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PRACTICAL GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF AMINO ACIDS IN HUMAN SERUM

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SUMMARY

Application of the gas—liquid chromatographic method previously reported by us was made to the analysis of the 22 amino acids including asparagine and glutamine in serum. The method permitted that aqueous serum samples obtained after deproteinization with perchloric acid were directly subjected to derivatization without any further clean-up procedure such as ion-exchange chromatography. The N-ethyloxycarbonyl methyl esters, which were prepared in the same manner as the N-isobutyloxycarbonyl methyl esters, were introduced for the determination of leucine, isoleucine, arginine and tyrosine. Both derivatives were prepared by two-step procedures involving alkyloxycarbonylation in aqueous media and esterification with diazomethane, and simultaneously analyzed by using the dual set of columns with the same thermal conditions. The advantages of this method are that the sample pretreatment and derivatization are very simple and rapid, and that both asparagine and glutamine along with other amino acids in serum can be determined.

INTRODUCTION

Extensive research investigations conducted for the development of amino acid analysis by gas—liquid chromatography (GLC) have resulted in the appearence of certain promising derivatives and derivatization methods. An enormous number of approaches for the preparation of volatile derivatives suitable for the quantitative analysis of amino acids has been reviewed [1—3]. The N-perfluoroacyl alkyl esters including the N-trifluoroacetyl (TFA) methyl esters [4—8], the N-TFA *n*-butyl esters [9—18], the N-heptafluorobutyryl (HFB) *n*-propyl esters [19—23], the N-HFB isoamyl esters [24,25], the N-HFB isobutyl esters [26—37] and the N-pentafluoropropionyl isopropyl esters [38,39] have been studied the most extensively, some of which have been practically employed for the successful quantitation of amino acids in biological samples.

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However, it has been recently pointed out that these derivatives possess certain negative aspects when applied to routine laboratory usage [40]. Notably, it would be supposed to be substantially disadvantageous that derivative preparations require conditions free from moisture and high reaction temperature, and that the amides, asparagine and glutamine, can not be differentiated from their corresponding acids due to hydrolysis in the drastic esterification phase. On the other hand, the N-acetyl *n*-propyl esters which are considered to be more stable to moisture than the N-perfluoroacyl alkyl esters have been successfully applied to biological samples [41—44]. Also in this case it was not possible to distinguish the amides from their acids.

The amides, especially glutamine, play important roles in a wide variety of physiological processes [45—49], so that a suitable method which allows simultaneous estimation of the amides and the free acids is desired. Therefore, the GLC method is inferior to the classical ion-exchange chromatographic method [50,51] because the latter allows determination of asparagine and glutamine in the presence of the free acids together with other amino acids. However, the ion-exchange chromatographic method is characterized by more lengthy analysis times if these amides should be analyzed, and some problems have been mentioned for analysis of glutamine [52]. The difficulty in the GLC methods described above still remains to be solved, although a few attempts using the esterification under restricted conditions have been made [45,48,53].

Recently, another approach has been proposed on the basis of the formation of 1,3-oxazolidinone derivatives of amino acids [40]. This method maybe has potential since it permits analyzing both asparagine and glutamine in the presence of all other serum amino acids.

Although the N-dinitrophenyl methyl esters possess more desirable features with regard to ease of derivative preparation, they have only been applied to analysis of limited amino acids in serum owing to their low volatility [54].

Some years ago, we proposed a new GLC approach [55], in which the volatile derivatives, N-isobutyloxycarbonyl (isoBOC) methyl esters were prepared under mild conditions by reaction with isobutyl chloroformate in aqueous alkaline media, followed by esterification with diazomethane. Recently, we have reported [56] that improvements of the previous method have made it possible to perform the determination of all the protein amino acids including asparagine and glutamine in sub-microgram amounts. In this paper, we describe the application of the method to the determination of amino acids in human sera.

