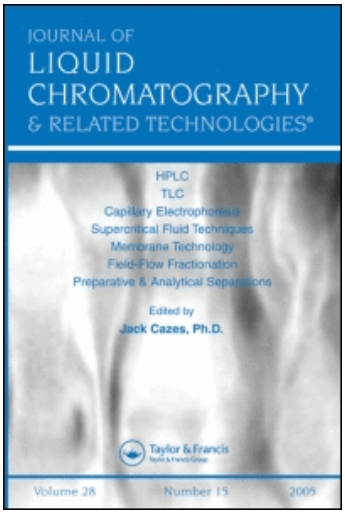

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High Performance Liquid Chromatographic Determination of Amino Acids in Biological Samples by Precolumn Derivatization with *O*-Phthaldialdehyde

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS IN BIOLOGICAL SAMPLES BY PRECOLUMN DERIVATIZATION WITH *O*-PHTHALDIALDEHYDE

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ABSTRACT

A suitable gradient system has been developed for rapid analysis of amino acids in biological samples using *O*-phthaldialdehyde as a precolumn derivatizing agent and fluorescence detection. Resolution of 21 amino acids has been accomplished with 3 μm Ultrasphere ODS column by using a multi-step gradient system of two solvents (0.1M sodium acetate, pH 7.2/methanol:tetrahydrofuran) in less than 1 hour. Within-assay and between-assay coefficients of variation of retention times and fluorescence yield show good reproducibility. The fluorometric detection response is linear from 25 to 500 pmoles with a minimum detection limit of less than 1 pmol. High resolution, rapid analysis and high sensitivity of this method facilitates amino acid analysis in samples of less than 1 mg of tissue.

INTRODUCTION

A considerable number of reports have been appeared to quantitate and separate wide spectrum of amino acids in complex biological samples using high performance liquid chromatography. There are many different methods for separation of amino acids such as ion-exchange chromatography followed by derivatization with nin-hydrin,[1] and other fluorogenic reagents,[2,3] derivatization with dansyl,[4] dabsyl,[5] phenylisothiocyanate,[6]

or *O*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (2-ME),[7-14] or ethanethiol.[15-18] The OPA/2-ME derivatization technique has become popular for the amino acid analysis using reversed phase HPLC because of obvious advantages such as high sensitivity, rapid quantitative analysis and good selectivity. Though there are different types of retention mechanisms using different mobile phases and different derivatization techniques depending on the application, choice of method, and final objective, very few methods have extracted and separated most of the biological amino acids. Inferior separation of glycine and threonine,[16-18] histidine, asparagine, glycine, citrulline and tyrosine,[15] and coelution of tyrosine with α-aminobutyric acid,[19] have been reported using OPA/ethanethiol (ET). Whereas poor resolution of asparagine and serine using OPA/2-ME,[20] incomplete resolution of asparagine from glutamate and tryptophane from methionine using OPA/2-ME-Electrochemical detection method, [21] and coelution of phenylalanine and tryptophane using OPA/3-mercaptopropionic acid (3-MPA),[22] have also been noted in earlier reports. Currently, relatively little information is available on the application of HPLC for amino acid analysis in animal tissues. The present study outlines a suitable method for the determination of biological amino acids in plasma and tissue samples using OPA-precolumn derivatization and fluorescence detection.

MATERIALS AND METHODS

Chromatographic system

The HPLC system (Waters Associates, MA, USA) consisted of a Model-680 automated gradient controller, a Model M45 solvent delivery systems, a model 420 fluorescence detector and a Rheodyne injection valve 7125 with a 20-μl filling loop. The fluorometric measurements were made at an excitation wave length of 338 nm and an emission wave length of 425 nm with a 12-μl flow cell and a fluorescent lamp.

