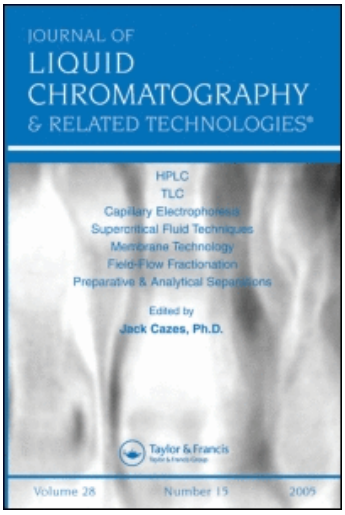

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QUANTITATIVE HPLC ANALYSIS OF PLASMA AMINO ACIDS AS ORTHOPHTHALDIALDEHYDE/ETHANETHIOL DERIVATIVES

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the analysis of plasma amino acids is described. The method employs pre-column derivatization with o-phthaldialdehyde using ethanethiol as the reducing agent. The analysis shows good linearity and reproducibility. An average overall difference of 12% was seen for results obtained by the HPLC method versus those obtained with an amino acid analyzer. The chromatographic parameters of buffer concentration and column temperature were also examined.

INTRODUCTION

More than 70 diseases associated with defects in amino acid metabolism have been reported (1). Since most of these conditions result in abnormal plasma concentrations of one or several amino acids, plasma amino acid concentration profiles have been useful in the diagnosis of these disorders.

Amino acids were first determined in physiological fluids by the classic method of Stein and Moore (2). This method utilized ion-exchange chromatography and the reaction with ninhydrin for the separation and detection of amino acids. Several modifications of this method have been developed (3,4); however, separation of amino acids in physiological fluids requires at least 4 1/2 hours.

Amino acids in hydrolysates of proteins have recently been analyzed by HPLC as phenylthiohydantoin (5,6), dansyl (7) and o-phthaldialdehyde/thiol derivatives (8,9). Hill et al.(10) and Lindroth et al.(8) have shown that amino acids in plasma can be determined in less than an hour by HPLC as o-phthaldialdehyde/ethanethiol (OPA/EtSH) or o-phthaldialdehyde/2-mercaptoethanol derivatives. The purpose of the present study was to characterize the optimal parameters necessary for the quantitation of plasma amino acids by HPLC as OPA/EtSH derivatives.

MATERIALS AND METHODS

Chromatographic System

Waters Associates Liquid Chromatographic Systems with two Model 6000A pumps were used for all experiments. One system utilized a Model 660 solvent programmer, a Model U6K injector and a Hewlett Packard HP3352D data system, whereas the other system utilized a Model 720 microprocessor solvent programmer, a WISP 710B automatic injector system and a Model

730 data module. Both systems were equipped with Schoffel FS-970 fluorometers adjusted to the following parameters: excitation wavelength - 229 nm; emission cut-off filter - 470 nm; time constant - 0.5 s. The sensitivity setting was adjusted so that tne photomultiplier tube had an absolute sensitivity of approximately 40 A/lm.

A μBondapack C_{18}, 30 cm x 3.9 mm i.d., reverse phase column along with a C_{18}/Corasil, 2 cm x 3.9 mm i.d. guard column was used for all analyses. New columns were conditioned before analysis of OPA/EtSH amino acids by equilibrating the column with 70% CH_3OH/H_2O and then programming to 100% CH_3OH in 15 min and allowing approximately 100 mL of CH_3OH to flow over the column. The solvent was then changed to 100% water over a 15 min period. The water was allowed to flow over the column for approximately 5 min. The solvent system was then changed to 0.1% (v/v) H_3PO_4 and approximately 200 mL of the acid was allowed to flow through the column. The column was flushed with water to remove the phosphoric acid prior to equilibrating with the solvent system described below. This procedure was found to be necessary to remove from the column an acid soluble component that reacted with the OPA/EtSH reagent and caused rapid degradation of the column. The acid wash of the columns was required only once.

The temperature of the column was maintained at 28° by circulating water through an aluminum water jacket that en-

circled the column. A Haake Model FJ heater/circulator and a Haake K11 cooling system was used to produce the required water temperature and pump the water through the column jacket.

A stock buffer of 300 mM phosphate/518 mM sodium was prepared by dissolving 11.59 g $NaH_2PO_4 \cdot H_2O$ and 30.66 g Na_2HPO_4 in reagent grade water and diluting to 1 L with reagent grade water. The buffer was filtered through a 0.45μ filter prior to preparing the HPLC solvent systems. Solvent system A (15.0 mM PO_4/25.9 mM Na) was prepared by diluting 50.0 mL of the 300 mM PO_4/518 mM Na stock buffer to 1 L with reagent grade water. Solvent system B_1 (15.0 mM PO_4, 25.9 mM Na, 45% H_2O/CH_3CN) was prepared by diluting 50.0 mL of 300 mM PO_4/518 mM Na stock buffer and 400 mL of reagent grade water to 1 L with CH_3CN. Solvent System B_2 (15.0 mM PO_4, 25.9 mM Na, 45% H_2O/tetrahydrofuran) was prepared by the same procedure described for solvent system B_1 except the solution was diluted to volume with tetrahydrofuran instead of acetonitrile.

