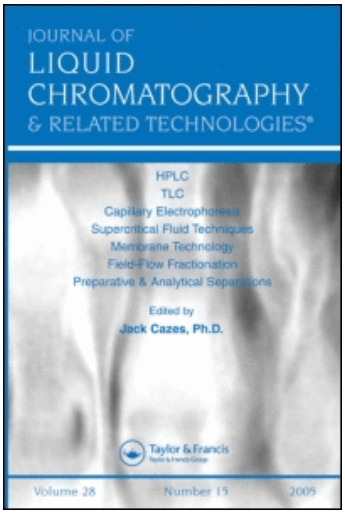

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Automatic Pre-Column Derivatization and Reversed-Phase High Performance Liquid Chromatography of Primary and Secondary Amino Acids in Plasma with Photo-Diode Array and Fluorescence Detection

H. G. Worthen[a]; H. Liu[a]
[a] Department of Pediatrics, Texas Scottish Rite Hospital for Children, Dallas, Texas

To cite this Article Worthen, H. G. and Liu, H.(1992) 'Automatic Pre-Column Derivatization and Reversed-Phase High Performance Liquid Chromatography of Primary and Secondary Amino Acids in Plasma with Photo-Diode Array and Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 15: 18, 3323 — 3341
To link to this Article: DOI: 10.1080/10826079208020887
URL: http://dx.doi.org/10.1080/10826079208020887

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(18), 3323-3341 (1992)

AUTOMATIC PRE-COLUMN DERIVATIZATION AND REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PRIMARY AND SECONDARY AMINO ACIDS IN PLASMA WITH PHOTO-DIODE ARRAY AND FLUORESCENCE DETECTION

H. G. WORTHEN AND H. LIU
Department of Pediatrics
Texas Scottish Rite Hospital for Children
2222 Welborn Street
Dallas, Texas 75219

ABSTRACT

An automatic pre-column double derivative procedure has been established for the determination of plasma amino acids by reversed-phase high performance liquid chromatography. O-phthaldialdehyde/3-mercaptopropionic acid and fluorenylmethyl-chloroformate were used as reagents for the derivatization. The optimal conditions for separation and detection and the stability of derivative were investigated. This method has the advantages of automatic pre-column derivatization and analysis; simple and economic mobile phase; shorter analysis time with optimal separation; high precision for retention time and peak area; higher sensitivity and reliable peak identification and simultaneous determination of both primary and secondary amino acids. Plasma amino acid levels in 160 healthy children were determined and the results showed a good agreement with reference values reported previously by using an ion-exchange chromatographic method. Patients suffering from several type of amino acid metabolic abnormality were detected in the screening of selected pediatric patients. This method appears suitable for application to the measurement of plasma amino acids in a clinical laboratory.

INTRODUCTION

In recent years, methods employing pre-column derivatization of amino acids, combined with reversed-phase high performance liquid chromatography have gained increasing importance and have partially replaced classical amino acid analyzer[1-6]. In comparison with the post-column derivatization used for nearly 30 years by most of ion-exchange chromatographic amino acid analyzers, pre-column derivatization has the advantages of reducing analysis times and enhancing sensitivity[4-10]. Among the methods of pre-column derivatization, O-phthaldialdehyde (OPA) has become the most popular one because the derivatization procedure is relatively easy and the reaction occurred rapidly at room temperature[11-17]. The OPA derivatives are less polar than the original amino acids and can be well separated from each other by reversed-phase chromatography[11,15]. One disadvantage of OPA derivatization has been the lack of stability of the adduct when 2-mercaptoethanol (2-ME) is used as sulfhydryl reagent[4,7]. Consequently, time differences between the reaction and injection during a manual procedure may cause significant errors in quantitation. An automated on-line OPA derivatization procedure is described in this paper. This procedure eliminates most of the errors due to the variation in reaction time and in volumes of sample or reagents. In addition, 2-ME is replaced by 3-mercaptopropionic acid (3-MPA) with considerable improvement in stability.

The other disadvantage of OPA derivatization is that it reacts only with primary amine, so secondary amino acids (imino acids) are not detected[3,4]. Following the suggestion of Schuster and Apfel[18], 9-fluorenylmethyl-chloroformate (FMOC-Cl) is incorporated to this automatic derivatization procedure as second reagent for the derivatization of secondary amino acids. In addition, Photo-diode array and programmable fluorescence detectors are used for the detection. Therefore, both primary and secondary amino acids can be detected simultaneously.

It appears from our studies that the combination of automatic on-line derivatization, the improvement in stability of the reaction from using 3-MPA, and the use of OPA and FMOC-Cl as dual derivatizing reagents, creates a pre-column derivatization procedure which can be used by HPLC to yield more rapid and sensitive results than that of classic amino acid analyzer with comparable accuracy and precision. This method has been used for determination of more than 1500 plasma samples during a period of four years with satisfactory results.

MATERIALS AND METHODS

Apparatus : An Hewlett-Packard HP 1090 M series HPLC system was used for this experiment. The system consists of a DR 5 solvent delivery system with 3 solvent channels, a variable volume auto-

injector and an auto-sampler, an HP 1040A photo-diode array UV detector and HP 1046 programmable fluorescence detector. Data were processed by an HP 79994A analytical workstation. Two HP Hypersil-ODS 5 μm columns (100*2.1 mm) and one guard column (20*2.1 mm) were used for the separation.

Reagent : Water, methanol and acetonitrile were HPLC grade (Curtin Matheson Scientific, Inc., Houston, Texas). Chemicals used were analytical grade including sodium acetate, glacial acetic acid, boric acid, sodium hydroxide, O-phthaldialdehyde, 3-mercaptopropionic acid and 9-fluorenylmethyl-chloroformate (all from Sigma Chemical Company, St. Louis, MO).