An Ultrasphere 3 μm ODS (7.5 cm X 4.5 mm ID) reversed phase column (Beckman Instrument, Inc., CA, USA) was used in conjunction with a stainless steel guard column (20 mm X 3.9 mm ID) which was packed with Waters Corasil C_{18} packing material and a precolumn filter (2 μm) (Waters Associates). The column was maintained at room temperature (21° C). Chromatographic peaks were recorded and integrated by a SP 4100 computing integrator system (Spectra Physics, San Jose, CA, USA)

Reagents

Individual crystalline amino acid standards were obtained from Sigma (St.Louis, MO, USA). HPLC grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ,

USA). All other chemicals used were analytical grade. 2-mercaptoethanol was from Eastman-Kodak (Rochester, NY, USA). Water used for the preparation of all reagents was distilled, deionized and passed through a "norganic cartridge" (Millipore, MA, USA) and filtered through 0.45 μm membrane filter (Millipore).

Preparation of amino acid standards

A Stock solution mixture of amino acids was prepared in HPLC grade water at a concentration of 1 mM and stored at -80 °C. A working standard was prepared by diluting the stock solution with water to yield a final concentration of 0.1mM. 100 μl of this working standard was added to 1ml capped vials and stored at -80° C until analyzed.

OPA/2-ME reagent

The derivatization reagent was prepared by dissolving 50 mg of anhydrous *O*-phthaldialdehyde in 2 ml of methanol. To this 8 ml 0.4 M borate buffer (pH adjusted to 9.5 with 4 N Sodium hydroxide) and 50 μl of 2-mercaptoethanol were added. The solution was stored in the dark and kept at room temperature for 24 hours before use. 20 μl of 2-mercaptoethanol was added every 3 days.

Preparation of biological samples

Heparinized blood, obtained from the aorta of Male Wistar rats, was centrifuged at 2000 g for 15 minutes. The plasma was transferred to 1 ml capped vials and stored at -80° C until analyzed. At the time of analysis 0.5 ml plasma was gently thawed and deproteinized with 1ml of methanol (HPLC grade) and centrifuged at 2000 g. The supernatant was collected and 0.5 ml of methanol was added to the residue and mixed on a rotary mixer. The test tube contents were then centrifuged and supernatant collected. This procedure was repeated with another 0.5 ml of methanol. The supernatants were pooled and stored at -80° C until analysis.

Tissue (Brain, Liver and Muscle), excised from rats at surgery, was immediately frozen in liquid nitrogen and homogenized (approximately 50 mg/ml) in methanol. The extraction was repeated three times as described for plasma to ensure complete extraction of amino acids. Supernatants were stored at -80° C until analysis.

CHROMATOGRAPHIC CONDITIONS

Solvent preparation

A stock 1M sodium acetate buffer (pH 7.2) was prepared by dissolving 82.03 g anhydrous sodium acetate in 1 liter of HPLC grade water and pH was adjusted with 1N

acetic acid. The buffer was filtered through 0.25 μm membrane filters (Millipore). Solvent A was prepared by diluting 1M sodium acetate buffer to yield a 0.1M concentration. Solvent B was prepared by mixing methanol and tetrahydrofuran in a ratio of 97:3 (V/V). Two solvents were degassed by vacuum and sonication before use.

Derivatization of amino acids

500 μl of OPA/2-ME reagent were added to the vial containing 100 μl of amino acid standard or sample. The vial was capped, shaken and after derivatization for 2 minutes 20-μl aliquots of the sample was introduced into HPLC column for analysis.

Quantitation

Resolution of peaks was accomplished using multistep gradient elution of two solvents with a flow rate of 1.5 ml/min. Fluorescence response was measured at a sensitivity dial setting of 4 on the detector. Amino acid concentration was quantified from the peak area under the chromatogram curve with SP4100 computing integrator against an external standard. Details of the gradient elution profile are shown in Figure 1. Within-run precision of the standard was estimated by injecting derivatized standard on the same day and between-run precision was determined by analyzing the same standard on different days. The linearity of response was calculated at various concentrations for 19 amino acids and regression equations were derrived from the response versus concentration curves. The recovery experiments were conducted by adding known quantities of amino acid standard to the plasma sample and the percentage recoveries were calculated.