Analysis of the OPA/EtSH amino acid derivatives on a new column resulted in a gradual, parallel decrease in the retention time of all the amino acid derivatives except arginine. This resulted in a loss of resolution between arginine and tyrosine. To regain this resolution, the concentration of the buffer in solvent A and B was increased by using an addi-

tional 10.0 mL of stock buffer. Additionally, 10.0 mL less water was added to solvent systems B so that the concentration of water remained 45%.

For separation of the amino acid derivatives (except Gly and Thr) a solvent program consisting of a linear gradient from 15% (or 10%) solvent B_1/solvent A to 75% solvent B_1/solvent A over a period of 60 min was used (System I). For the separation of OPA/EtSH Gly and Thr derivatives an isocratic system of 30% B_2/A was used. After 12 min the solvent was changed to 75% B_2/A to remove the other amino acid derivatives from the column (System II). A flow rate of 2.0 mL/min was used for both solvent programmed systems. The columns were reequilibrated to initial solvent conditions by a 10 min reverse linear gradient.

Preparation of Standards

Individual crystalline samples of L-amino acids were obtained from Pierce Chemical Co. (AMAC Standard Kit, No. 20065), Aldrich Chemical Co. (DL citrulline, No. 85,572-3; DL-p-fluorophenylalanine, No. Fl-380-D; Taurine, No. 15,224-2) and Sigma Chemical Co. (β-alanine, No. A-7752; DL-α-aminoadipic acid, No. A-0637; L-α-amino-n-butyric acid, No. A-1879: DL-β-aminoisobutyric acid, No. A-8504; L-1-methylhistidine, No. M-9005; L-3-methylhistidine, No. M-3879; L-ornithine, No. O-2375). A mixture of 0.4μmol/mL of each of the following amino acids was

prepared in 0.1 N HCl: Ala, Arg, Asn, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Ser, Tau, Thr, Trp, Tyr and Val. Five mL (2.00 μmol each amino acid) of this solution was placed in several 150 mm x 14 mm test tubes, lyophylized to dryness and glass sealed under the original vacuum. Fresh standards were prepared daily by dissolving the residue from one of the lyophylized standards with 4.0 mL of 0.1 N HCl to produce a 500 nmol/mL stock standard. Other amino acids standards were prepared by diluting aliquots of this solution with 0.1 N HCl.

A stock solution of the internal standard (2.00 mol/mL fluorophenylalanine) was prepared by dissolving 37.7 mg of 97% DL-fluorophenylalanine in 0.1 N HCl and diluting to 100 mL with 0.1 N HCl. The working internal standard solution (20.0 nmol/mL fluorophenylalanine) was prepared by diluting 1.0 mL of the 2.00 μmol/mL fluorophenylalanine solution to 100 mL with methanol.

Preparation of OPA/EtSH Amino Acid Derivatives

Two methods, a macro and a micro method were used to prepare OPA/EtSH derivatives of amino acids in plasma and standards. In the macro preparation, 2.00 mL of methanol or internal standard solution were placed into a 15 mL glass-stoppered centrifuge tube. Three-tenths mL of standard amino acid solution or plasma (or serum) was slowly added to the methanol

with intermittent mixing. The solution was mixed on a vortex-type mixer for 15 s and then centrifuged at 1200 x g for 5 min. One mL of the supernatant phase was transferred to a 5.00 mL volumetric flask. The reaction was initiated by adding, in order, 0.5 mL of saturated borate buffer (pH 9.5), 1.0 mL of 20 µL/mL ethanethiol (in methanol) and 1.0 mL 20 mg/mL o-phthaldialdehyde (in methanol). The total was diluted to 5.00 mL with methanol and allowed to remain at room temperature for at least 2 min for completion of reaction before analysis of 5 to 10 µL on the HPLC system.

In the micro method, 30 µL of amino acid standard or plasma (or serum) sample were added to 200 µL of internal standard solution in a 5 mL glass-stoppered centrifuge tube. The solution was mixed on a vortex-type mixer for 15 s and then centrifuged at 1200 x g for 5 min. One hundred µL of the supernatant phase was transferred to a 15 mL centrifuge tube. The reaction was initiated by adding, in order, 20 µL saturated borate buffer (pH 9.5) and 10 µL/mL ethanethiol/20 mg/mL o-phthaldialdehyde in methanol. The solution was allowed to stand at room temperature for at least 2 min before analysis of 5 to 10 µL on the HPLC system.

Quantitative Analysis

Mixtures of three or four of the standard amino acids were analyzed on the HPLC system at concentrations ranging

from 500 nmol/mL to 10 nmol/mL. The peak area (A_s) of each amino acid derivative was divided by the concentration (c_s) to produce the response factor (k). The response factor for each concentration of a given amino acid was averaged to determine the k that was used to calculate the concentration of the amino acid in the sample (C_u) as follows:

$$C_u = \frac{A_u}{k}$$

A_u= Area of amino acid derivative in sample

Optimization of OPA/EtSH Reagent Concentration

A plasma sample was prepared for amino acid analysis by mixing 1.5 mL of plasma with 10.0 mL of methanol in a culture tube. Following centrifugation at 1200 x g for 5 min, 1.0 mL of the supernatant phase, 1.0 mL of 300 nmol/mL amino acid standard and 0.5 mL saturated borate buffer (pH 9.5) were added to six 5.00 mL volumetric flasks. One of the following volumes of a solution containing 100 mg OPA/100 µL EtSH per mL methanol was added to one of the reaction flasks: 0.005, 0.025, 0.050, 0.100 or 1.00 mL. The solutions were diluted to 5.00 mL with methanol. Five µL of each sample were analyzed by the described HPLC system 2 min after initiating the reaction. To determine the optimal amount of OPA/EtSH necessary for complete reactivity with the amino acids in plasma, the

sum of the area of the peaks for all amino acids in a profile was plotted against the concentration of OPA/EtSH.