EXPERIMENTAL

Reagents and materials

Standard amino acids and kainic acid as an internal standard were obtained from Nakarai Chemicals (Kyoto, Japan). Two standard solutions, one containing the 21 amino acids except ornithine listed in Table I and the other containing the 21 amino acids plus ornithine, were prepared in 0.1 *M* hydrochloric acid at individual concentrations of 50 μg/ml. An internal standard solution (50 μg/ml) was prepared in water. Ethyl and isobutyl chloroformates were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The reagents (anhydrous sodium sul-

phate, sodium chloride, reagents for generation of diazomethane), arginase solution and water were the same as in the previous paper [56]. It is recommended that freshly distilled, peroxide-free diethyl ether is used to protect the amino acids easily oxidized, especially methionine. All other chemicals and solvents were the purest grades available from standard commercial sources.

Sample preparation

Human blood was drawn from the cubital vein, left to stand for 10 min in an ice-bath and then centrifuged to remove blood particles.

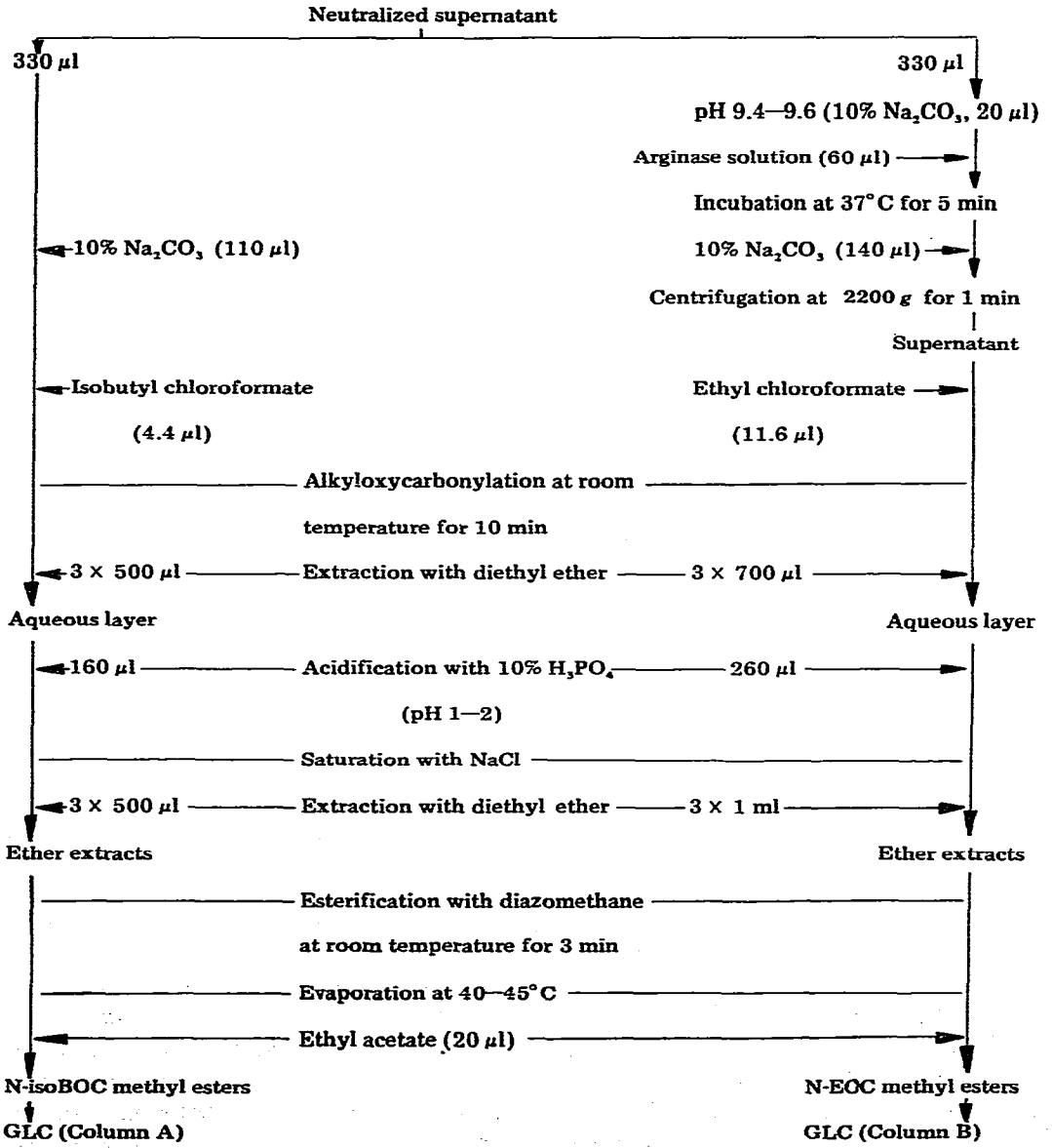

Fig. 1. Schematic flow diagram of the procedure.

To 200 μl of serum in a 2-ml glass tube (6.5 cm × 9 mm I.D.) were successively added 100 μl of the internal standard solution, 100 μl of water and 80 μl of 10% orthophosphoric acid. The solution was gently mixed for 10 sec with an equal volume of chloroform with a Vortex-type mixer and centrifuged at 2200 *g* for 5 min. The upper layer was transferred to another tube, taking care not to collect any chloroform droplets, and 80 μl of 12% perchloric acid were added with gentle swirling to precipitate proteins. Vigorous mixing should be avoided at this stage. After centrifugation for 1 min at 2500 *g*, the supernatant was carefully transferred to a small glass tube and neutralized with 10% sodium carbonate (ca. 120 μl).

Each 330-μl portion of the neutralized supernatant was taken in two 2-ml vials fitted with PTFE-lined screw caps (Wheaton No. 224950, Wheaton Scientific, Millvilles, NJ, U.S.A.) and they were treated according to the flow diagram shown in Fig. 1. Leucine, isoleucine, arginine and tyrosine were determined as their N-ethyloxycarbonyl (EOC) methyl esters, the reason for which is described in the Results and Discussion section. Since the colloidal precipitates were produced when 10% sodium carbonate was added to the incubation mixture obtained after arginase treatment, they were removed by centrifugation at 