RESULTS AND DISCUSSION

The chromatogram of amino acids in the standard mixture is shown in Figure 1. The elution order confirms that of an earlier report which used a similar column and derivatization method.[11] However a notable difference from the method of Jones and Gilligan [11] was the addition of tetrahydrofuran (THF) in solvent B instead of solvent A. By adopting mobile phase conditions as reported by Jones and Gilligan [11] (solvent A-THF:methanol:0.1 M sodium acetate buffer; 5:95:900 and solvent B-methanol) we could not separate methionine and valine. Hence 3% THF was added to solvent B (97:3; methanol:THF) to obtain optimum separation of methionine/valine pair without affecting the resolution of other amino acids. All the 21 compound (amino acids and related compounds) used in the present study were clearly resolved. Inspite of good separation of lysine and ornithine, quantitation of these compounds were not performed in the

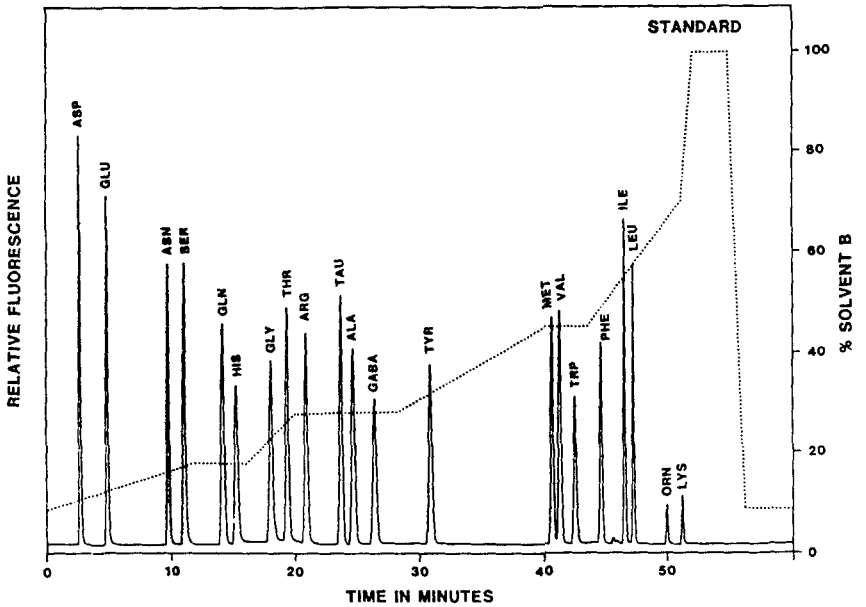

Figure 1. Chromatogram of pre-column OPA-amino acid standard derivatives (333 pmol each) on Ultrasphere ODS (75 X 4.5 mm ID), 3 μ particle size. Operating conditions : Flow rate, 1.5 ml/min; Solvent A, 0.1M Sodium acetate buffer pH=7.2; Sovent B, Methanol: Tetrahydrofuran (97:3); Gradient program, linear gradient from 10% (initial) to 18% B for 12 min, isocratic elution step at 18% B for 4 min, linear step to 28% B for 4 min, isocratic step at 28% B for 8 min, linear step to 45% B for 12 min, isocratic step at 45% B for 3.5 min, linear step to 70% B for 7.5 min, linear step to 100% B for 1 min, isocratic step at 100% B for 3 min, linear step to 10% B (initial) for 1 min, isocratic at 10% (initial) for 10 min; Sample volume 20 μl; Excitation at 338 nm; Emission at 425 nm. Peaks : ASP= Aspartate; GLU= glutamate; ASN= asparagine; SER= serine; GLN=glutamine; HIS=histidine; GLY= glycine; THR= threonine; ARG= agrinine; TAU= taurine; ALA= alanine; GABA= γ-aminobutyric acid; TYR= tyrosine; MET= methionine; VAL= valine; TYP= tryptophane; PHE= phenylalanine; ILE= isoleucine; LEU= leucine; ORN= ornithine; LYS= lysine. Dotted line indicates solvent gradient program.

present study since the fluorescent response of these compounds was poor. Brij 35 may be added to increase the sensitivity of the method for lysine if it were to be quantified.[23]

Other amino acids such as proline, hydroxyproline and cysteine could not be quantified in the present system since the secondary amino acids (proline and hydroxyproline) do not react with OPA/2-ME,[24,25] whereas cysteine can be derivatized in the presence of iodoacetic acid.[26] The ammonia peak was detected after tyrosine but was not quantified because of its very low fluorescence response within the present detection limits.