Effect of Buffer Concentration on Retention of OPA/EtSH Amino Acids

Six sets of A and B_1 solvent systems were prepared containing different concentrations (3.00/5.16, 6.00/10.3, 9.00/15.5, 12.0/20.6, 15.0/25.8 and 18.0/31.0 mM PO_4/mM Na) of sodium phosphate buffer, while maintaining a 45% concentration of water in solvent B_1 and the same pH in all systems. A sample of OPA/EtSH derivatized amino acids (19.6 pmoles each) was analyzed by the described HPLC system using each set of A and B_1 solvent systems in turn. The effect of the change in buffer concentration was visualized by plotting the retention time for each amino acid derivative against the buffer concentration.

Effect of Column Temperature on the Retention of OPA/EtSH Amino Acids

Using the described HPLC system, 19.6 pmol of OPA/EtSH amino acid standard was analyzed using column temperatures of 15°, 20°, 25°, 28°, 30° and 40°. The effect of column temperature on the retention of each amino acid derivative was observed by plotting the retention time of each amino acid against the column temperature.

Comparison of Plasma Amino Acid Concentrations Determined by an Amino Acid Analyzer and by the Proposed Method

Samples of plasma were collected from six healthy rats and were analyzed for Thr, Ser, Pro, Glu, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, Lys, His and Arg on a Beckman 119C Amino Acid Analyzer. The samples were also analyzed by the procedure described in the present study. The data from both methods were compared by taking the absolute difference in the values obtained by the two methods and dividing by the value obtained by the amino acid analyzer. This number was multiplied by 100 to obtain the percent difference.

RESULTS AND DISCUSSION

Figures 1 and 2 show chromatograms of OPA/EtSH derivatives of standard amino acids, using system I and system II respectively. Attempts were made to resolve the Gly and Thr derivatives in one system along with the other amino acid derivatives shown separated in Figure 1. As indicated by Larsen *et al.* (11), Jones *et al.* (9) and Figure 2, the use of tetrahydrofuran in the solvent system is effective in resolving Gly and Thr, however, small amounts of tetrahydrofuran added to either solvent A or solvent B_1 in system I resulted in either the loss of resolution between Ser and Gln or Ile and Trp or the loss of resolution between both sets of these amino acids. Since system II gives baseline resolution between Gly and Thr, it was decided to sacrifice the

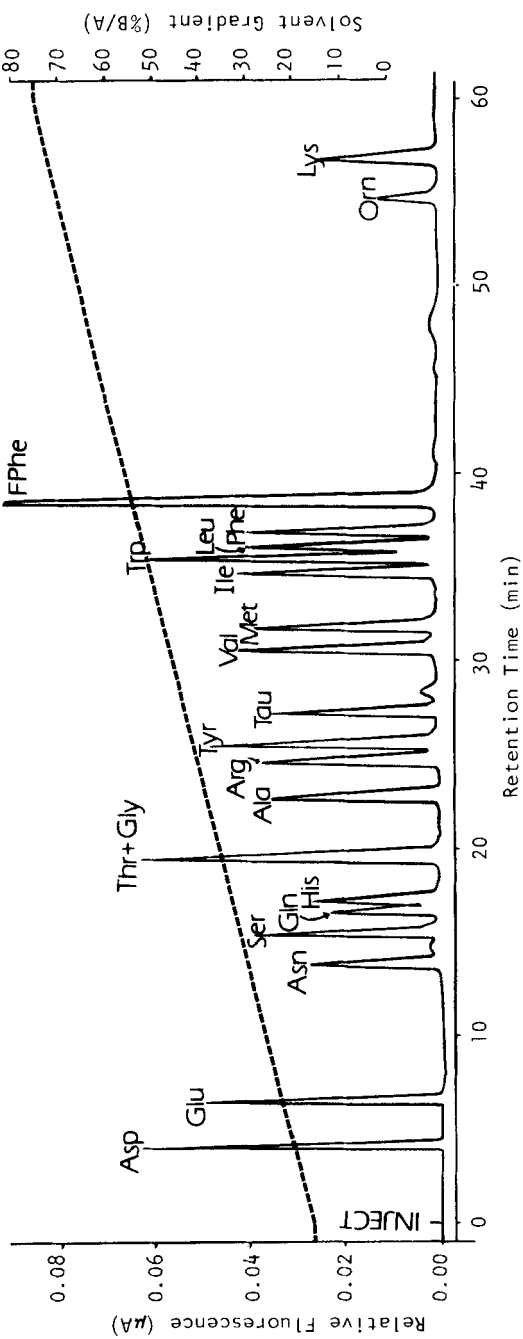

Figure 1. Chromatogram of OPA/EtSH Amino Acid Standards. Chromatographic System I. (FPhe = Fluorophenylalanine, Internal Standard)

