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≪Original≫ Quantitative analysis of Free Amino Acids in Human Blood Serum by Gas-Liquid Chromatography

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Abstract

The quantitative analysis of various kinds of free amino acids contained in blood serum of patients with chronic mandible ostities, epidermic hemorrhagic fever, chronic renal failure and liver cirrhosis were measured with the gas-liquid chromatography (G. L. C.).

The results compared with the quantity of free amino acids of healthy persons. It was found that the quantity of free amino acids were differently contained in blood serum in accordance with kinds of patients.

요 약

만성 하악골염, 유행성출혈, 만성 신부전 및 간 경변증을 가진 환자의 혈청중에 포함된 여러가지 종류의 유리아미노산의 정량분석을 gas-liquid chromatography (G. L. C.)로 측정하였고, 건강인들의 유리아미노산의 정량과 비교한 결과 환자의 종류에 따라서 혈청중에 포함된 유리아미노산의 양에 차이가 있음을 발견 하였다.

1. Introduction

Qualitative and quantitative analysis of mixture of free amino acids are very important in various fields such as biology, pharmacology, foodstuff industry and biochemistry. Of many processes with regard to the study of free amino acids, the method using chromatography is most wildly adopted. In quantitative analysis, particularly, the ion-exchange chromatography method which has been developed by Moore *et al.* [1,2] Hamilton[3] and Piez & Morris[4] and the gas-liquid chromatography (G. L. C.) method explored by Gehrke *et al.*[5] are popularly used.

The G. L. C. method, compared with the ion-exchange chromatography method, can reflect a high degree of sensitivity and shorten the time of analysis. The ion-exchange chromatography method can be used for direct analysis of amino acids but in the G. L. C. method, derivatization must first be made in order to enhance the volatility of amino acids.

In this regard, research has been made by many research workers. Among them, Blau[6], Weinstein[7], McBride & Klingman[8], synthesized various derivatives of amino acids and based upon them, conducted study which can

be suitably applied to the analysis of amino acid derivatives. Furthermore, Smith and Sheppard[9], Klebe *et al.*[10] and Rühlmann & Giesecke[11] succeeded in synthesizing trimethylsilyl derivatives.

In 1965, Lamkin & Gehrke[12], for the purpose of quantitative analysis of amino acids by the G. L. C. method, made important studies on the volatility and chomatographic properties of amino acid derivatives.

In this study, using the OV-17 single column of G. L. C., the authors established a method to clean up and make quantitative analysis of various kinds of free amino acids contained in patient's blood serum and healthy person's.

The results compared with the quantity of free amino acids in blood serum of the patients of various diseases and healthy person's.

2. Experiment

1) Reagents

a) Amino acid (E. Merck. GR); Chromatographically pure amino acid was used.

b) Methanol; 500ml of anhydrous methanol (E. Merck. GR) was first refluxed with 5 gr. of Mg and redistilled.

c) Methanol-HCl; Anhydrous HCl gas was passed through a H_2SO_4 drying tower and saturated.

d) n-Butanol(E. Merck. GR)-HCl; Anhydrous HCl gas was passed through a H_2SO_4 drying tower and saturated.

e) Methylene chloride (E. Merck. GR); This was first refluxed with 13 gr. of anhydrous calcium chloride in 30 min. and redistilled.

f) Trifluoroacetic anhydride (T. F. A. A.) (E. Merck. GR);

g) Support material; Acid washed 80/100 mesh chromsorb G was dried at 550±50°, 15 hrs. and cooled at 200° and then stored in a desiccator over P_2O_5.

h) Ion exchange resin;
Cation (Dowex-50W, 50×8, 400 mesh)
Anion (Dowex-1, 1×10, 400 mesh)

2) Standard amino acid stock solution

This solution consisted of an aqueous solution (0.1 N-HCl) containing nineteen kinds of amino acids at individual concentration of 5 mg./100ml.

3) Instruments and chromatographic conditions

a) Instruments

Gas Chromatograph; Varian aerograph Model 1800
Recorder; Varian aerograph Model 20

b) Chromatographic conditions

Column; 6 w/w%, OV-17, 80/100 mesh chromsorb G, 6 feet x 1/8 inch.
Column temperature; Initial 120°C, Final 300°C.
Program rate; 4°C/min.
Detector temp.; 300°C.
Sensitivity; 32×10^{-10}
Carrier flow; Nitrogen (N_2) gas 6.7ml/min.
Chart speed; 10 inch/hr.

