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Note

Analysis of γ-amino-β-hydroxy butyric acid (GABOB) by chromatography and electrophoresis

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γ-Amino-β-hydroxy butyric acid (GABOB) has been found in the brains of mice, rabbits, cattle, and humans [1], but it has not been found in normal urine and plasma. In Japan, however, GABOB has been tested as a drug for the treatment of convulsions and mental retardation.

Urine from a patient with mental retardation was examined for inborn errors of metabolism. Sodium buffer column chromatography showed a large unknown peak corresponding to tyrosine, but the tyrosine metabolites were normal. Lithium buffer was added to the urine and the unknown peak appeared, like homocystine between phenylalanine and β-alanine. By using paper chromatography and electrophoresis however, tyrosine and homocystine were eliminated as possibilities. Then it was disclosed that the patient had been given GABOB and by adding pure GABOB to the urine, the unknown peak increased correspondingly for all four methods of chromatography.

MATERIALS AND METHODS

Urine and blood samples were obtained from a 21 year old man with anophthalamus and mental retardation. He was given 1300 mg of GABOB (Ono Pharmaceuticals, Tokyo, Japan) daily. The day before the examination, fruit and vegetables were limited, and fasting urine and blood samples were collected early in the morning.

Procedure

For the determination of the urinary amino acid, the urine was deproteinized by adding 5 mg sulfosalicylic acid per ml of urine. The blood was collected in a heparinized centrifuge tube and shaken several times. The heparinized blood was immediately centrifuged (1620 *g*, 5 min) and the plasma was carefully removed from the upper layer. 120 mg dry sulfosalicylic acid were added to 4 ml of plasma, vigorously shaken and then centrifuged (1620 *g*, 5 min) [2].

Two dimensional paper chromatography

Whatman No. 1 paper, 20 × 20 cm, was used in Shandon equipment for ascending chromatography at 23°. Run 1: the solvent was pyridine—water—acetone—ammonia (45:30:20:5). Run 2: the solvent was isopropanol—formic acid—water (75:12.5:12.5). Both runs were 16 h.

Spray reagents: 3 g ninhydrin solution in 950 ml isopropanol and 50 ml collidin.

Hydrolysis

A volume (2 ml) of urine was hydrolyzed with an equal volume of 6 *N* HCl for 24 h at 110°. The hyrolyzates were dried in an evaporator at 55° and dissolved in 0.01 *N* HCl.

High-voltage paper electrophoresis was carried out with Pherograph-Original (Frankfurt, G.F.R.) using Paper No. 214, 35 × 40 cm from Macherey Nagel (Düren, G.F.R.). The buffer was pH 2 (1.5 *M* formic acid—2 *M* acetic acid, 1:1) and the electrophoresis was run at 100 mA and 2000—2500 V for 1.5 h. The paper was dried at 50°, and then used at right angles with isopropanol—acetic acid—water (8:1:1) as the solvent, for ascending chromatography at 23° for 21 h.

Liquid column chromatography

The amino acids were examined with the Technicon and Hitachi Model KLA-5 amino acid auto-analyzers. The technicon analyzer was equipped with a column (140 × 0.6 cm I.D.) filled with Chromobeads B resin. The gradient buffer solution contained lithium instead of sodium, according to Perry et al. [3]. The column was operated at 35° until glutamine had been eluted, then the temperature was raised to 70°. The Technicon integrator—calculator was used to quantitate all the amino acid peaks. Norleucine was used as internal standard. The concentration of the ninhydrin positive substances is expressed in μmole/l of plasma and μmole/mg creatinine in urine. The Hitachi analyzer had a column (25 × 0.9 cm I.D.) filled with Hitachi custom ion-exchange resin

No. 2615, and with 0.38 *N* sodium citrate buffer and was kept at 60° for basic amino acids. For acid and neutral amino acids, a column (55 × 0.9 cm I.D.) filled with Hitachi custom ion-exchange resin No. 2614 and with 0.2 *N* sodium citrate buffer, was used between 32° to 60°.

RESULTS

Two dimensional paper chromatography (urine)

Fig. 1 illustrates the positions of ninhydrin positive substances in two dimensional paper chromatography. Fig. 1(1) shows one unknown strongly ninhydrin positive spot near the alanine in the urine from the patient receiving GABOB. Fig. 1(2) shows the same ninhydrin positive spot to be present, two days after oral administration of GABOB was withdrawn. Fig. 1(3) shows the typical amino acid pattern of normal urine. Fig. 1(4) shows the spot of 10 μg pure GABOB added to the sample used in Fig. 1(3). GABOB shows up in the same position as the unkown compound in Fig. 1(1).