Using the present chromatographic conditions the resolution of glycine/threonine, asparagine/serine, tyrosine and γ-aminobutyric acid could be achieved which remain unresolved by previous methods.[15-22] In addition to the amino acids shown in the chromatogram of Figure 1 citrulline was eluted between threonine and arginine. 1-methylhistidine and 3-methylhistidine were eluted after arginine which is in accordance with the reports of Jones and Gilligan .[11]

Under the present analytical conditions it takes 50 minutes for a complete run which gives excellent and reproducible separation of 21 amino acids commonly measured in biological samples. An additional period of 10 minutes was allowed after return to the initial conditions before injection of the next sample to minimize variation of retention times between samples. The average coefficient of variation in retention time for amino acids in standard, plasma and brain samples was 0.189%. Relative retention times were measured for repeated analysis on the same day and for several samples on different days during a two month period (Table 1). The average between-day coefficient of variation was slightly higher (1.53%) than the within-day variation (0.194%). The coefficient of variation of individual amino acids was less than 4 % for between-day assays and less than 1 % for within-day assays which shows that the present system is highly reproducible.

Time dependent loss of fluorescence and different stabilities of fluorescence for various amino acids have been reported by several workers.[9,13, 16, 18, 27] In order to minimize run-to-run variability of fluorescence as a function of derivatization time and maximize reproducibility it has been suggested that the time between derivatization and injection onto the column should be kept constant.[9] Accordingly, two minutes were allowed for derivatization and the derivatized sample was injected exactly at 2 minutes. Calculated apparent fluorescent constants, FK', and the relative fluorescence (relative to glycine) for each amino acid are listed in Table 2. The FK' value is the ratio of fluorescence yield to 1 pmol concentration of the respective amino acid derivative. The fluorescence yield was measured from the peak area of each amino acid and relative fluorescence was calculated by normalizing the peak area of individual amino acid with that of glycine. In general relative fluorescence of each amino acid was similar to the value of the corresponding amino acid obtained by Lindroth and Mopper .[9] Average within-day variation of the fluorescence constant, relative fluorescence and between day variation of fluoresence constant were 1.98, 1.17 and 3.32 respectively. By injecting serially diluted standard mixtures until a zero standard signal, the lower detection limit was observed to be less than 1 pmol under the present detection conditions . However sensitivity may be increased by changing the detector settings.