2200 *g* for 1 min and the supernatant was transferred to another vial with a PTFE-lined screw cap. This procedure contributed to obtaining reproducible results in the determination of tyrosine. Ethyl and isobutyl chloroformates (11.6 μl and 4.4 μl, respectively), were taken up by means of 10-μl syringes (Hamilton, Reno, NV, U.S.A.). Both isobutyloxycarbonylation and ethyloxycarbonylation were performed with a shaker (Iwaki KM Shaker VS Type, KK Iwaki, Tokyo, Japan), which was operated at 300 rpm (up and down). Extraction with diethyl ether was achieved with a Vortex-type mixer for 30 sec. After the first ether extracts were discarded, the reaction mixture was brought to pH 1—2 with 10% orthophosphoric acid and then saturated with sodium chloride. The combined ether extract was esterified with diazomethane [57]. The residue obtained after evaporation of the solvent was dissolved in 20 μl of ethyl acetate and thereafter a few grains of anhydrous sodium sulphate were added. From the resulting solution two 4—5 μl aliquots were taken and each was injected simultaneously into the gas chromatograph with two analytical columns, Column A and B.

Gas—liquid chromatography

The Shimadzu 4CM gas chromatograph used in this study was equipped with dual hydrogen flame ionization detectors, a double-column oven with on-column injection ports and a temperature programmer, and each electrometer was individually connected to a one-pen recorder. Two columns (each 1 m × 3 mm I.D.) were employed consisting of silanized glass tubes packed with 100—120 mesh re-silanized Uniport HP coated with 1.605% Poly-I-110/Poly-A-101A/FFAP (2000:1300:105, w/w/w) mixed phase (Column A) and 1.0% Poly-A-101A/FFAP (1:1, w/w) mixed phase (Column B), and were simultaneously operated. Materials for GLC were obtained as follows: the polyamide stationary phases Poly-I-110 and Poly-A-101A and the polyester stationary phase FFAP from Applied Science Labs. (State College, PA, U.S.A.), and the support Uniport HP from Gasukuro Kogyo (Tokyo, Japan). The column packings were

prepared and conditioned exactly as described in the previous paper [56]. The GLC conditions for analyses were identical for both columns with the exception of the nitrogen flow-rates. The columns were programmed from 80°C to 280°C at 10°C/min and maintained at 280°C for 5 min. The nitrogen flow-rates for Columns A and B were 35 and 25 ml/min, respectively. Quantitation of leucine, isoleucine, arginine and tyrosine was performed on Column B, and that of the other amino acids on Column A.