After hydrolysis, the ninhydrin positive GABOB did not disappear.

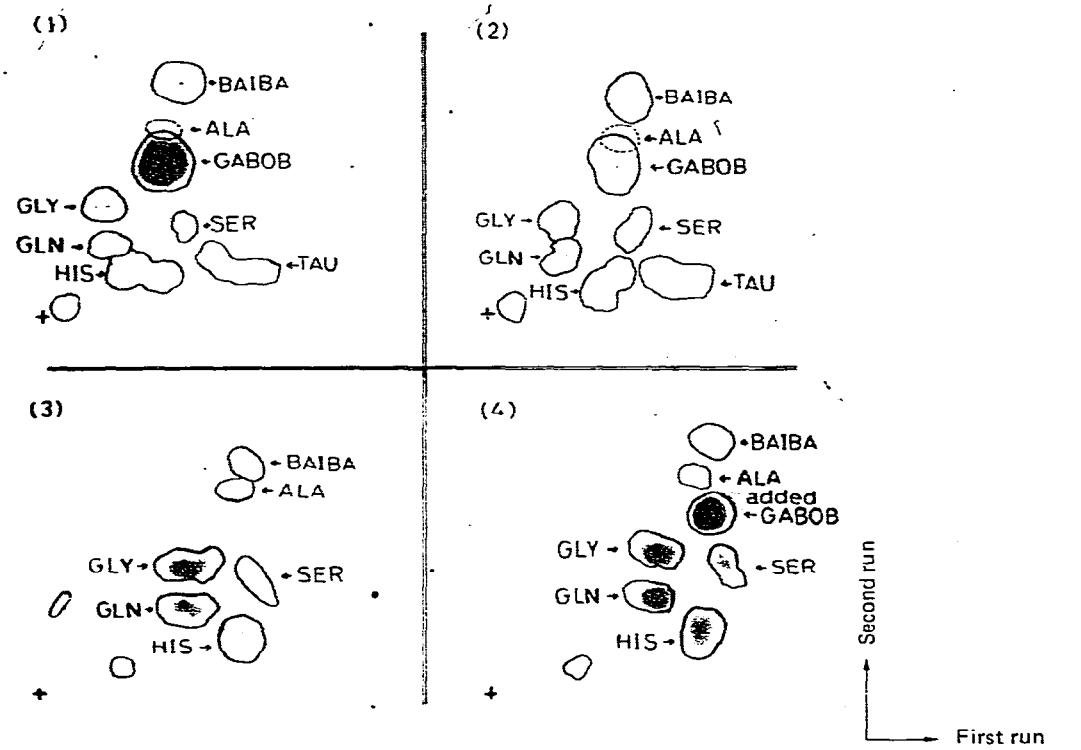

Fig. 1. Two dimensional paper chromatography of amino acids. Solvent pair: pyridine—water—acetone—ammonia (45:30:20:5) and isopropanol—formic acid—water (7.5:12.5:12.5). Detection reagent is ninhydrin. Chromatograms: (1) and (2) = urine samples from a patient receiving GABOB; (3) = normal urine sample; (4) = normal urine sample with added GABOB.