TABLE 1. REPRODUCIBILITY OF RETENTION TIMES AND RELATIVE RETENTION TIMES OF PRE-COLUMN OPA AMINO ACID DERIVATIVES

Amino acid	Retention time			Relative retention time[3]					
	With-in			With-in			Between		
	Mean[1]	±SD	C.V. (%)	Mean[1]	±S.D.	C.V. (%)	Mean[2]	±S.D.	C.V. (%).
ASP	2.15	0.015	0.69	0.045	0.0004	0.88	0.057	0.002	3.51
GLU	3.86	0.019	0.49	0.081	0.0004	0.49	0.102	0.003	3.43
ASN	8.64	0.030	0.35	0.182	0.0005	0.27	0.206	0.004	1.89
SER	10.12	0.030	0.29	0.213	0.0008	0.37	0.238	0.006	2.52
GLN	13.15	0.035	0.27	0.277	0.0005	0.18	0.304	0.005	1.64
HIS	14.61	0.047	0.32	0.308	0.0009	0.29	0.325	0.007	2.34
GLY	17.20	0.051	0.29	0.363	0.0007	0.19	0.384	0.007	1.90
THR	18.73	0.039	0.21	0.395	0.0007	0.18	0.409	0.003	0.83
ARG	21.28	0.027	0.13	0.449	0.0004	0.09	0.450	0.005	1.13
TAU	23.20	0.013	0.06	0.489	0.0005	0.10	0.502	0.005	1.10
ALA	24.32	0.025	0.10	0.513	0.0008	0.15	0.521	0.004	0.73
GABA	25.81	0.026	0.10	0.545	0.0007	0.13	0.565	0.014	2.42
TYR	30.71	0.020	0.06	0.648	0.0006	0.09	0.656	0.012	1.90
MET	40.15	0.016	0.04	0.847	0.0005	0.06	0.857	0.007	0.79
VAL	40.66	0.017	0.04	0.858	0.0005	0.06	0.875	0.008	0.95
TRP	41.66	0.019	0.04	0.879	0.0005	0.06	0.896	0.009	1.04
PHE	43.48	0.024	0.05	0.917	0.0005	0.05	0.943	0.007	0.77
ILE	46.40	0.014	0.03	0.979	0.0005	0.05	0.984	0.001	0.13
LEU	47.36	0.015	0.03	1.000	0.0000	0.00	1.000	0.000	0.00

1 Calculated from 6 measurements on the same day.
2 Calculated from 30 measurements over a two month period.
3 Values are compared with retention time of leucine.

TABLE 2. APPARENT FLUORESCENCE CONSTANT (FK'), RELATIVE FLUORESCENCE AND PRECISION OF DETERMINATION

Amino acid	FK' (Area/pmol.10^2) Mean[1]	±S.D.	C.V. (%)	Relative fluorescence[2] Mean[1]	±S.D.	With-in* C.V. (%)	Between** C.V. (%)
ASP	8.989	0.157	1.74	1.849	0.039	2.110	3.34
GLU	8.538	0.159	1.86	1.757	0.034	1.930	3.50
ASN	7.367	0.059	0.80	1.516	0.022	1.450	4.25
SER	6.513	0.114	1.75	1.339	0.099	0.672	3.05
GLN	7.380	0.097	1.31	1.518	0.099	0.593	3.61
HIS	6.526	0.091	1.39	1.343	0.011	0.819	2.35
GLY	4.859	0.078	1.60	1.000	0.000	0.000	0.00
THR	6.218	0.097	1.56	1.279	0.005	0.391	1.92
ARG	5.947	0.111	1.87	1.222	0.007	0.573	4.43
TAU	5.397	0.146	2.70	1.108	0.014	1.260	3.66
ALA	5.947	0.091	1.53	1.222	0.005	0.410	2.78
GABA	3.923	0.128	3.26	0.806	0.015	1.861	2.41
TYR	6.593	0.122	1.85	1.353	0.011	0.813	3.33
MET	6.062	0.123	2.03	1.246	0.016	1.284	3.47
VAL	7.392	0.115	1.56	1.509	0.016	1.053	3.85
TRP	4.252	0.142	3.33	0.873	0.019	2.017	4.71
PHE	5.722	0.142	2.48	1.176	0.018	1.615	4.09
ILE	6.711	0.158	2.35	1.379	0.024	1.769	4.31
LEU	5.588	0.152	2.72	1.148	0.019	1.655	4.11

[1] Calculated from 6 measurements on the same day. [2] Defined as the ratio between peak area relative to glycine.

* With-in run coefficient of variation is based on 6 injections of the standard on the same day.

** Between run coefficient of variation is based on the mean coefficient of variation of six days over a two month period