Calibration graphs, recovery rates and calculations

For the construction of calibration graphs, the proportional amounts of the standard corresponding to 0.5—10 μg (10—200 μl of the standard solution) and 100 μl of the internal standard (10 μg) were put into 2-ml vials, and the total volume of each vial was made up to 330 μl with water. The solutions were subjected to the procedure shown in Fig. 1. The standard solution not containing ornithine was employed only in the procedure involving the arginase treatment. The peak height ratios of amino acids with respect to the internal standard were calculated and were plotted against the quantities of amino acids.

To evaluate the recovery rates, five 200-μl portions of the serum sample, supplemented with 5—15 μg of each amino acid, were analyzed by the present procedure. The parent serum sample was processed at the same time. The recovery rates were calculated by subtracting the amounts obtained from the parent serum from those obtained from the same serum fortified with amino acids.

The calibration graph for ornithine obtained on Column B was used for the determination of total ornithine obtained after arginase treatment because the conversion yield of arginine to ornithine was above 95% in the range 0.5—10 μg of arginine under the conditions used. The concentration of arginine was determined by the following equation:

$$\text{Arginine (mg/dl)} = (\text{Orn}_{\text{total}} - \text{Orn}) \times 1.318 \times 500$$

where $\text{Orn}_{\text{total}}$ = amount of total ornithine obtained using Column B after arginase treatment, and Orn = amount of ornithine obtained using Column A without arginase treatment.

In the analysis of serum amino acids, one standard point, usually at the 5-μg level of amino acids, was incorporated in each series of determinations and the peak height ratios obtained from the standard were employed for the calculation of amino acid concentrations in sera.

RESULTS AND DISCUSSION

Generally, in most of the GLC methods proposed for the determination of amino acids in serum, the denaturants such as picric acid [12,17,39,58], sulfosalicylic acid [33] and trichloroacetic acid [15] were employed to remove proteins by precipitation. An alternative technique, based on dilution of the sample with acetic acid and without involving protein precipitation, has also been used [40,42,44,58,59]. However, in all cases a clean-up procedure using the ion-exchange resin column prior to derivatization was necessary, not only to exclude the excess of denaturants which usually interfere with the following GLC analysis, but also to isolate the amino acids.

On the other hand, our derivatization method has the advantage that amino acids in aqueous solution can be easily converted to their N-isoBOC amino acids, which are selectively extracted from the acidified reaction mixture into diethyl ether, without any step to exclude water (moisture) being necessary prior to derivatization. Taking this into account, attempts were carried out to develop a less time-consuming and less laborious sample pretreatment. Inorganic denaturants would be expedient for this purpose since it is not necessary in our method to remove them. The use of perchloric acid finally proved to be convenient for the effective and reliable removal of serum proteins in combination with prior extraction of lipids with chloroform from the acidified serum. Not only did this improve the clean-up of amino acids from serum but it was also satisfactory with respect to time requirements.

In the preliminary experiments, we tried to analyze all of the serum amino acids as their N-isoBOC methyl esters. However, it was found that the recovery of tyrosine from serum was not satisfactory, being below 70%. A similar phenomenon was also observed even when serum was subjected to the picrate precipitation followed by cation-exchange column chromatography according to the method of Zumwalt et al. [12] and the amino acids thus obtained were analyzed as their N-isoBOC methyl esters. Therefore, it may be tentatively concluded that the incomplete derivatization of tyrosine mainly resulted from its adsorption to some specific components which were not removed by these clean-up processes. This problem was solved by introducing the N-EOC methyl ester derivatives, which could be prepared by the reaction with ethyl chloroformate in the same fashion as in the N-isoBOC methyl esters. The recovery rates of tyrosine were sufficiently improved (see Table II), suggesting that ethyl chloroformate is more reactive than isobutyl chloroformate. As a consequence