Preparation of amino acid standard solution : An amino acid standard solution containing 29 amino acids was prepared by adding crystalline O-phospho-L-serine (OPS), reduced glutathione (GSH), asparagine, glutamine, citrulline, taurine, α-amino-N-butyric acid (AABA), tryptophan, ornithine, hydroxy-proline (Hyp) and sarcosine to a commercial AA-S-18 Amino Acid Standard Solution (All from Sigma). Nor-Valine (N-Val) was tested as an internal standard (United States Biochem. Corp., Cleveland, Ohio). The concentration was 500 μmol/L for each amino acid. The mixture solution was kept in -76^{0}C. Argininosuccinic acid (barium salt) was purchased from Sigma and used for the identification and quantitation of argininosuccinic acid (ASA) in a plasma sample from a patient suffered from argininosuccinase deficiency.

Preparation of OPA, 3-MPA and FMOC-Cl derivatization solution : OPA (3 mg) was dissolved in 50 μl of methanol and 450 μl of 0.5 M sodium borate buffer (pH 10.2), to which 5 μl of 3-MPA was added. This solution was kept in dark at 4^{0}C. Fresh solution was prepared each week. FMOC-Cl solution was prepared by dissolving 1 mg of FMOC-Cl in 1 ml of acetonitrile and stored at 4^{0}C.

Plasma sample : Plasma samples were collected from 160 children (age from 1 month to 21 years old, 75 boys and 85 girls) Most of them were orthopedic patients. None had a history of liver or kidney diseases, or diseases known to affect amino acids metabolism. The routine blood and urine tests were normal.

Ultrafiltration : Heparinized plasma samples were ultrafiltrated by using Centrifree System (Amicon, beverly, MA). Plasma of 0.2 ml was put in the sample reservoir, then place the device in a centrifuge with a fixed-angle rotor. About 60 μl of ultrafiltrate was collected after centrifuge at 1500 g for 15 minutes.

Chromatographic condition : Pre-column derivatization was performed by an injector program (Table 1). After drawing from sample or reagents, the needle of the injector was always dipped

TABLE 1. Injector Program

Line#	Function	Amount	Vial No.	Reagent
1	Draw :	0.0 µl from :	Vial#:4	(Water)
2	Draw :	2.5 µl from :	Vial#:5	(OPA)
3	Draw :	0.0 µl from :	Vial#:4	
4	Draw :	2.5 µl from :	Vial#:X	(Sample)
5	Mix :	5.0 µl cycles 2		
6	Draw :	0.0 µl from :	Vial#:4	
7	Draw :	1.0 µl from :	Vial#:8	(FMOC-Cl)
8	Mix :	6.0 µl cycles 2		
9	Wait :	2.5 minutes		
10	Inject			

into water for cleaning. The separation of amino acids was done by a gradient elution according to chromatographic time table (Table 2). Flow rate was 0.3 ml/minute and stop time was 30 minutes following the injection. Two HP Hypersil-ODS 5 µm columns were put in series in a thermostatically controlled column compartment for the separation, preceded by a guard column. The column temperature was set at 40^0C. For the detection and identification of amino acid derivatives, the photo-diode array detector was set at three sample wavelengths of 338, 266 and 230 nm with bandwidths 10, 4 and 4 nm respectively, the reference wavelength was 550 nm with bandwidth 100 nm. The initial parameters for fluorescence detector were excitation wavelength (Ex) 230 nm and emission wavelength (Em) 450 nm. After 20 minutes of the injection, the Ex and Em were changed to 260 nm and 315 nm respectively by an automatic program.

Quantitative analysis : Amino acid peaks were identified with reference to retention times of standard amino acids injected separately and supplemented with appropriate amount of amino acid standards to plasma to yield a larger peak. The linearity of response was estimated by injecting of derivatized amino acids with different concentrations and constructing regression equations of UV and fluorescence response-concentration curves. External method was used for the quantitation. The precision of analysis was observed from the reproducibility of the retention time and peak areas of eight consecutive injections of 29 amino acid mixture solution. The accuracy of measurement was tested by adding known quantity of amino acid standards to a plasma sample, then the sample was ultrafiltrated and derivatized, the analytic recovery rate of each amino acid was calculated after HPLC analysis. The detection limit was determined by injecting amino acid derivatives from serially diluted standards until the ratio of signal to noise was 2.

TABLE 2. Time Table for Gradient Elution

Time(Minute)	Solvent A%	B%	C%
0.05	100	0	0
15.00	60	40	0
18.50	57.5	42.5	0
22.00	45	55	0
25.00	0	0	100
30.00	0	0	100

Solvent A : 0.015 M NaAc (pH 6.8)
Solvent B : Methanol
Solvent C : 0.010 M NaAc (pH 6.8)

Statistic analysis : Two sample test was used to test the difference between the mean values of amino acids in children with different age and sex.

RESULTS

Chromatograms of an amino acid standard mixture or a representative plasma sample are demonstrated in figures 1 and 2 or figures 3 and 4. These chromatograms showed a satisfactory separation of 29 primary and secondary amino acids.

The stability of OPA and FMOC-Cl derivative of each amino acid was determined by varying the waiting time in injector program after mixing, then analyzed by identical chromatographic condition and detected at UV 338 nm and 266 nm. The results are shown in Table 3. The peak areas are normalized to waiting time of 2.5 minutes. When waiting time was increased beyond 2.5 minutes, the stability of various amino acid was different. The UV absorbency of OPS and GSH increased, whereas asparagine, glutamine, glycine, histidine, taurine, ornithine and lysine decreased. Others were unchanged.