TABLE-3 : STANDARD CURVE DATA

Amino acid	Regression Equation	Correlation Coefficient (r)	Standard error of the estimation
ASP	y = 0.026 x + 0.207	0.995	0.438
GLU	y = 0.027 x - 0.078	0.998	0.307
ASN	y = 0.025 x - 0.382	0.996	0.355
SER	y = 0.025 x - 0.536	0.996	0.356
GLN	y = 0.027 x - 0.408	0.997	0.321
HIS	y = 0.023 x - 0.281	0.997	0.261
GLY	y = 0.020 x - 0.442	0.998	0.211
THR	y = 0.024 x - 0.447	0.998	0.249
ARG	y = 0.023 x - 0.448	0.997	0.262
TAU	y = 0.023 x - 0.611	0.997	0.262
ALA	y = 0.022 x - 0.319	0.998	0.230
GABA	y = 0.017 x - 0.513	0.997	0.213
TYR	y = 0.023 x - 0.332	0.998	0.262
MET	y = 0.021 x - 0.396	0.999	0.159
VAL	y = 0.025 x - 0.313	0.999	0.159
TRP	y = 0.016 x - 0.521	0.998	0.156
PHE	y = 0.020 x - 0.398	0.999	0.159
ILE	y = 0.023 x - 0.373	0.998	0.218
LEU	y = 0.019 x - 0.338	0.998	0.218

Regression equation is $y = mx + c$, where y is the peak area ($X10^3$), m is the slope, x is the amino acid concentration and c is the y intercept.

As suggested by Lindroth and Mooper [9], excessive amounts of OPA/2-ME was used for optimum derivatization to obtain linearity of fluorescence response. Linear regression analysis of peak area of each amino acid versus concentration of amino acid throughtout a range of 25 to 500 nmols/ml gave a correlation coefficient between 0.995 to 0.999 for all amino acids which is in accordance with earlier reports (Table 3).[14,28]

Recovery experiments were conducted in order to assess reliability of the extraction procedure of the method. The percentage recovery of individual amino acids was calculated by adding known quantities of amino acid standard to the plasma sample which was then extracted with methanol. Methanol was used as a deproteinizing agent in the present method since use of acetonitrile as a protein precipitation reagent results in proteolysis[22] producing erroneously high amino acid concentrations in the biological samples. Deproteinization with SSA gives a large sovent peak which obscures aspartic acid in biological samples.[22] The present extraction procedure results in recovery ranging from 91.87 to 99.34% and an average recovery of amino acid is 97.05%. The

TABLE 4. AMINO ACID CONCENTRATION IN DIFFERENT TISSUES OF WISTAR RAT
(Values are mean, ± S.D. of five samples)

Amino acid	Plasma[1]		Brain[2]		Liver[2]		Gastrocnemius muscle[2]	
	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.
ASP	65.84	4.87	2.077	0.156	0.432	0.089	0.111	0.015
GLU	77.07	3.10	9.399	0.587	1.116	0.285	1.107	0.237
ASN	7.30	0.19	0.093	0.009	0.060	0.011	0.135	0.016
SER	216.44	18.30	1.010	0.080	0.677	0.113	0.615	0.071
GLN	557.37	28.82	6.389	0.593	3.268	0.625	3.068	0.430
HIS	63.61	6.53	0.025	0.009	0.290	0.032	0.099	0.020
GLY	366.77	30.66	0.800	0.078	2.240	0.567	2.456	0.480
THR	284.84	33.70	0.398	0.026	0.393	0.059	0.448	0.060
ARG	187.33	18.35	0.304	0.052	—	—	0.082	0.030
TAU	286.64	62.30	6.790	0.593	3.354	0.765	14.340	2.930
ALA	636.59	27.50	0.831	0.061	3.261	0.445	2.040	0.180
GABA	—	—	2.314	0.148	—	—	—	—
Tyr	101.77	7.60	0.066	0.005	0.064	0.015	0.076	0.020
MET	61.75	4.90	0.038	0.010	0.051	0.011	0.063	0.009
VAL	253.47	23.20	0.095	0.010	0.263	0.043	0.188	0.021
TRP	104.73	12.20	0.016	0.002	0.055	0.004	0.035	0.008
PHE	76.93	3.90	0.057	0.007	0.082	0.014	0.054	0.005
ILE	115.91	12.70	0.053	0.004	0.143	0.024	0.076	0.010
LEU	199.78	15.90	0.114	0.011	0.257	0.042	0.131	0.017