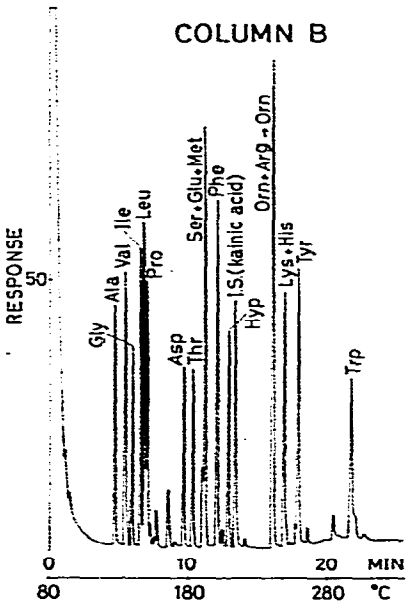

Fig. 2. Gas chromatographic separation of the N-EOC methyl esters of amino acids on Column B. Sample preparation and GLC conditions as described in Experimental.

of these observations, we decided to adopt the N-EOC methyl esters for the determination of leucine, isoleucine and arginine along with tyrosine in serum on Column B since in our method the former three amino acids essentially required a separate analysis [56]. Fortunately, it was found that the N-EOC methyl esters of these four amino acids provided good separations on Column B, as shown in Fig. 2. Alternatively, an attempt to use the N-EOC methyl esters for the simultaneous analysis of all serum amino acids failed because the N-EOC amino acids (not being esterified) of threonine, serine and hydroxyproline could not be extracted quantitatively from the acidified aqueous reaction mixture, particularly when working with the low levels. In addition asparagine and glutamine only gave negligible peaks presumably due to higher solubility of their N-EOC amino acids in aqueous solution.

After trying a number of compounds, kainic acid was chosen as the internal standard. Both derivatives of kainic acid were well-separated from those of the amino acids both on Column A and B, as shown in Figs. 3 and 4, and no peak was observed in the serum samples with the same retention time as that of the internal standard. Addition of the internal standard to serum at the first step in the overall procedure made the method reliable.

The total reaction volume in the alkyloxycarbonylation was reduced to below 1 ml while the concentration of sodium carbonate was maintained at