4) Samples

a) In this experiments, amino acid analysis was carried out with 3-4 ml. serum of healthy persons, patients of chronic mandible ostitis, epidermic hemorrhagic fever, chronic renal failure, and liver cirrhosis.

b) At first, protein was removed from blood serum with picrate[13] (0.1% aqueous picric acid) and picrate was removed with 0.02 N-HCl by cation and anion exchange

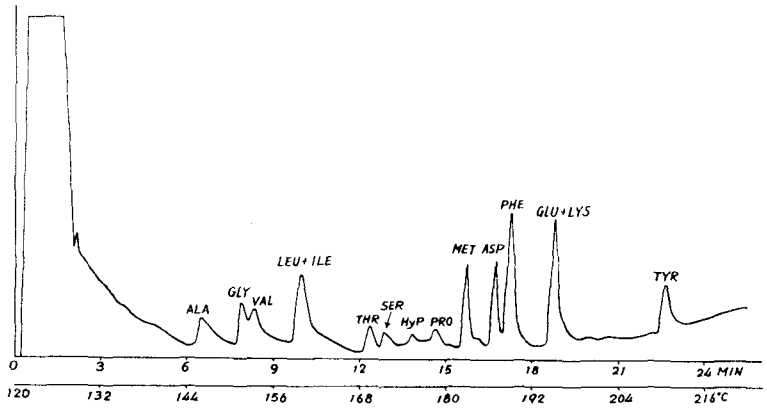

Fig. 1. Chromatogram obtained by G. L. C. of standard amino acid. (Sensitivity: 32×10^{-10}, Injected 4μl/Solvent 0.2ml)

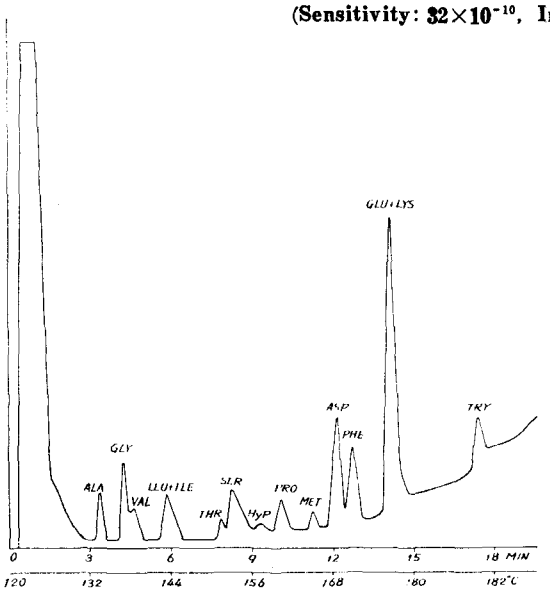

Fig. 2. Chromatogram obtained by G. L. C. of healthy blood serum cleaned by cation and anion exchange. (Sensitivity: 8×10^{-10}, Injected 0.2μl/ Solvent 0.05 ml)

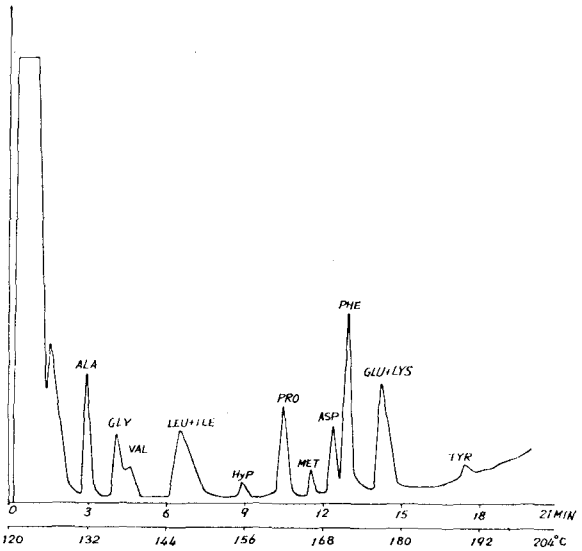

Fig. 3. Chromatogram obtained by G. L. C. of chronic mandible ostitis blood serum cleaned by cation and anion exchange. (Sensitivity: 16×10^{-10}, Injected 2 μl/ Solvent 0.2 ml)

chromatography.

The effluent and washings were evaporated to dryness on a rotary evaporator at 60°C constant temperature water bath. From the residue were derivatized N-trifluoroacetyl(N-T. F. A.) n-butyl esters of anino acids and the derivatized sample was then analyzed by G. L. C.

c) Standard amino acid

10 ml. stock solution was evaporated just to dryness on a rotary evaporator at 60°C water bath and N-trifluoroacetyl n-butyl esters of amino acids was derivatized.