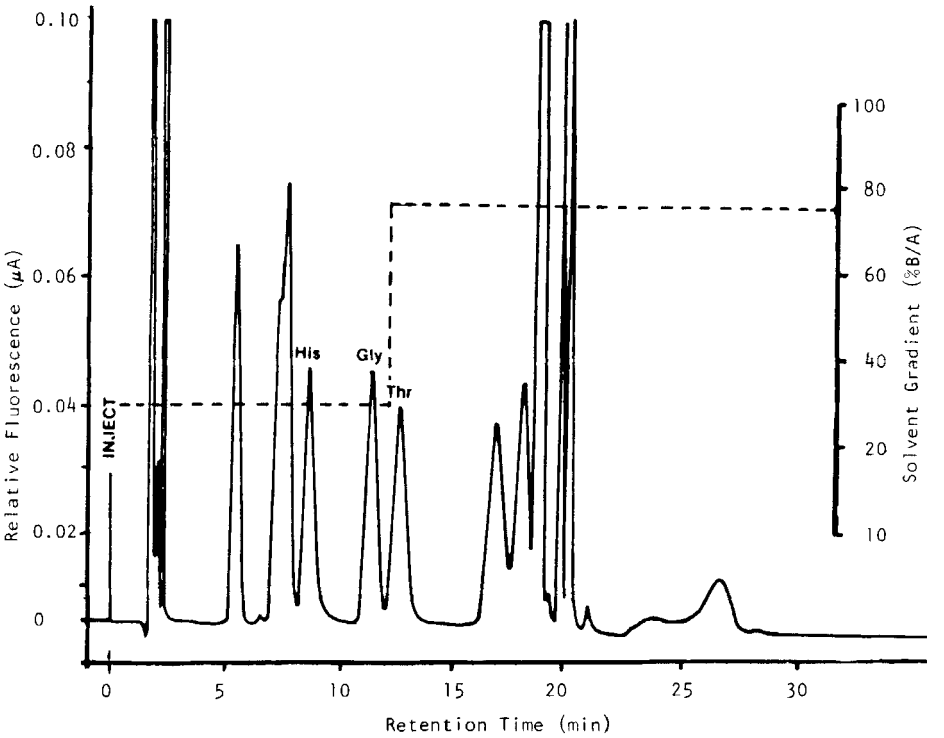

Figure 2. Chromatogram of OPA/EtSH Amino Acid Standards. Chromatographic System II.

separation of this pair of amino acids in system I, in order to maintain good baseline resolution of all other amino acids and to use system II to analyze Gly and Thr separately.

In addition to those amino acids shown in the chromatogram in Figure 1, the elution order of several other amino acids in system I is listed in Table 1. A comparison of retention times of amino acids relative to fluorophenylalanine is listed. These sets of data were obtained six months

TABLE 1

Elution Order of OPA/EtSH Amino Acids on Bondapak C_{18} in System I

Amino Acid	RR_t [1,2]	RR_t [1,3]
Aspartic Acid	0.101	0.106
Glutamic Acid	0.166	0.168
αAminoadipic Acid	0.226	---
Asparagine	0.355	0.357
Serine	0.398	0.399
Glutamine	0.426	0.430
Histidine	0.448	0.445
Citrulline	0.490	---
Threonine	0.505	0.506
Glycine	0.505	0.506
1-Methylhistidine	0.541	---
Alanine	0.588	0.590
Arginine	0.610	0.639
β-Alanine	0.646	---
Tyrosine	0.664	0.663
Taurine	0.702	0.706
β-Aminoisobutyric Acid	0.702	---
α-Amino-n-butyric Acid	0.702	---
Valine	0.789	0.795
Methionine	0.817	0.826
Isoleucine	0.891	0.902
Tryptophan	0.914	0.920
Leucine	0.926	0.939
Phenylalanine	0.947	0.957
p-Fluorophenylalanine	1.000	1.000
Ornithine	1.385	1.427
Lysine	1.437	1.483

[1] RR_t = Retention time of OPA/EtSH amino acid relative to the retention time of OPA/EtSH p-fluorophenylalanine.

[2] RR_t of OPA/EtSH amino acids determined on column number 113134 on 11/80.

[3] RR_t of OPA/EtSH amino acids determined on column number 139094 on 5/81.