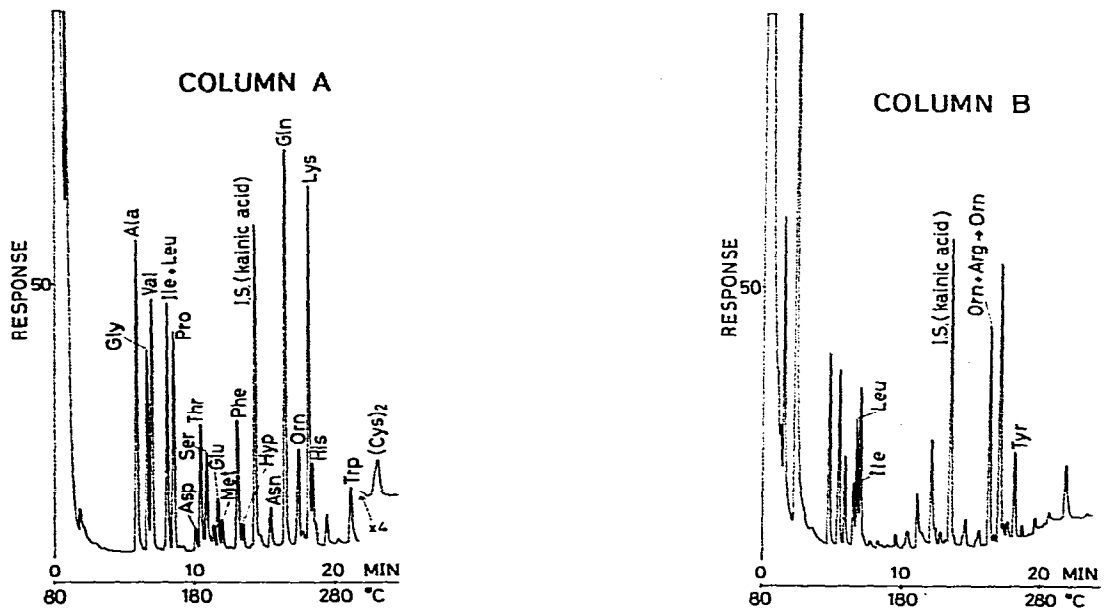

Fig. 3. Representative gas chromatogram of the N-isoBOC methyl esters of amino acids in a serum sample analyzed on Column A. Sample preparation and GLC conditions as described in Experimental.

Fig. 4. Representative gas chromatogram of the N-EOC methyl esters of amino acids in a serum sample analyzed on Column B. Prior to derivatization, a serum sample was treated with arginase, and only the peaks labelled by their names were determined. Sample preparation and GLC conditions as described in Experimental.

2.5% (w/v). From the experiments with the standard mixture containing 30 μg of each amino acid, it was found that the amounts of reagents equivalent to ca. 1% (v/v) for isobutyl chloroformate and ca. 2% (v/v) for ethyl chloroformate against the total reaction volume were enough to accomplish the reaction quan-

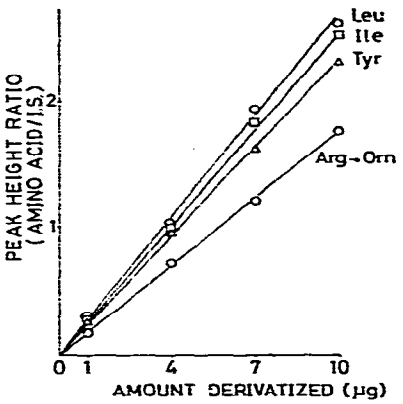

Fig. 5. Calibration graphs for the amino acids analyzed as their N-EOC methyl esters on Column B after arginase treatment followed by derivatization. Internal standard: 5 μg of kainic acid.

TABLE I

OVERALL REPRODUCIBILITY OF ANALYSES OF AMINO ACIDS IN HUMAN SERUM

Five 200-μl aliquots of serum were individually analyzed throughout the entire procedure.

Amino acid*	Abbreviation	Mean (mg/dl)	C.V. (%)
Alanine	Ala	2.81	1.67
Glycine	Gly	1.84	2.83
Valine	Val	2.57	2.98
Proline	Pro	2.41	3.69
Aspartic acid	Asp	0.23	6.15
Threonine	Thr	1.28	4.80
Serine	Ser	1.35	4.84
Glutamic acid	Glu	0.76	3.88
Methionine	Met	0.23	5.68
Phenylalanine	Phe	1.20	1.51
Hydroxyproline	Hyp	0.29	3.93
Asparagine	Asn	0.89	6.74
Glutamine	Gln	11.0	4.40
Ornithine	Orn	0.50	4.60
Lysine	Lys	2.02	5.37
Histidine	His	1.31	4.23
Tryptophan	Trp	0.35	5.50
Cystine	$(Cys)_2$	1.03	7.27
Isoleucine	Ile	0.65	4.74
Leucine	Leu	1.22	4.02
Arginine	Arg	2.47	1.65
Tyrosine	Tyr	1.45	1.43

*Prior to derivatization, arginine was converted to ornithine by treatment with arginase, and the total ornithine (ornithine from arginine plus ornithine originally present) together with isoleucine, leucine and tyrosine were analyzed as their N-EOC methyl esters on Column B. The other amino acids were analyzed as their N-isoBOC methyl esters on Column A.

titatively and reproducibly. It is, therefore, apparent from the above experiments that quantitative analysis was not affected even by more elevated amino acid levels in sera under the experimental conditions described.

The use of large excess of arginase (ca. 24 units/vial) in this study shortened the incubation time to 5 min, while maintaining the quantitative conversion of arginine to ornithine. However, in this case the precipitates were produced by subsequent addition of sodium carbonate solution, and this should be centrifuged off in order to ensure the quantification of tyrosine.