5) Synthesis of N-T. F. A. n-butyl ester

a) To ensure complete removal of water

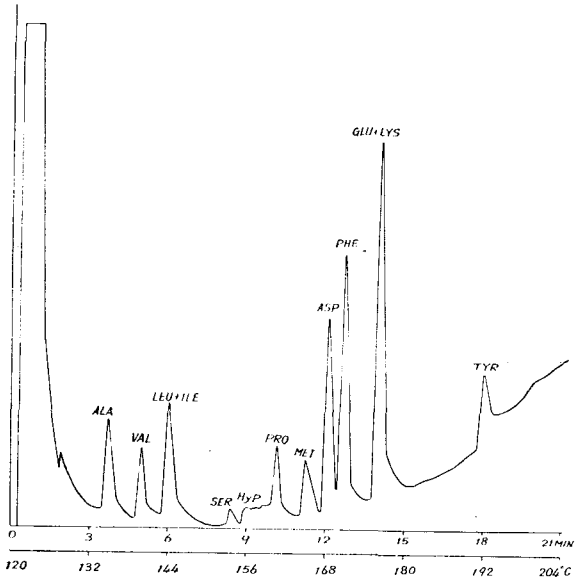

Fig. 4. Chromatogram obtained by G. L. C. of epidermic hemorrhagic fever blood serum cleaned by cation and anion exchange.
(Sensitivity: 8×10^{-10}, Injected 2μl/Solvent 0. 2ml)

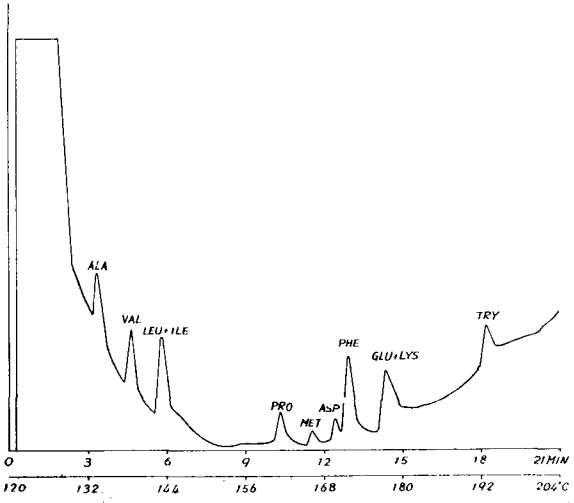

Fig. 5. Chromatogram obtained by G. L. C. of chronic renal failure blood serum cleaned by cation and anion exchange.
(Sensitivity: 8×10^{-10}, Injected 2 μl/Solvent 0. 2ml)

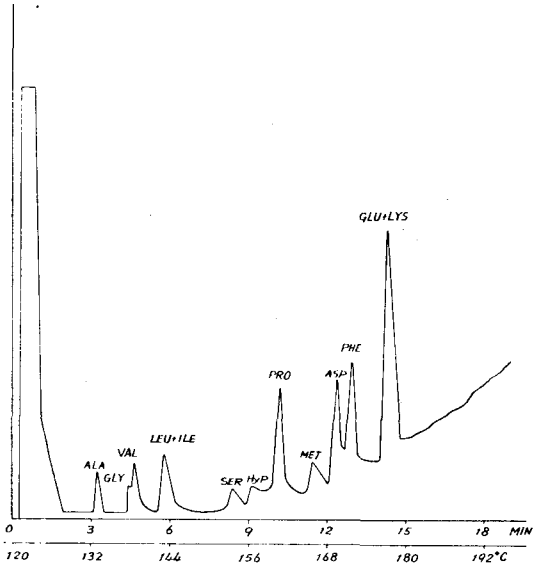

Fig. 6. Chromatogram obtained by G. L. C. of liver cirrhosis blood serum cleaned by cation and anion exchange.
(Sensitivity: 8×10^{-10}, Injected 2μl/Solvent 0. 1ml)

from the sample, 10 ml. of CH_2Cl_2 was added and then evaporated to dryness on a rotary evaporator at 60°C water bath.

b) To form amino acid methyl esters, in a 50 ml. culture tube, the residue was tightly capped with 10 ml. CH_3OH-HCl and allowed then to stand 30 min. at room temperature and the excess CH_3OH-HCl was evaporated at 60°C with nitrogen.

c) The interesterification to form amino acid n-butyl esters was performed by adding 20 ml. n-butanol-HCl and sample were heated 5 min. at 150°C oil bath and then at 100°C for 1 hr. sand bath. The excess n-butanol-HCl was evaporated from the sample with the 100°C sand bath and nitrogen gas to dryness on a rotary evaporator.

d) The amino acid n-butyl esters were acylated by adding 5 ml. of CH_2Cl_2 and 0.5 ml. of T. F. A. A.; the acylation tube was then securely capped, and the solution was completely mixed. To complete the acylation, the tube was placed in a 150°C constant temperature oil bath for 5 min. and then at 100°C for 1 hr. After being allowed to cool to room temperature, the samples were just dried at room temperature on a rotary evapo-

Table 1. Amino acid analysis of human blood serum. mg./100ml. of serum.