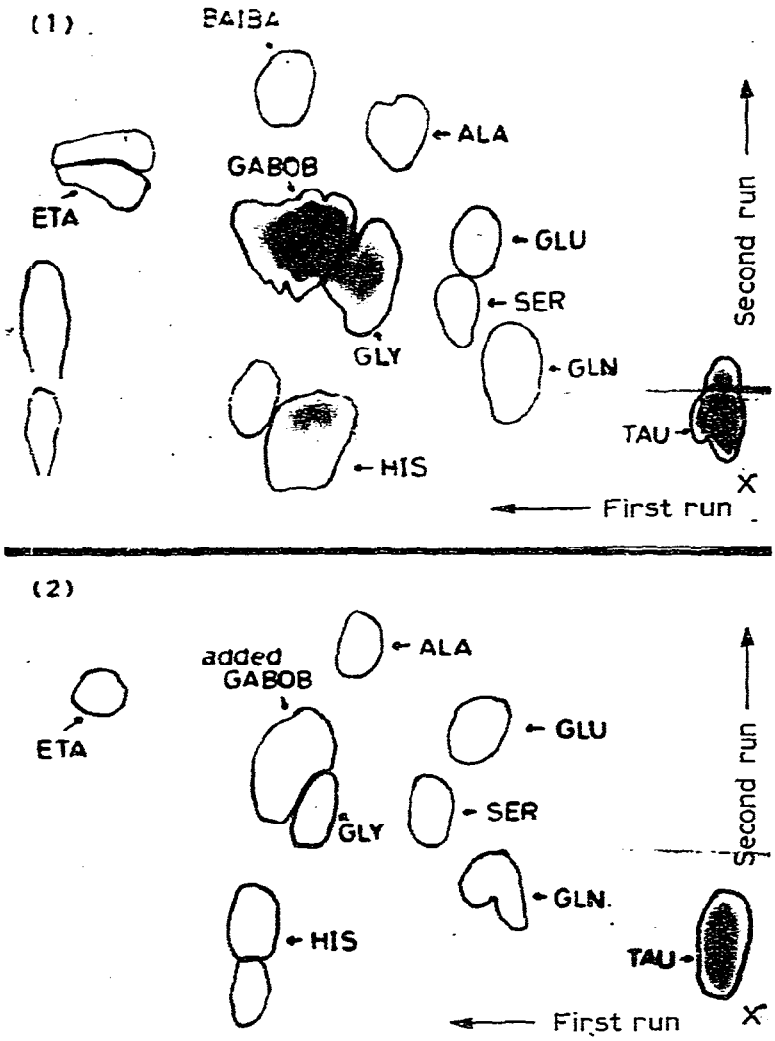

Fig. 2. Amino acids in a two dimensional separation using high voltage electrophoresis in the first run and isopropanol—acetic acid—water (8:1:1) for the second run. Chromatograms: (1) = urine sample from a patient receiving GABOB; (2) = normal urine sample with added GABOB.

High voltage paper electrophoresis (urine)

Fig. 2 shows the position of ninhydrin positive substances in the two-dimensional separation, using electrophoresis in the first dimension and isopropanol—acetic acid—water for the second dimension. Fig. 2(1) shows one strongly ninhydrin positive spot near the glycine in the urine from the patient receiving GABOB. Fig. 2(2) shows a similar ninhydrin positive spot in the same place after adding 20 μg of GABOB to the normal urine.

Even if the chromatogram was heated for 2—3 min in an oven at 105°, the colour did not change.

Liquid column chromatography with detection by amino acid auto-analyzer

(a) Lithium buffer method. Fig. 3 shows the chromatograms of ninhydrin positive substances from the Technicon amino acid auto-analyzer. Fig. 3(1) shows the ninhydrin positive GABOB peak 42 min after phenylalanine was added to the urine. Fig. 3(2) shows the peak from plasma in the same position; both samples were from one patient receiving GABOB. Two days after the withdrawal of oral administration of GABOB, the GABOB peak was still present in the urine, but not in the plasma.

(b) Sodium buffer method. Fig. 4 shows the chromatograms of ninhydrin positive substances from the Hitachi Model KLA-5 amino acid auto-analyzer. Fig. 4(1) shows the ninhydrin positive GABOB peak including leucine between isoleucine and tyrosine. One day after the withdrawal of oral administration of GABOB, the GABOB peak was not found in the plasma, as shown Fig. 4(2).

DISCUSSION

γ-Aminobutyric acid (GABA) has been shown to be converted to GABOB by beta oxidation and also to glutamic acid by transamination between GABOB and α-ketoglutaric acid [4]. Takada et al. [5] found two GABOB metabolites in the urine of rats receiving ^{14}C-GABOB. One was the γ-acetoamino-β-hydroxybutyric acid (N-acetyl GABOB) and the other was an unknown metabolite in low concentration.

Quantitative determination of amino acid in physiological fluid with the automatic amino acid analyzer is being used as a routine procedure for detecting inborn errors of metabolism [6, 7]. It is, however, very dangerous to diagnose metabolic diseases using only one method of amino acid analysis. Our case was originally diagnosed as tyrosinosis using the Hitachi auto-analyzer, because the GABOB peak appeared at the expected place for tyrosine. When using the Technicon analyzer, GABOB appeared close to homocystine. Paper chromatography and high voltage electrophoresis, however, ruled out these amino acids and identified GABOB.