The reproducibility of retention time and peak areas for each amino acid was tested by eight consecutive injections of 29 amino acid mixture. The results showed that both retention time and peak areas were highly reproducible. The coefficients of variation for retention time ranged from 0.02% to 0.97%, with a mean of 0.26%$\pm$0.21 S.D.. The coefficients of variation for peak areas ranged from 0.78% to 2.84%, with a mean of 1.73%$\pm$0.67 S.D.. The high precision of this method would allow analysis without an internal standard for quantitation.

Table 4 shows the recovery rate of amino acid standard added to plasma. The recovery rates for most amino acids were between 95% to 107%. Tryptophan and GSH showed lower recovery rate (81% and 32% respectively). So the multiplier in calibration form was set at 1.19 for the quantitation of plasma tryptophan. No effort has been made for the determination of GSH in plasma.

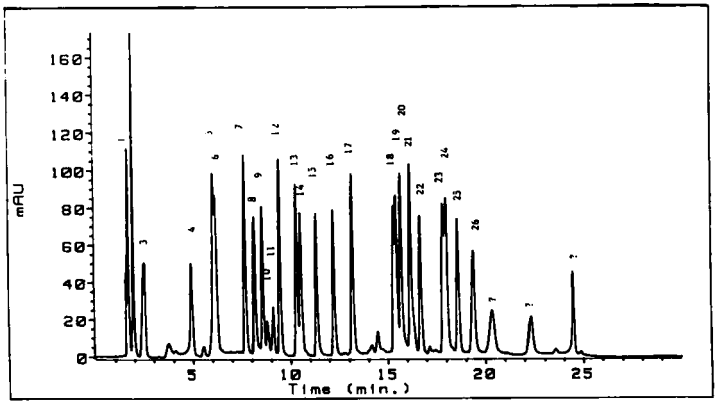

FIGURE 1. Signal from UV detection at 338 nm.

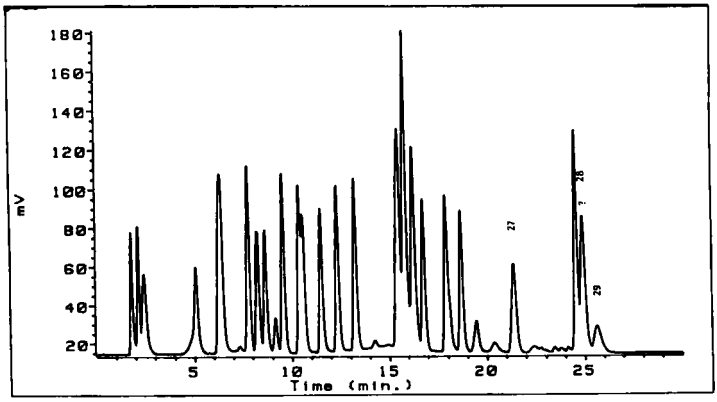

FIGURE 2. Signal from Fluorescence detection. Typical Chromatograms Showing the Separation of 29 OPA/3-MPA and FMOC-Cl Derivatized Standard Amino Acids (500 µmol/L). For Chromatographic conditions see Materials and Methods. Peaks : 1 = O-phospho-L-serine, 2 = Aspartic Acid, 3 = Glutamic Acid, 4 = Glutathione (reduced), 5 = Asparagine, 6 = Serine, 7 = Glutamine, 8 = Glycine, 9 = Threonine, 10 = Histidine, 11 = Cystine, 12 = Citrulline, 13 = Taurine, 14 = Alanine, 15 = Arginine, 16 = Tyrosine, 17 = Alpha-amino-N-butyric Acid, 18 = Methionine, 19 = Valine, 20 = Nor-Valine, 21 = Tryptophan, 22 = Phenylalanine, 23 = Isoleucine, 24 = Ornithine, 25 = Leucine, 26 = Lysine, 27 = Hydroxy-proline, 28 = Sarcosine, 29 = Proline.

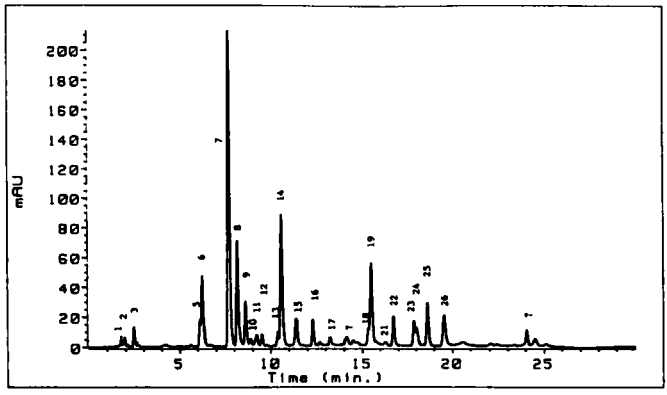

FIGURE 3. Signal from UV detection at 338 nm.

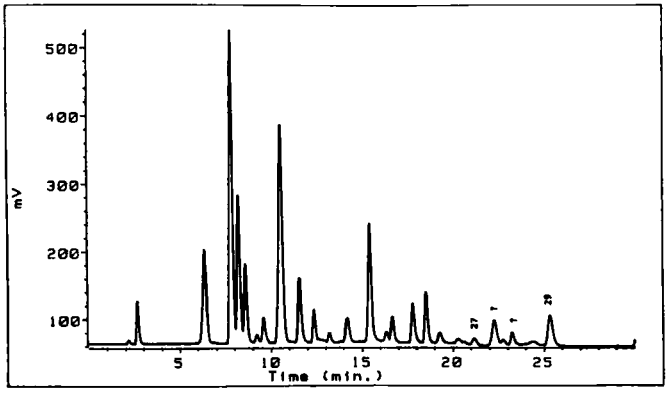

FIGURE 4. Signal from Fluorescence detection. Chromatograms of a Representative Plasma Sample Under the Identical Experimental Conditions as in Figure A and B. Peak numbers are identified in Figure A and B.