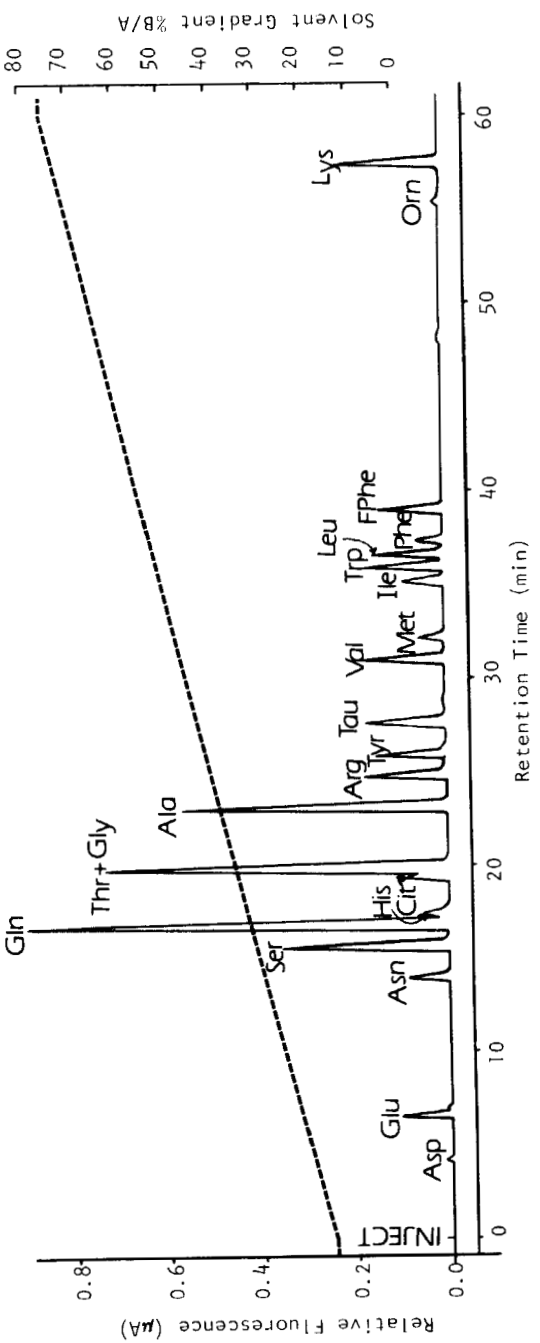

Figure 3. Chromatogram of Rat Plasma Amino Acids as OPA/EtSH Derivatives. Chromatographic System I. (FPhe = Fluorophenylalanine, Internal Standard)

apart on two columns containing different batches of μBondapak C_{18} column material. Relative retention times between the two runs are proximal. Note that Gly and Thr co-elute. α-Amino-n-butyric acid and β-aminoisobutyric acid co-elute with Tau.

A chromatogram of OPA/EtSH derivatives of amino acids extracted from a normal rat plasma is shown in Figure 3. The amino acid derivatives were identified by comparing the relative retention times of the peaks in the sample profiles to the relative retention times of the amino acid standards.

The average variation in the retention time of the 20 amino acids in the standard solution when analyzed six times was 1.9% CV (range 1.6% to 2.4%). For the same set of samples the average relative retention time varied by 0.3% CV (range 0.1% to 0.6%). The retention data suggest that the identification of amino acids should be evaluated on the basis of the relative retention time.

Linear regression analysis of the peak area of each amino acid versus the amino acid concentration over a range of 500 to 5 nmol/mL gave a correlation coefficient of 0.999 or better for all the amino acids in the standard solution. Figure 4 shows a standard curve for 3 representative amino acids. The curve illustrated for Ala is representative of the slope that was obtained for most of the amino acids. The curves obtained for Orn and Lys had a lower slope value than the other amino