Samples / Amino acid	Normal range+	Healthy person	Chronic mandible ostitis	Epidermic hemorrhagic fever	Chronic renal failure	Liver cirrhosis
Alanine	1.2—6.0	2.75	19.25	12.33	5.50	3.67
Glycine	0.9—4.2	4.13	9.13	--	—	1.17
Valine	1.1—3.7	2.00	5.25	8.00	5.33	4.17
Leucine	0.7—2.3	1.75*	6.50*	7.50*	4.83*	4.33*
Isoleucine	0.4—1.3					
Threonine	0.8—2.9	3.38	—	—	—	—
Serine	0.8—2.6	10.38	—	3.00	—	7.50
Hydroxyproline	—	2.75	4.75	5.17	—	6.17
Proline	1.3—5.7	5.38	26.38	15.67	5.17	25.50
Methionine	0.2—0.5	0.88	1.50	3.00	0.33	1.83
Aspartic acid	0.0—0.7	4.25	5.38	11.17	0.67	6.00
Phenylalanine	0.4—1.3	1.88	9.25	9.50	3.00	4.00
Glutamic acid	0.3—5.1	8.75♯	5.63♯	3.17♯	2.00♯	8.83♯
Lysine	1.1—3.9					
Tyrosine	0.4—1.8	3.70	1.38	6.50	3.17	—
Total		51. 98	94.40	95.01	30.00	73.17

+: See references 13 to 16.
*: The mixture of leucine and isoleucine to be not separated.
♯: The mixture of glutamic acid and lysine to be not separated.

rator and redissolved in 0.2 ml. acetone and then analyzed by G. L. C.

3. Results and Discussion

The chromatogram of standard amino acid is revealed by Fig. 1. Serum amino acid values are listed in table 1. The levels obtained in the healthy person agree with these previously reported[13-16]. As shown in the figure 1. leucine is not completly separated from isoleucine and likewise glutamic acid not from lysine and furthermore, arginine, histidine, tryptophane and cystine are not shown in the chromatogram.

From Figures 1 to 6 represent chromatograms of standard amino acid and amino acids from patients of various diseases.

As indicated by the table, the blood serum of a healthy person contains 51.98mg. of 13 kinds of free amino acids. On the other hand the blood serum of a patient of chronic mandible ostitis has 11 kinds of amino acids amounting to 94.40 mg., lacking threonine and serine, that of a patient of epidermic hemorrhagic fever 11 kinds amounting to 95.01mg. lacking glycine and threonine, that of a patient of chronic renal failure 30.00mg. in 9 kinds, lacking glycine, threonine, serine and hydroxyproline, and patients of liver cirrhosis showed 73.17mg. in 11 kinds, lacking threonine and tyrosine.

The value of which leucine and isoleucine was contaminated with each other as well as the couple of glutamic acid and lysine. According to normal range, the measured levels of the above mentioned amino acids were nearly agree with the mean value of the each couples. The levels of alanine, valine, hydroxyproline, methionine in the patient serums

were more increased than healthy person's. However, threonine was not detected in the over-all patient serum, glycine, serine, hydroxyproline and tyrosine were irregularly revealed in the patient serums.

There is no known cause for the abnormalities in serum amino acids concentrations reported in this study. Two possibilities which seem unlikely are a defect in renal tubular absorption of amino acids, since the urinary clearances were normal, and extensive hepatic necrosis accompanied by a generalized release of amino acids into blood, a diagnosis unsupported by the clinical, laboratory, and histological findings.

The authors expect medical doctor to explan the above results by clinical study and to apply it to medical examination.

4. Conclusion

In making a quantitative analysis of free amino acids in human blood serum, the authors attempted a new clean-up method, a micro method in handling samples, and analysis by a single column method, and were able to analyze quantitatively 13, 11 and 9 kinds of free amino acids depending upon diseases.

As described above, compared with the blood serum of healthy persons, that of sick people contain a smaller number of amino acids, but have a larger quantity of free amino acids, and, in particular, it is known that the lack of threonine is common to all patients. It is presumed that the difference in quantity of amino acids in blood serum will be conductive to the diagnosis and treatment of patients.

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