TABLE 3. Stability of OPA/3-MPA and FMOC-Cl Amino Acid Derivative

Amino Acid	0.5 min.*	2.5 min.**	5.0 min.	15.0 min.	30.0 min.
OPS***	0.97	1.00	1.05	1.10	1.34
Asp	0.98	1.00	1.01	1.01	0.99
Glu	1.14	1.00	1.03	1.05	1.03
GSH	1.00	1.00	1.29	1.41	1.41
Asn	1.04	1.00	1.02	0.99	0.92
Ser	1.00	1.00	1.06	1.06	1.00
Gln	1.02	1.00	1.03	0.98	0.91
Gly	1.03	1.00	1.02	0.88	0.63
Thr	1.00	1.00	1.02	1.01	0.96
His	0.97	1.00	1.07	0.89	0.58
Cys	1.05	1.00	1.04	1.05	0.98
Cit	1.02	1.00	1.04	1.04	1.00
Tau	1.02	1.00	1.02	0.95	0.78
Ala	1.01	1.00	1.01	0.99	0.93
Arg	1.02	1.00	1.02	1.02	0.99
Tyr	1.02	1.00	1.01	1.05	1.05
AABA	1.01	1.00	1.02	1.02	1.00
Met	1.00	1.00	1.01	1.00	0.98
Val	0.99	1.00	0.99	1.00	1.03
N-Val	1.00	1.00	1.02	1.06	1.05
Trp	1.01	1.00	1.00	1.01	1.02
Phe	1.03	1.00	1.02	1.03	1.04
Ile	0.99	1.00	0.99	1.00	1.00
Orn	1.05	1.00	0.96	0.72	0.48
Leu	1.05	1.00	1.02	1.01	1.05
Lys	1.03	1.00	0.94	0.70	0.43
Hyp	1.02	1.00	1.01	1.01	0.98
Sar	1.00	1.00	1.08	0.96	0.93
Pro	0.93	1.00	1.14	1.04	1.08

* Waiting time before injection.

** Peak areas from UV signals at 338 and 266 nm were normalized to 2.5 minutes.

*** OPS = O-phospho-L-serine, Asp = Aspartic Acid, Glu = Glutamic Acid, GSH = Glutathione (reduced), Asn = Asparagine, Ser = Serine, Gln = Glutamine, Gly = Glycine, Thr = Threonine, His = Histidine, Cys = Cystine, Cit = Citrulline, Tau = Taurine, Ala = Alanine, Arg = Arginine, Tyr = Tyrosine, AABA = Alpha-amino-N-butyric Acid, Met = Methionine, Val = Valine, N-Val = Nor-Valine, Trp = Tryptophan, Phe = Phenylalanine, Ile = Isoleucine, Orn = Ornithine, Leu = Leucine, Lys = Lysine, Hyp = Hydroxy-proline, Sar = Sarcosine, Pro = Proline.

TABLE 4. Recovery of Amino Acid Standard Added to Plasma

Amino Acid*	Plasma alone(μmol/L)	Plasma+250μmol/L (expected)	Actual	Recovery Rate%
Ops	32.65	282.65	271.87	97
Asp	18.98	268.98	273.34	101
Glu	28.76	278.76	290.15	104
GSH	----	250.00	80.73	32
Asn	35.89	285.89	284.52	100
Ser	128.27	378.27	401.06	106
Gln	538.74	788.74	757.19	96
Gly	219.74	469.74	465.04	100
Thr	132.14	382.14	384.32	100
His	31.71	281.71	290.16	103
Cys	64.12	314.12	307.83	98
Cit	18.36	268.36	287.14	107
Tau	38.85	288.85	291.73	101
Ala	376.32	626.32	613.79	98
Arg	103.50	353.50	352.22	100
Tyr	62.38	312.38	303.01	97
AABA	29.09	279.09	287.46	103
Met	19.60	269.60	258.82	96
Val	170.04	420.04	407.73	97
N-Val	----	250.00	252.36	101
Trp	14.26	264.26	214.05	81
Phe	49.82	299.82	297.42	99
Ile	60.21	310.21	313.08	100
Orn	50.18	300.18	285.17	95
Leu	100.92	350.92	349.15	100
Lys	137.24	387.24	385.69	99
Hyp	24.49	274.49	277.23	101
Pro	180.86	430.85	439.46	102

* OPS = O-phospho-L-serine, Asp = Aspartic Acid, Glu = Glutamic Acid, GSH = Glutathione (reduced), Asn = Asparagine, Ser = Serine, Gln = Glutamine, Gly = Glycine, Thr = Threonine, His = Histidine, Cys = Cystine, Cit = Citrulline, Tau = Taurine, Ala = Alanine, Arg = Arginine, Tyr = Tyrosine, AABA = Alpha-amino-N-butyric Acid, Met = Methionine, Val = Valine, N-Val = Nor-Valine, Trp = Tryptophan, Phe = Phenylalanine, Ile = Isoleucine, Orn = Ornithine, Leu = Leucine, Lys = Lysine, Hyp = Hydroxy-proline, Sar = Sarcosine, Pro = Proline.