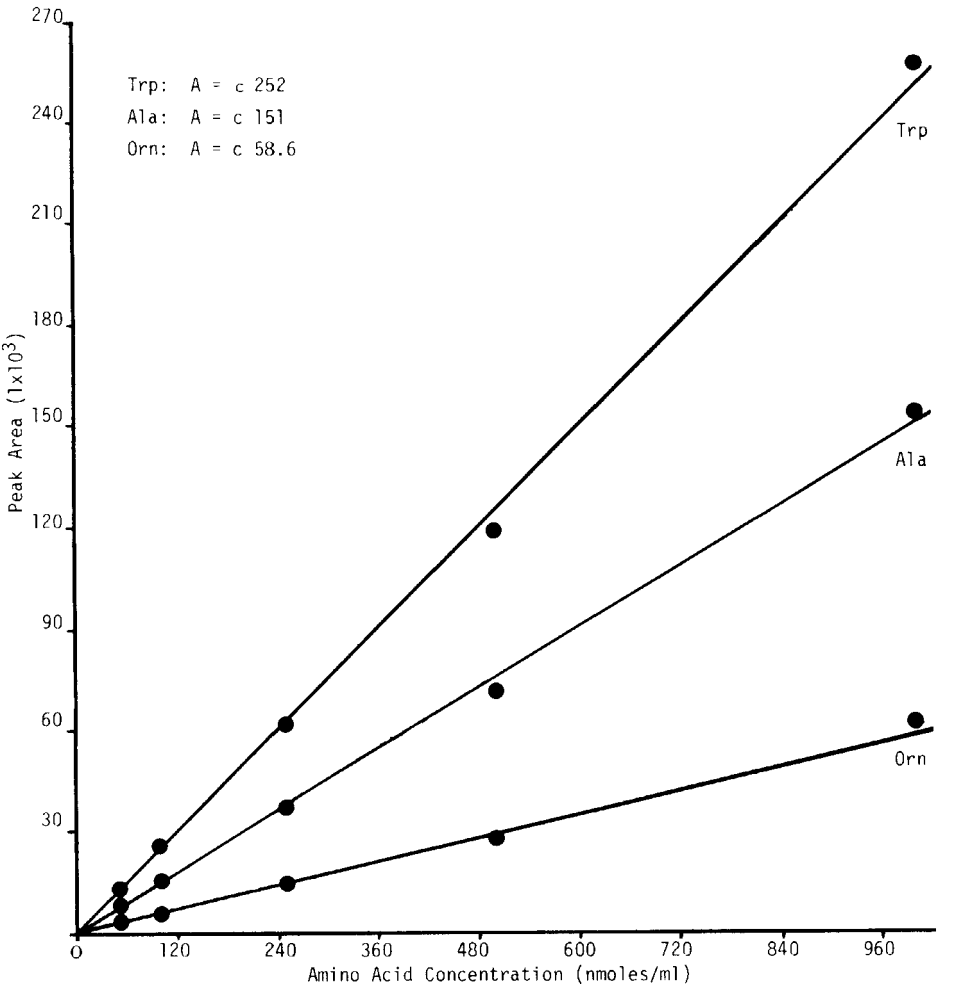

Figure 4. Representative Standard Curves for OPA/EtSH Amino Acid Response versus Concentration.

acid derivatives. This has been observed by other investigators in both post-column and pre-column derivatization with OPA/mercaptoethanol (8, 9, 12, 13). The cause may be due to the presence of two fluorescent isoindole structures, as occurs for both Orn and Lys, causing an internal quenching of the fluroescence of each structure. The fact that the slope for Lys was greater than the slope for Orn suggests that a greater distance between the two isoindole structures results in less internal quenching effects. The slope for Trp has a larger value than that of the other amino acid derivatives. This was probably due to the natural fluorescence of Trp enhancing the fluorescence of the adduct.

The relationship between the peak area response and the amount of derivatizing reagent used in the reaction of a plasma extract containing 2.3 μmol/mL additional concentration of each amino acid is shown in Figure 5. For the proposed derivatization procedure (utilizing 0.3 mL of plasma), the data indicated that 1 mL of a solution containing 5 mg OPA/5 μL EtSH was sufficient for complete reactivity of all amino acids in plasma even when present at a concentration several times normal. In order to be sufficiently upon the plateau of the reactivity curve a concentration of 20 mg OPA/20 μL EtSH per mL was chosen for routine applications. The ratio of OPA/EtSH was maintained at approximately 1:2 to insure

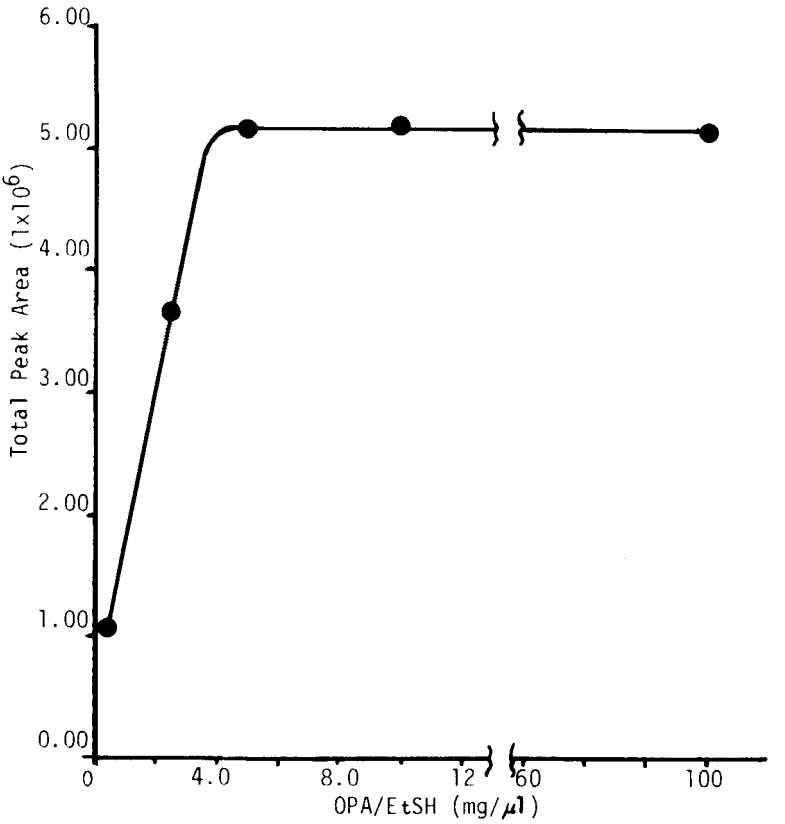

Figure 5. Optimum Concentration of o-Phthaldialdehyde and Ethanethiol for Complete Reaction of Amino Acids in Plasma.

that ethanethiol was the only thiol present in sufficient quantity to form the fluorescent derivative.

Hill, et al. (10) demonstrated that a change in the buffer concentration in the HPLC system affected the retention of the Tau and Tyr OPA/EtSH derivatives but not the retention of Arg. It was suggested that the sodium ion was re-

sponsible for the effect. Lindroth et al. (8) demonstrated that changing the buffer concentration in the solvent system affected the retention of all the OPA/mercaptoethanol amino acid derivatives except Arg or ammonia. Data were presented that suggested that the phosphate ion was responsible for the effect and not the sodium ion. Figure 6 shows the effect of changing the buffer concentration on the retention time of several of the OPA/EtSH amino acid derivatives. As observed for the OPA/mercaptoethanol amino acid derivatives an increase in buffer concentration results in an increase in the retention time of all OPA/EtSH amino acids except the Arg derivative. Additionally, Larsen et al. (11) have shown this same effect to occur by increasing the concentration of the counter ion triethylamine in the solvent system.

