | GABA |
| Liver | 20 | 96.5±9.2 | 93.6±8.9 | 96.4±9.5 |
| (n=3) | 50 | 100.9±9.9 | 96.0±2.6 | 99.0±7.2 |
| | 100 | 100.8±5.7 | 99.7±5.0 | 100.0±8.9 |
| Urine | 50 | 105.6±10.0 | 103.4±5.7 | 101.7±5.2 |
| (n=3) | 100 | 101.5±4.3 | 106.0±4.1 | 98.3±1.2 |
| | 200 | 100.8±2.3 | 101.6±4.1 | 100.5±2.1 |

[a]Authentic amino acids (nmol per 0.5 ml of TCA extract of liver or 2 ml of 1:10 diluted urine) were added and analysis was performed as described in Section 2.

Table 2
Contents of β-alanine (BALA), β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA) in tissues and urine of intact and (aminooxy)acetate (AOA)-treated rats

| Tissue | Contents in tissues (μmol $g^{-1}$), blood plasma (nmol $ml^{-1}$) and urine (μmol $kg^{-1}$ b.m. per day) | | | | | |
|---|---|---|---|---|---|---|
| | BALA treatment | | BAIBA treatment | | GABA treatment | |
| | None | AOA | None | AOA | None | AOA |
| Liver | 0.177±0.046 | 1.810±0.128[a] | 0.000±0.000 | 0.413±0.030[a] | 0.000±0.000 | 0.221±0.003[a] |
| Kidney | 0.123±0.025 | 0.567±0.082[a] | 0.000±0.000 | 0.062±0.026[b] | 0.048±0.015 | 0.026±0.010 |
| Brain | 0.082±0.021 | 0.099±0.016 | 0.000±0.000 | 0.000±0.000 | 3.844±0.342 | 4.794±1.608 |
| Blood plasma | 6.400±3.991 | 13.352±6.592 | 0.000±0.000 | 1.451±0.196[a] | 0.700±0.096 | 0.959±0.225 |
| Urine | 9.13±1.55 | 234.94±16.50[a] | 5.81±0.69 | 96.35±6.54[a] | 5.02±1.24 | 46.44±2.34[a] |

BALA, BAIBA and GABA in rats were determined as described under Experimental. Values are expressed as mean±S.D. of data obtained from 3 (tissues, μmol $g^{-1}$ of fresh tissue; blood plasma, nmol $ml^{-1}$) or 5 (urine, μmol $kg^{-1}$ of body mass per day) animals. AOA was injected at a dose of 15 mg $kg^{-1}$ of body mass and tissue extracts were prepared 2 h later. Urine was collected for 24 h after AOA administration. Statistical significance was assessed by Student's *t*-test: [a]$P<0.001$; [b]$P<0.02$.

with those obtained by other methods [9,15–17]. Löscher et al. [18] reported significant increase in GABA content in the rat brain after the intraperitoneal injection of 30 mg of AOA per kg of body mass. However, in the present study, in which 15 mg of AOA per kg of body mass was injected subcutaneously, the GABA content increased, but the change was not statistically significant probably because of the difference in dose and administration method. In an experiment, where brain extracts were analyzed at 4 h after the AOA administration, GABA increased significantly from 3.8 to 7.5 μmol per gram of fresh brain. In the urine, a nine-fold increase in GABA content occurred by AOA injection, but this increase was much smaller than those of BALA and BAIBA contents.

BALA is a metabolite of uracil and is contained in coenzyme A and β-alanyl dipeptides, carnosine and anserine [19]. The increase in BALA excretion after AOA injection in the present study and others [15] indicates that BALA is actively metabolized through transamination reaction [15,19]. Further study on BALA metabolism is in progress.

BAIBA is a metabolite of thymine and of L-valine [19]. This amino acid was not detected in liver, kidney, brain and blood plasma under the present analytical conditions. However, an amount corresponding to that of BALA was excreted in the urine, suggesting a rapid urinary excretion of this amino acid. Human urine contains *R*-BAIBA almost exclusively which is formed from thymine, whereas the plasma contains *S*-BAIBA formed from L-valine [19,20]. Although further study is needed on the chiral structure of BAIBA in rats, the present results seem to suggest that the increased excretion after AOA treatment resulted from the inhibition of valine metabolism.

GABA is produced from L-glutamic acid mainly in the brain and acts as an inhibitory neurotransmitter [19]. It is contained in the brain in high concentrations and a GABA-containing dipeptide, homocarnosine, is also contained in the brain. GABA contents in the brain did not change significantly by the subcutaneous administration of 15 mg of AOA per kg of body mass. However, its excretion in the urine increased considerably. This finding might suggest that the increased excretion was due to the inhibition of extracerebral metabolism of GABA such as that in the kidney, pancreas and liver [19].

In conclusion, the present method seems to be useful for the study of metabolism of these amino acids and pyrimidines in animal tissues.

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