TABLE 5. Free Plasma Amino Acid Levels (μmol/L) of 160 Children

Amino Acid*	Girls (N=85) Mean	± SD	Boys (N=75) Mean	± SD
Asp	6.19	1.86	7.62**	2.13
Glu	39.28	13.95	48.23	17.84
Asn	53.99	10.16	56.53	10.92
Ser	126.30	23.57	133.50	28.79
Gln	532.80	82.00	540.70	93.75
Gly	242.50	47.29	262.60	59.35
Thr	142.40	27.86	146.20	28.87
His	96.09***	16.51	85.07	16.95
Cys	88.96	14.91	87.21	15.29
Cit	24.29	4.10	26.00	4.15
Tau	41.57	7.73	44.35	11.53
Ala	423.30	82.15	425.10	96.85
Arg	95.05	18.18	100.60	19.52
Tyr	74.88	15.45	73.99	13.51
AABA	18.23	3.52	18.66	3.93
Met	23.88	2.60	25.17**	3.65
Val	221.80	39.27	235.20	49.11
Trp	5.67	2.73	6.38	3.33
Phe	64.94	11.15	65.32	5.96
Ile	66.36	15.35	74.28**	19.63
Orn	42.11	12.76	45.03	14.53
Leu	137.90	25.92	144.30	32.03
Lys	130.00	25.99	126.10	26.91
Hyp	18.96	4.20	21.34**	5.65
Pro	153.00	42.24	149.90	45.66

* Asp = Aspartic Acid, Glu = Glutamic Acid, Asn = Asparagine, Ser = Serine, Gln = Glutamine, Gly = Glycine, Thr = Threonine, His = Histidine, Cys = Cystine, Cit = Citrulline, Tau = Taurine, Ala = Alanine, Arg = Arginine, Tyr = Tyrosine, AABA = Alpha-amino-N-butyric Acid, Met = Methionine, Val = Valine, Trp = Tryptophan, Phe = Phenylalanine, Ile = Isoleucine, Orn = Ornithine, Leu = Leucine, Lys = Lysine, Hyp = Hydroxy-proline, Pro = Proline.

** Significantly higher than girls ($P < 0.01$).
*** Significantly higher than boys ($P < 0.001$).

TABLE 6. Comparison of Amino Acid levels (μmol/L)
Between Girls and Boys Below and Above 6 Years Old

Amino Acid@	Girls < 6 Yr. Mean ± SD		Girls > 6 Yr. Mean ± SD		Boys < 6 Yr. Mean ± SD		Boys > 6 Yr. Mean ± SD	
Asp	6.98	3.56	6.74	3.34	6.81	3.11	8.21	4.65
Glu	39.30	24.40	41.86	28.70	40.76	26.52	48.48	40.11
Asn	52.86	18.75	54.96	21.44	55.08	21.95	56.30	21.13
Ser	123.60	51.13	130.30	45.72	118.40	39.77	138.00	63.51
Gln	507.30	144.3	548.80	169.1	486.80	144.5	552.70	192.6
Gly	216.60	81.34	253.10	96.86	226.80	64.31	270.60*	120.6
Thr	125.30	54.87	153.70*	54.23	127.50	44.59	151.00*	60.44
His	82.87	29.57	104.60#	30.08	79.56	28.99	94.62*	33.52
Cys	82.40	27.68	90.46	30.29	83.42	30.22	94.52	26.85
Cit	23.54	8.35	24.25	7.95	23.55	7.34	26.40	8.61
Tau	38.90	10.98	44.11	22.01	36.47	11.92	45.18*	23.00
Ala	383.10	106.9	451.20*	171.2	355.20	143.5	455.30&	205.1
Arg	91.01	39.30	97.53	34.57	84.81	29.69	112.30#	40.17
Tyr	76.38	34.38	76.72	31.30	67.39	19.20	80.45*	30.38
AABA	19.04	8.13	17.81	6.43	16.95	6.68	20.18	7.90
Met	23.09	4.69	22.69	5.21	24.49	6.19	25.09	7.63
Val	230.30	89.92	228.38	77.76	207.50	86.38	276.90#	79.16
Trp	9.18	4.40	9.24	3.02	10.94	4.39	10.33	3.62
Phe	65.20	21.65	66.25	23.74	59.57	23.40	72.05*	27.02
Ile	66.89	33.64	69.22	31.08	66.05	29.57	77.56*	41.49
Orn	33.74	15.87	45.52	28.00	41.77	24.74	57.74&	26.95
Leu	135.00	55.47	143.90	52.81	129.60	55.29	173.10#	56.06
Lys	120.40	49.19	131.90	48.79	127.80	46.14	123.00	53.71
Hyp	17.45	6.68	19.57	7.55	21.07	8.50	23.59	9.67
Pro	156.10	65.94	152.00	81.10	151.10	74.20	171.10	63.69

@ Asp = Aspartic Acid, Glu = Glutamic Acid, Asn = Asparagine, Ser = Serine, Gln = Glutamine, Gly = Glycine, Thr = Threonine, His = Histidine, Cys = Cystine, Cit = Citrulline, Tau = Taurine, Ala = Alanine, Arg = Arginine, Tyr = Tyrosine, AABA = Alpha-amino-N-butyric Acid, Met = Methionine, Val = Valine, Trp = Tryptophan, Phe = Phenylalanine, Ile = Isoleucine, Orn = Ornithine, Leu = Leucine, Lys = Lysine, Hyp = Hydroxy-proline, Pro = Proline.

* = $P < 0.05$, & = $P < 0.01$ and # = $P < 0.001$, the mean values of girls (n=56) or boys (n=52) above 6 years old compare with girls (n=29) or boys (n=23) below 6 years old.