It was observed that as samples or standards were analyzed, the retention time of each amino acid, except Arg, decreased. The resolution between all amino acid derivatives remained constant except between Arg and Tyr which deteriorated. As suggested by the data in Figure 6, increasing the concentration of the Na/PO_4 buffer in the solvent system resulted in regaining the original retention time of all the amino acids as well as the resolution between Arg and Tyr. Changing the buffer concentration to maintain Arg-Tyr resolution is described in the Materials and Methods section. The similarity in the effects of column aging and decreasing

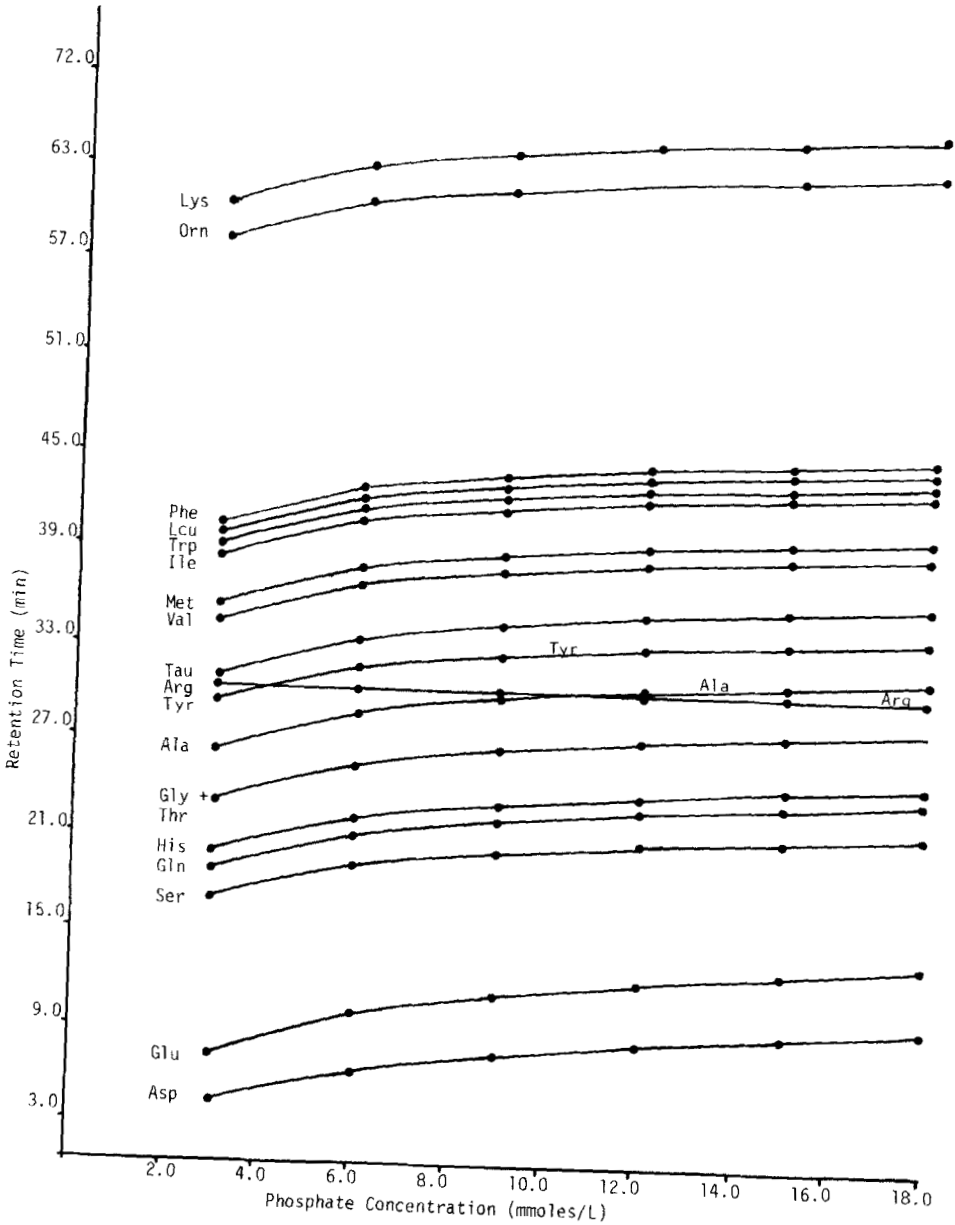

Figure 6. Effect of Buffer Concentration on the Retention of OPA/EtSH Amino Acids on a μC_{18} Column.