1 Values are expressed as nmoles/ml of plasma.
2 Values are expressed as nmoles/mg of tissue.

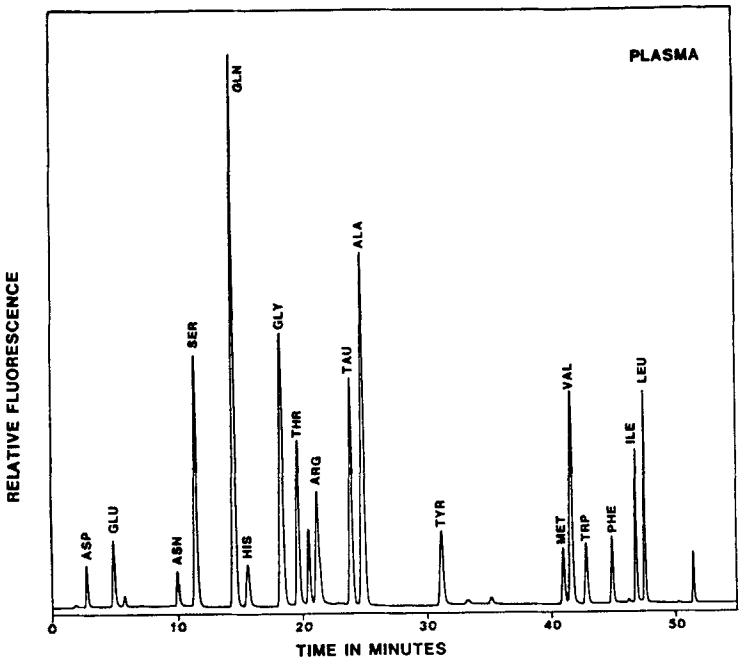

Figure 2. Chromatogram of rat plasma amino acids as OPA–mercaptoethanol derivatives. Conditions same as Figure 1. Unmarked peaks were not identified.

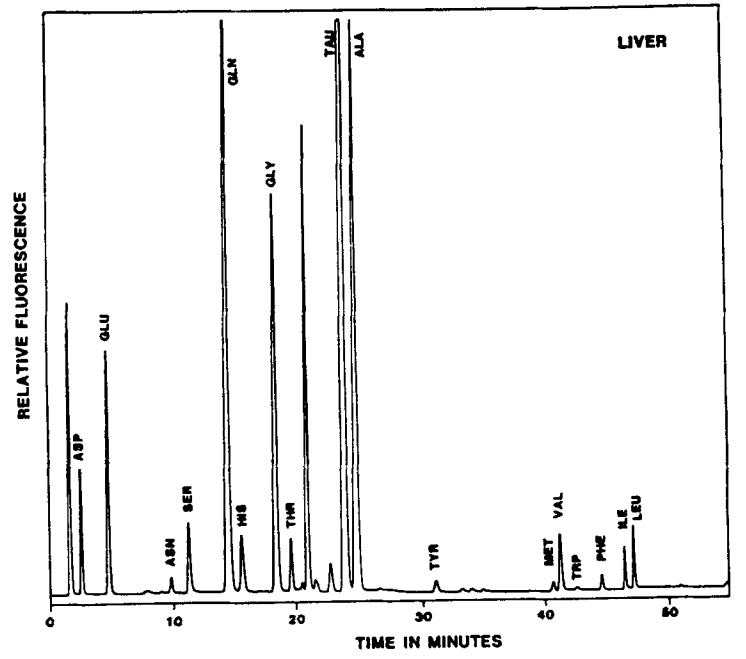

Figure 3. Chromatogram of rat liver amino acids as OPA–mercaptoethanol derivatives. Conditions same as Figure 1. Unmarked peaks were not identified.