Calibration linearity for leucine, isoleucine, arginine and tyrosine obtained in the presence of all other amino acids was satisfactory as depicted in Fig. 5 and its between-run and within-run reproducibility throughout the overall procedure consisting of arginase treatment, derivatization and GLC analysis on Column B was sufficient enough to perform quantitative analyses. Similarly good results were obtained for other amino acids which were analyzed on Column A as their N-isoBOC methyl esters.

To check the reproducibility of this method, five 200-μl portions of the same serum sample were taken through the complete procedure. Table I lists the results with the mean concentration and the relative standard deviation for

TABLE II

RECOVERY RATES OF AMINO ACIDS ADDED TO HUMAN SERUM

Serum samples fortified with the known amounts (μg/200 μl of serum) were analyzed throughout the entire procedure.

Amino acid*	Recovery (%)						Mean	C.V. (%)
	5 μg		10 μg		15 μg			
	A**	B	A	B	A	B		
Alanine	101.9	94.2	98.4	93.1	98.4	101.7	98.0	3.75
Glycine	104.3	96.1	98.8	90.9	100.1	100.6	98.5	4.63
Valine	98.1	91.0	97.1	92.4	97.6	99.4	95.9	3.54
Proline	95.3	92.5	97.6	93.0	99.9	97.7	96.0	3.03
Aspartic acid	103.7	98.9	97.3	96.9	96.5	101.7	99.2	2.95
Threonine	103.9	99.2	99.3	100.7	106.6	102.5	102.0	2.84
Serine	105.2	102.9	98.1	106.1	95.1	106.1	102.3	4.52
Glutamic acid	94.2	98.9	101.2	93.8	101.5	99.6	98.2	3.46
Methionine	95.8	91.2	96.5	90.5	105.4	99.8	96.5	5.76
Phenylalanine	89.1	83.9	94.3	90.2	99.7	96.4	92.3	6.14
Hydroxyproline	100.3	103.7	103.7	100.8	98.1	105.5	102.0	2.69
Asparagine	103.4	110.8	95.9	108.9	94.8	108.6	103.7	6.70
Glutamine	101.7	100.0	92.3	92.2	100.2	106.5	98.8	5.67
Ornithine	99.0	93.7	100.8	97.3	92.1	102.5	97.6	4.14
Lysine	94.6	90.6	101.1	95.6	93.6	99.6	95.9	4.06
Histidine	106.9	108.4	102.5	109.2	106.3	101.4	105.8	3.00
Tryptophan	83.2	81.2	95.4	84.8	99.4	94.0	89.7	8.40
Cystine	101.4	110.5	110.4	106.7	107.5	104.2	106.8	3.33
Isoleucine	102.9	101.3	101.4	99.1	94.8	98.7	99.7	2.87
Leucine	93.0	98.6	102.2	101.1	96.0	92.5	97.2	4.20
Arginine	99.8	101.9	102.2	102.7	96.4	104.2	101.2	2.71
Tyrosine	102.7	100.5	95.6	99.0	96.3	94.2	98.1	3.30

*See footnote to Table I.

**A and B are different serum samples.

each amino acid. As can be seen, the method allows the determation of amino acids in serum with a relatively high degree of reproducibility.

When serum samples fortified with cysteine in addition to the 22 amino acids were analyzed by the present method, the cysteine peak mostly disappeared and the recovery of cystine was increased. It was proved by almost all investigators that cysteine could be easily oxidized to cystine, but not quantitatively, and this would explain the increase of recovery rate for cystine. Therefore, in this study cysteine was excluded. The recovery rates were determined by comparison with their respective standards and the results are shown in Table II. Good recoveries with reasonable relative standard deviations could be obtained for all amino acids investigated.