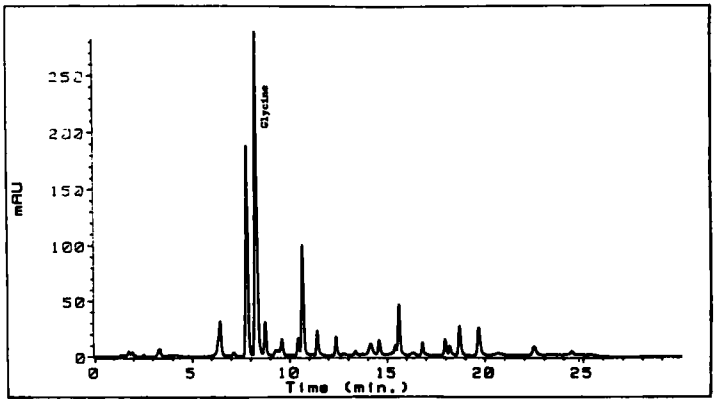

FIGURE 5. Analysis of a Plasma Sample from Patient Suffered From Nonketotic Hyperglycinemia (UV 338 nm).

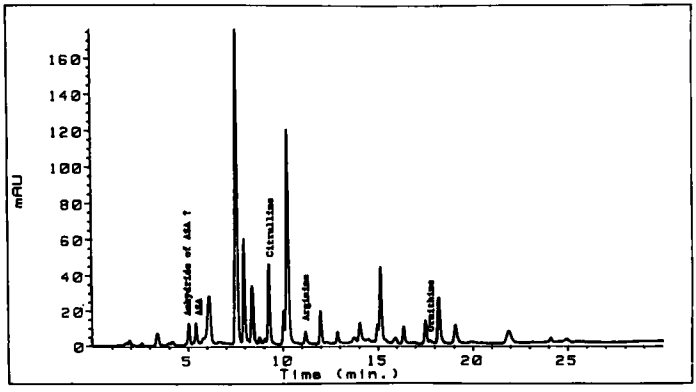

FIGURE 6. Analysis of a Plasma Sample from Patient Suffered From Argininosuccinase Deficiency (UV 338 nm).

The linear relationship of concentration to peak areas for each amino acid in standard solution was determined by analyzing each standard amino acid at concentrations from 31.25 to 500 μmol/L (N=5) by serial dilution. These concentrations covered the normal range of most plasma amino acids. For plasma, the original plasma sample and plasma samples diluted by HPLC water to 75%, 50% and 25% (N=4) of the plasma were analyzed. The linear regression analysis showed satisfactory coefficients of correlation (> 0.99) between the peak areas of each amino acid from UV and fluorescent signals and the concentrations in both standard amino acid and in plasma samples.

The levels of 25 plasma amino acid from 75 boys and 85 girls are listed in Table 5. In general, amino acid levels of boys were higher than that of girls. The mean values of aspartic acid, methionine, isoleucine and hydroxy-proline in boys were significantly higher than that in girls ($P < 0.01$). The girls had significant higher level of histidine than boys ($P < 0.001$). Table 6 is a comparison of plasma amino acid levels between girls and boys below and above 6 years old. Compared with boys below 6 years old (N=23), boys over 6 years old (N=52) showed significant higher value of glycine, threonine, histidine, taurine, alanine, arginine, tyrosine, valine, phenylalanine, isoleucine, ornithine and leucine. For girls over 6 years old (N=56), there were only threonine, histidine and alanine were significantly higher than that girls below 6 years old (N=29).

Patients suffering from several type of amino acid metabolic abnormality were detected in the screening of selected pediatric patients. Figure5 was a chromatogram from a patient with nonketotic hyperglycinemia. Analysis of a plasma from a patient suffered from argininosuccinase deficiency showed argininosuccinic acid and its anhydride could be well separated. Other urea cycle related amino acids abnormalities such as high citrulline, low arginine and ornithine were detected at the same analysis (Figure 6).

DISCUSSION

Derivatization : The OPA pre-column derivatization procedure is an attractive method among several methods for pre-column amino acid derivatization. OPA amino acid derivatives are formed rapidly and quantitatively in aqueous solution and at ambient temperature. No laborious purification procedures are required[1-4]. OPA reacts at alkaline pH with primary amine to form a fluorescent molecule. The reaction takes place in the presence of a thiol, such as 2-ME[3,4]. The products are not stable and have a short half life, possibly due to a spontaneous intramolecular rearrangement, with sulfur being displaced by oxygen from the ethanolic portion[4]. When 2-ME was replaced by ethanethiol, the derivatives of lysine and ornithine were stabilized but eluted as double peaks and glycine was still

very unstable[4]. In a kinetic investigation, 3-MPA was suggested as a more stable reagent and also revealed a better fluorescent signal[19]. In addition, 3-MPA had the advantage of being nonvolatile and less toxic because of its carboxylic moiety[3]. This reagent was therefore chosen by us for this studies. The other major cause of variability in the pre-column derivatization system appears to come from errors in reaction time and in volume of sample and reagents. By using an auto-injector, the exact time of each step from the beginning of reaction to injection can be controlled according to injector program. The precision of the time and volume eliminates human errors which may occur during manual derivatization. Although the stability of OPA/3-MPA derivative is better than OPA/2-ME derivative[4,7,11,12], the OPA/3-MPA derivative of various amino acid does not have the same stability (table 3). When the waiting time was increased beyond 2.5 minutes, the stability of various amino acid was different. The UV absorbency of GSH and OPS increased, whereas asparagine, glutamine, glycine, histidine, taurine, ornithine and lysine decreased. Others were unchanged. So the precise control of reaction time is still important in the derivatization using OPA/3-MPA as reagents. The results from Table 3 showed OPA/3-MPA derivatives were relative stable during the waiting time from 0.5 to 5 minutes. So the waiting time was set at 2.5 minutes for subsequent studies.