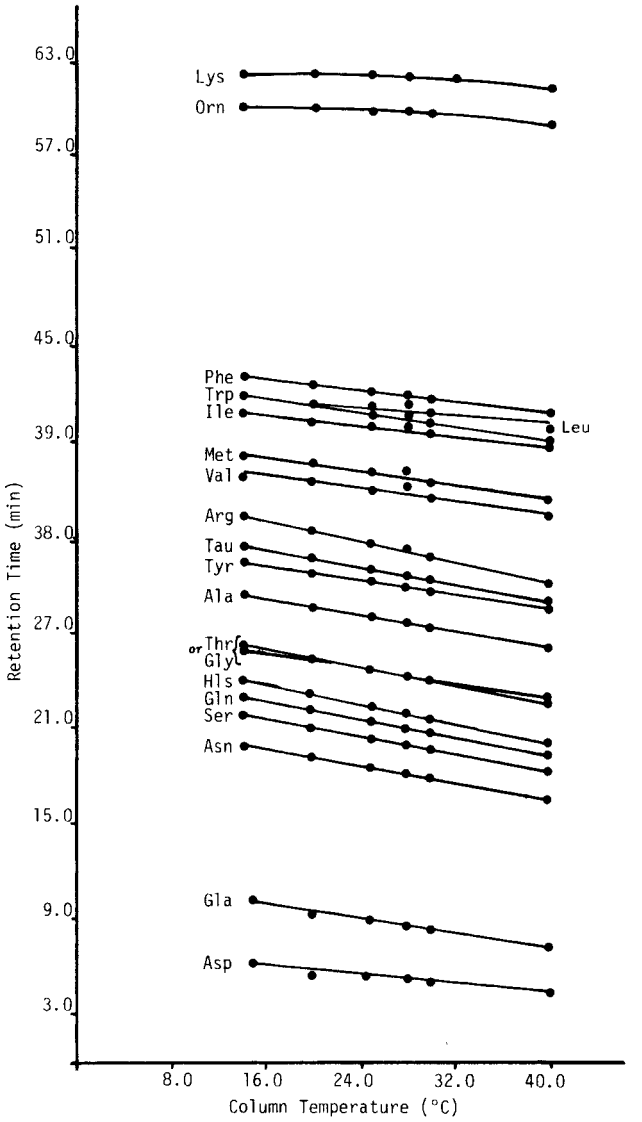

Figure 7. Effect of Column Temperature on the Retention of OPA/EtSH Amino Acids on a μC_{18} Column.

buffer concentration on the retention of the amino acid derivatives suggested that the two phenomena were related.

As expected, increasing the temperature of the column (Figure 7) caused a decrease in the retention of all the amino acids; however, there was little change in the resolution between most peaks. Exceptions to this were in the resolutions of Trp and Leu which tend to co-elute at low temperatures; Phe and Leu, and Trp and Ile which tend to co-elute at high temperatures; and Thr and Gly which begin to separate at either high or low temperatures. The Thr-Gly resolution was slight and losses in resolution of the other amino acid pairs prevented high column temperature from being used to resolve Thr-Gly in a total system. Temperatures low enough to resolve Thr and Gly resulted in peak broadening and loss of resolution between the Trp-Leu amino acid pair. A temperature of 28° appeared to be optimal for resolving the greatest number of amino acid pairs.

Table 2 lists a comparison between rat plasma amino acid concentrations obtained by the amino acid analyzer and the HPLC method presented in this study. Of the amino acids studied, the values differed by as much as 31% and as little as 0%. The average difference for all the amino acids was 12.3%.

Recently, Fernstrom and Fernstrom (14) reported reasonable correlation between amino acid profiles obtained by the

TABLE 2

Comparison of Plasma Amino Acid Concentrations Determined by Amino Acid Analyzer and HPLC/OPA Methods

Amino Acid	Plasma 1		Plasma 2		Plasma 3		Plasma 4		Plasma 5		Plasma 6		Average
	OPA[a]	IE[b]	OPA	IE	OPA	IE	OPA	IE	OPA	IE	OPA	IE	%DIF[c]
Ala	465	538	551	609	548	552	651	651	528	564	525	571	6.4
Arg	177	229	221	256	178	192	205	231	161	207	159	200	16.3
Gly + Thr	802	963	990	1061	990	1056	993	1061	747	902	748	869	11.6
His	74	80	87	89	93	90	94	80	67	72	85	95	7.9
Ile	71	82	83	91	112	117	92	86	76	89	87	93	9.1
Leu	123	148	147	166	162	168	156	157	134	148	144	160	8.7
Lys	352	511	346	466	454	535	352	535	282	363	417	503	24.3
Met	54	64	55	59	78	78	62	69	54	65	51	57	10.2
Phe	57	62	60	64	71	67	61	56	58	63	59	62	6.9
Ser	296	374	328	396	390	407	410	430	283	330	330	397	13.0
Tyr	129	119	95	99	152	123	167	132	125	114	101	90	14.1
Val	152	196	183	213	225	254	181	233	167	224	185	233	19.4

a. Plasma concentrations in nmoles/mL obtained by the HPLC/OPA method described in the present study

b. Plasma concentrations in nmoles/mL obtained by amino acid analyzer

c. $\% \text{ DIF} = \frac{\Sigma \left| 100(\text{OPA}-\text{IE})/\text{IE} \right|}{N}$; where N = number of samples analyzed

HPLC method presented here with those obtained by an amino acid analyzer. Additionally, they have demonstrated that amino acid concentration profiles obtained by the HPLC method on plasma from rats with experimental diabetes was diagnostic for the disease.

The amino acid analysis presented in this study offers a rapid and sensitive alternative to the time-consuming ion-exchange methods currently in use for determining plasma amino acid concentrations. Further studies are in progress to determine the accuracy of the HPLC method.

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