Ten human serum samples obtained from normal adult volunteers, who were not restricted in food intake, were analyzed according to the present method to discern whether the levels of amino acids determined are in agreement with those reported by others using an amino acid analyzer technique [42,60] and a GLC method [42]. The results are given in Table III, and typical chromatograms both on Column A and B obtained from a serum sample are illustrated in

TABLE III

AMINO ACID ANALYSIS OF HUMAN SERUM BY THE PRESENT METHOD AND COMPARISON WITH THE LITERATURE VALUES

Samples were taken from ten normal adult subjects and each was analyzed in duplicate. Values for the amino acid analyzer and GLC methods were from refs. 60 and 42, respectively.

Amino acid	Concentration (mg/dl)				
	Present method*		Amino acid analyzer		GLC method
	Range	Mean	Range	Mean	Range
Alanine	2.45—4.64	3.32	2.31—4.65	3.46	2.9—4.3
Glycine	1.75—2.70	2.15	1.37—2.30	1.84	0.9—2.8
Valine	2.20—3.14	2.50	2.34—3.63	2.80	2.1—4.1
Proline	1.31—2.77	1.86	–	2.36**	1.9—3.0
Aspartic acid	0.17—0.50	0.32	0.13—0.28	0.17	0.2—1.5***
Threonine	0.96—2.25	1.58	1.31—2.38	1.87	1.0—1.7
Serine	0.96—1.88	1.33	0.95—1.79	1.30	0.7—1.9
Glutamic acid	0.52—1.03	0.75	0.22—1.27	0.43	4.8—12.9***
Methionine	0.27—0.47	0.34	0.39—0.73	0.55	0.3—0.8
Phenylalanine	0.96—1.48	1.19	0.74—1.34	0.97	0.3—2.2
Hydroxyproline	0.17—0.38	0.26	–	–	0.1—0.8
Asparagine	0.67—1.09	0.81	0.63—1.19	0.82	–
Glutamine	9.60—11.4	10.6	7.31—12.1	9.21	–
Ornithine	0.61—1.09	0.85	0.46—1.15	0.74	0.1—1.2
Lysine	1.68—3.10	2.26	1.68—3.95	2.51	1.9—4.1
Histidine	1.11—1.59	1.29	0.90—1.72	1.33	0.6—1.8
Tryptophan	0.62—1.17	0.87	–	1.11**	0.5—2.4
Cystine	0.24—1.60	0.73	1.20—1.97	1.44	0.6—1.9
Isoleucine	0.66—1.07	0.88	0.70—1.34	0.96	0.3—1.7
Leucine	1.45—1.99	1.64	1.36—2.40	1.80	0.7—2.1
Arginine	0.95—2.87	2.05	1.24—2.26	1.65	0.9—2.7
Tyrosine	0.72—1.23	1.12	0.94—2.07	1.40	0.6—1.5

*See footnote to Table I.
**Values cited from ref. 61.
***Values include the corresponding amides.

Figs. 3 and 4. The ranges of serum amino acid values found by the present method correspond reasonably well with the results reported by others, although the population of individuals taking part in this study was rather small. Only a few and small extraneous peaks are detected on the chromatograms, thus indicating that the procedure provides adequate purification and separation of amino acids in sera. This method is by no means inferior to other GLC methods, as it required only small volumes of serum (200 μl).

In most GLC methods proposed to date, asparagine and glutamine are completely converted to their corresponding acids during acid-catalyzed esterification, so that no distinction between the amides and the acids is possible, whereas in our method both of the amides emerged as symmetrical and well-separated peaks, and could be determined separately from the respective acids and also from all other amino acids. Purification of the support is most important for a precise analysis of both amides since their derivatives still keep the intact amide groups in their molecules. The purification procedure for Uniport HP used has been reported in a previous paper [56].

CONCLUSION

We have developed a GLC method for the determination of the 22 amino acids including asparagine and glutamine in human serum, in which a serum treatment, involving neither ion-exchange column chromatography nor subsequent eluate evaporation, was introduced so that the first step of our derivattization method could be carried out in aqueous medium. Only 20 min was needed for the alkyloxycarbonylation and esterification, and the GLC analysis could be performed in 25 min. This method has the advantage of determining both asparagine and glutamine in serum. Recently, another method has been reported which also allows determination of asparagine and glutamine in serum [40].

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