FMOC-Cl is a highly reactive reagent that has been used as an amino-protective group in peptide synthesis. In 1983, Einarsson applied FMOC for the first time to pre-column derivatization of amino acids[20]. It was as sensitive as OPA but reacts with secondary as well as primary amine. Derivatives produced were stable and highly fluorescent. This method has been used to measure hydroxyproline, sarcosine and proline in serum, cerebrospinal fluid and urine[21]. However, excess FMOC-Cl had to be removed by pentane extraction because FMOC-Cl itself was fluorescent and may obscure some amino acid peaks[20,21]. Subsequent to the beginning of our studies, Betner and Foldi published a modified pre-column derivative procedure using FMOC-Cl, in which the excess FMOC-Cl remaining after derivatization was reacted with hydrophobic amine 1-aminoadamantane (ADAM) to form an amine-FMOC complex[22]. The retention time of this complex was longer than any amino acid and is eluted at the end of chromatographic system without interfering with amino acid measurements. This modification overcame the problem of extraction steps, but analysis time might be delayed for the total elimination of hydrophobic amine-FMOC complex from column at the end of chromatographic separation. We obviated this problem by using double derivative technique and using both photo-diode array and programmable fluorescence detectors. In this way we took advantage of different absorption and fluorescent spectra of OPA and FMOC-Cl derivatives. For the photo-diode array detection, sample wavelength 266 nm was particularly used for FMOC-Cl derivatives of secondary amino acids. For the fluorescence measurement, we set Ex. 230 nm and Em. 450 nm during the first 20 minutes for the measurement of

OPA derivatives of primary amino acids, then switched to Ex. 266 nm and Em. 315 nm for the determination of FMOC-Cl derivatives of secondary amino acids, since hydroxyproline, sarcosine and proline were all eluted after 20 minutes in our chromatographic system. In our experiment, we did not observe interference from unreacted derivatization reagents or the degradation products.

Optimal separation : Several combinations of the organic solvents and buffer and the pH of the buffer in mobile phase were tested in this study. Also, different patterns of gradient elution and column temperature were investigated. Originally, acetonitrile was used as solvent B instead of methanol, because column pressure was lower and analysis time was shorter. However, the separation was not as good as when methanol was used (data not shown). In particular, the separation between three closely eluting pairs of amino acids : asparagine-serine, taurine-alanine and valine-methionine were better when methanol was used as solvent B. When 0.01 M concentration of acetate buffer was used for gradient elution, early eluted amino acids were well separated but leucine and ornithine merged. At higher concentration of 0.015 M acetate buffer, leucine and ornithine separated well, but OPS, aspartic acid and glutamic acid merged. The gradient elution was therefore designed to start with low concentration of acetate buffer (0.01 M, solvent C) and almost immediately switch to a higher concentration (0.015 M, solvent A) for the gradient elution. At 25 minute of the elution, solvent A was switch back to solvent C for reequilibrium. Comparison of buffer pH from 6.4 to 7.2 were made with all other conditions held constant. The pH at 6.8 appeared to give optimal separation. Column temperature was also tested for its effect on separation. When column temperature was maintained at 30^0C, the separation of amino acids at front half of the chromatogram were better than that at temperature of 35^0C or 40^0C, but the separation of later half was not satisfactory. Increase the temperature to 40^0C gave better separation for amino acids eluted later and the overall results were the best, together with a lower column pressure. We therefore set the column temperature at 40^0C for all subsequent studies. Other advantages of our method are simple mobile phase composition, low ion concentration and moderate pH of the buffer in mobile phase, which should add to the longevity of column and pump system. Initially, a single 10 cm small bore column was used, but we found later that put two columns in series could increase the efficiency of column and improve the separation. The small bore column we used has the advantage of enhancing detection sensitivity and reducing solvent consumption. Its disadvantage is decreased column life and higher back pressure. Using two columns in series allowed us to eliminate part of the problem of decreased column life. When contamination was detected (baseline drift during the gradient elution) or separation deteriorated and the change of guard column could not correct the problem, the first column (next to the guard column) was

discarded, second one moved to the first position, and a new column was added at second position. Under normal situation, the guard or analytical column can be used for analysis up to 50 or 300 plasma samples respectively.

Qualitation and quantitation : The HPLC detection system we used consists of a photo-diode array detector, so the OPA/3-MPA and FMOC-Cl derivatives can be detected at several different UV wavelengths and some special function such as peak purity, spectral comparisons and ratio signals may be used for the identification of amino acid in addition to retention time. We also compared the spectra of different primary amino acids to see if the difference of spectra could be used to identify different amino acid. No notable difference in the spectra could be found among primary amino acids. It appears to us that this method can not be used to distinguish various primary amino acid. However, it is useful in identify non-amino acid from amino acid and has been proved useful in detecting previously unsuspected amino acids in the screening analysis. Since OPA/3-MPA and FMOC-Cl derivatives were detected by a photo-diode array detector at three different sample wavelengths, the intensity of the signal, baseline noise and interference might differ at various wavelengths setting. We compared several sample and reference wavelengths. At sample wavelength of 338 nm, we compared two reference wavelengths at 390 nm and 550 nm. Although the signal was stronger when 390 nm was used as a reference wavelength, the baseline was more stable at 550 nm as reference wavelength. At sample wavelength of 266 nm for the detection of secondary amino acids, 550 nm as reference wavelength produced stronger signal and better baseline than that at 390 nm. So 550 nm was used as reference wavelength for the photo-diode array detector. For the fluorescence detector we used to detect OPA and FMOC-Cl derivatives, both 230 nm and 340 nm were evaluated as Ex. for OPA primary amino acid derivatives. The results showed that the detection of OPA primary amino acid derivatives at Ex 230 nm gave a response over 7 times stronger than that at 340 nm, with an acceptable baseline. For the detection of FMOC-Cl secondary amino acid derivatives, three excitation wavelengths of 254, 260 and 266 nm were compared. the results showed 260 nm had the strongest signal. Under the chromatographic conditions we had chosen, there was no interfering peak on either UV or fluorescence signals during a blank solvent gradient elution. When HPLC water was used as a blank sample, there was no peak interfering with amino acid peak at UV sample wavelength 338 nm and fluorescence signals. But at UV 266 nm, and especially at 230 nm, peaks were observed that were large enough and close enough to interfere with about 10 amino acid peaks. Although detection at UV 230 nm was more sensitive than at 338 nm, the interference could seriously compromise the quantitation. For this reason, UV 338 nm is recommended as the main sample wavelength for the quantitation of primary amino acids. Extremely high sensitivity is not required for the analysis of plasma amino acids,