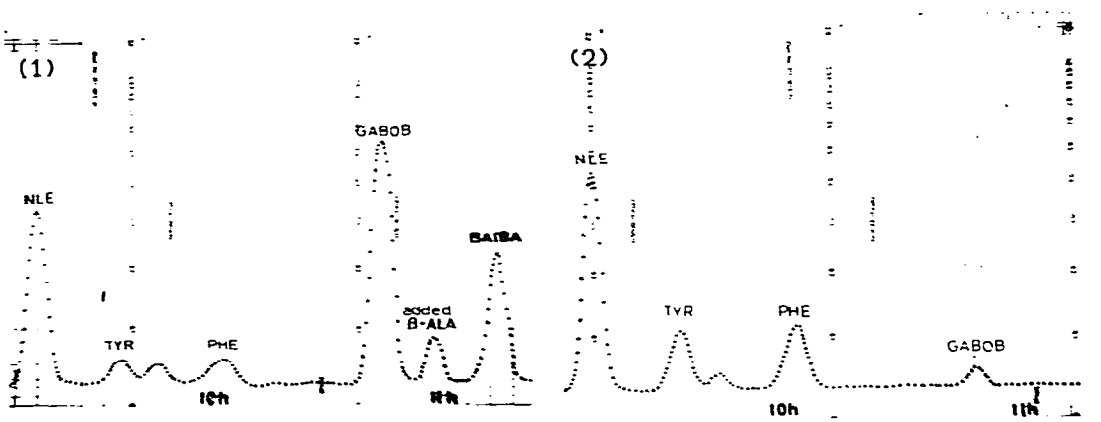

Fig. 3. Chromatograms from Technicon amino acid auto-analyzer using lithium buffer solution: (1) = urine sample from a patient receiving GABOB with added β-alanine; (2) = plasma sample from the same patient receiving GABOB.

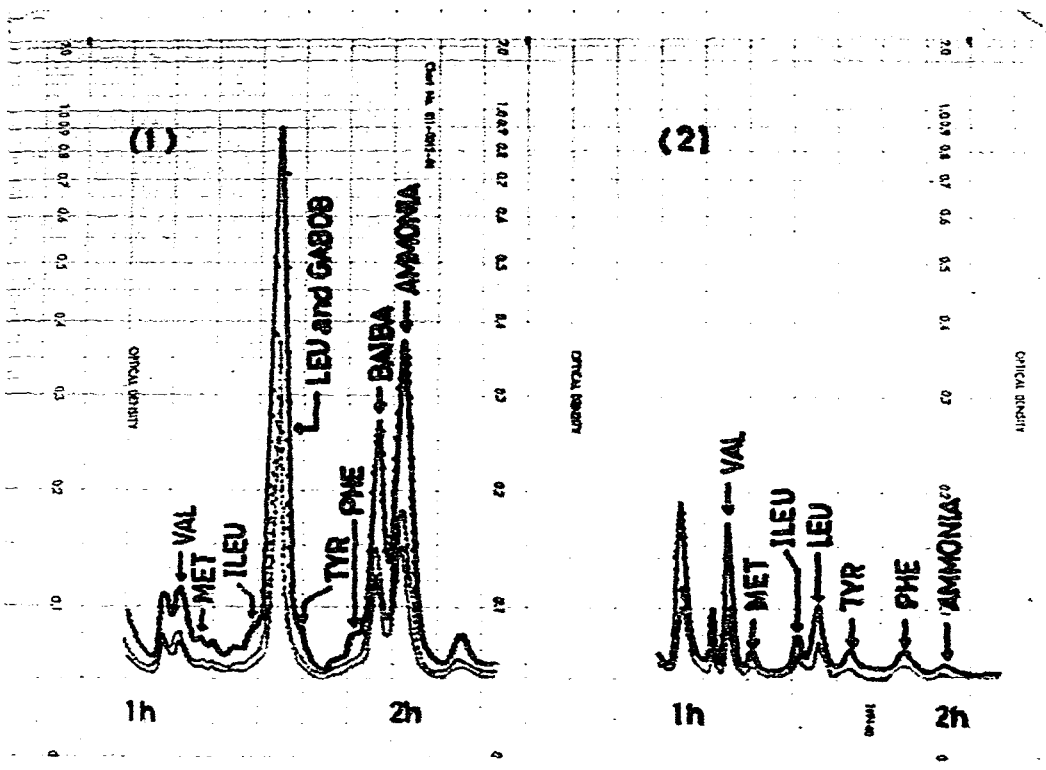

Fig. 4. Chromatograms from Hitachi Model KLA-5 amino acid analyzer using sodium buffer solution: (1) = urine sample from the patient receiving GABOB; (2) = plasma sample 1 day after withdrawal of GABOB.

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