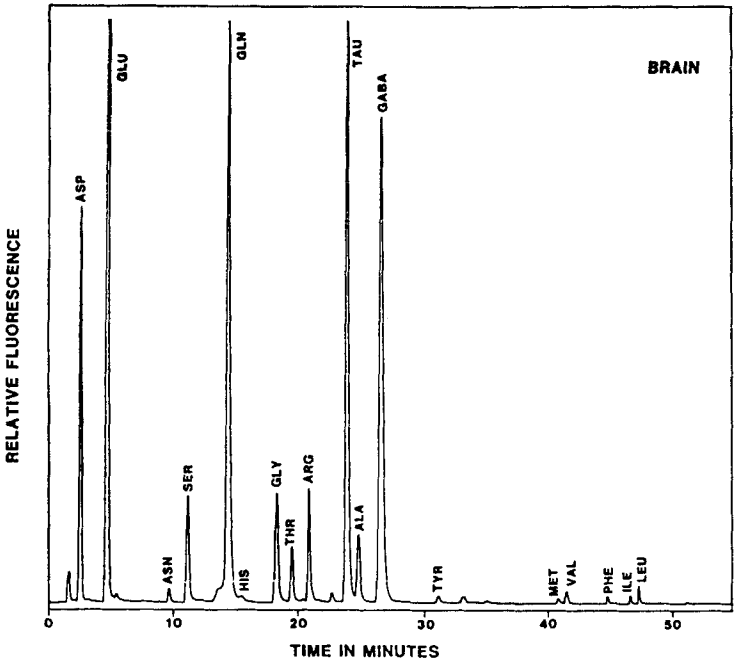

Figure 4. Chromatogram of rat brain amino acids as OPA-mercaptoethanol derivatives. Conditions same as Figure 1. Unmarked peaks were not identified.

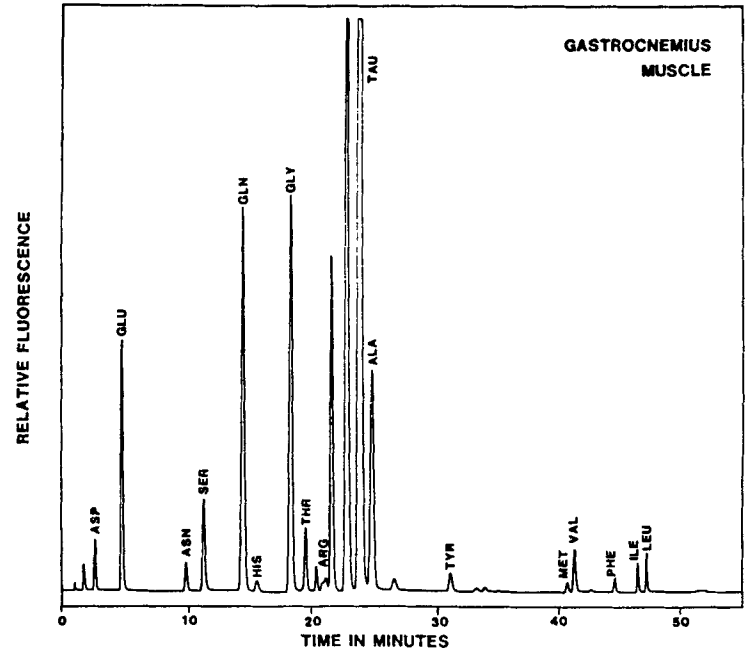

Figure 5. Chromatogram of rat gastrocnemius muscle amino acids as OPA-mercaptoethanol derivatives. Conditions same as Figure 1. Unmarked peaks were not identified.

concentration of individual amino acids estimated in plasma, brain, liver and gastrocnemius muscle of Wistar rats in the present method confirms those of earlier reports (Table 4; Figure 2 -5).[29-33] Satisfactory response was obtained by using $\leq$1 mg of tissue and $\leq$ 5-10 μl of plasma samples per injection in the present system instead of using $\geq$10 mg for a conventional analytical methods .

The use of a 3 μm particle Ultrasphere ODS column for quantitative analysis of primary amino acids in biological samples is highly advantageous because of rapid analysis, high sensitivity and minimum band broadening. Because of this high versatality, the technique may be adopted for routine analysis of other biological samples such as cerebrospinal fluid, amniotic fluid, protein hydrolysates and animal feeds with only minor change in the gradient elution program.

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