since the limitation is not sample size, but the amount of plasma required for deproteinization procedure (such as ultrafiltration). The detection limit of our method was 10 picomole for histidine and cystine and 5 picomole for other primary and secondary amino acids by UV detection. Although a large increase of sensitivity can be achieved by fluorescence detection, the purity of the reagent was important for extending detection limit. A previously unused and aged reagent (> 24 hour old) was necessary for high level sensitivity detection[4]. The sensitivity depended mainly on the ability to eliminate or subtract background levels and reduce the interfering substances present in solvents and reagents.

Applications : About 1500 plasma samples have been analyzed by this method during a period of four years. This procedure has been proved as a reliable and reproducible method. The plasma amino acid levels of 160 children obtained by this method showed a good agreement with the reference values reported previously by using ion-exchange chromatography[23]. The findings about the difference between amino acid levels of boys and girls and between the children with different age were also similar to the results observed by Armstrong and stave[23,24]. Patients suffered from hyperlysinemia, nonketotic hyperglycinemia, disorders of branched chain amino acid metabolism and argininosuccinase deficiency have been detected by this technique and confirmed by further investigation. Since this method can detect both argininosuccinic acid and its anhydride as well as other urea cycle related amino acids, it is very useful for the screening of patient suspected suffering from urea cycle disorders.

The automatic on-line pre-column derivatization, the combination of double-derivative technique with photo-diode array and fluorescence detection, and the optimized gradient elution program with simple mobile phase, along with the high degree of precision and good linearity, render this method suitable for the quantitative analysis of plasma amino acids in a clinical laboratory. Similar methods have been developed and used in the determination of amino acid in biological, pharmaceutical, plant and food samples and in protein structure determination by Schuster[25] and Blankenship et al[26].

ACKNOWLEDGEMENTS

Presented in part to The 12th International Symposium on Liquid Chromatography, Washington, D.C., June 1988.

This study was supported by the Research Committee of Texas Scottish Rite Hospital for Children.

REFERENCES

1. H.P. Fiedler and A. Plaga, J. Chromatogr., 386: 229-241 (1987)
2. C.R. Krishnamurti, A.M. Heindze and G. Galzy, J. Chromatogr., 315: 321-331 (1984)
3. G. Ogden and P. Foldi, LC-GC 5: 28-40 (1987)
4. P. Lindroth and K. Mopper, Anal. Chem., 51: 1667-1674 (1979)
5. G.A. Qureshi and A.R. Qureshi, J. Chromatogr., 491: 281-289 (1989)
6. P. Furst, L. Pollack, T.A. Graser, H. Godel and P. Stehle, J. Chromatogr., 499: 557-569 (1990)
7. H. Liu, Z.M. Liu, W.N. Zhu, Y.H. Li, X.S. Jiang and S.D. Wang, Chinese J. Chromatogr., 1: 83-87 (1984)
8. R.H. Buck and K. Krummen, J. Chromatogr., 303: 238-243 (1984)
9. R.L. Cunico and T. Schlabach, J. Chromatogr., 266: 461-470 (1983)
10. M.W. Dong and J.R. Gant, J. Chromatogr., 327: 17-25 (1985)
11. B.N. Jones and J.P. Gilligan, J. Chromatogr., 266: 471-482 (1983)
12. M.J. Winspear and A. Oaks, J. Chromatogr., 270: 378-382 (1983)
13. M.O. Fleury and D.V. Ashley, Anal. Biochem., 133: 330-335 (1983)
14. D.C. Turnell and J.D.H. Cooper, Clin. Chem., 28: 527-531 (1982)
15. G.A. Qureshi, L. Fohlin and J. Bergstrom, J. Chromatogr., 297: 91-100 (1984)
16. S.S.O. Hung and T.W. Moon, J. Chromatogr., 269: 114-118 (1983)
17. K. Venema, W. Leever, J.O. Bakker, G. Haayer and J. Korf, J. Chromatogr., 260: 371-376 (1983)
18. R. Schuster and A. Apffel, Hewlett-Packard HPLC Application Note No. 12-5954-6257 (1986)
19. H. Godel, T. Graser, P. Foldi, P. Pfaender and P. Furst, J. Chromatogr., 297: 49-61 (1984)
20. S. Einarsson, B. Josefeeon and S. Lagerkvist, J. Chromatogr., 282: 609-618 (1983)
21. S. Einarsson, J. Chromatogr., 348: 213-220 (1985)
22. I. Betner and P. Foldi, LC-GC 6: 832-840 (1988)
23. M.D. Armstrong and U. Stave, Metabolism, 22: 561-569 (1973)

24. M.D. Armstrong and U. Stave, Metabolism, 22: 571-578 (1973)

25. R. Schuster, J. Chromatogr., 431: 271-284 (1988)

26. D.T. Blankenship, M.A. Krivanek, B.L. Ackermann and A.D. Cardin, Analytical Biochemistry, 178: 227-232 (1989)

Received: March 16, 1992
Accepted: October 28, 1992