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Amino acid determination in biological fluids by automated ion-exchange chromatography: performance of Hitachi L-8500A

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The Hitachi L-8500A is a newly available apparatus for amino acid (AA) analysis that allows automatic on-line mixing of the ninhydrin reagent. The within-run precision (human plasma pools at three different concentrations) showed CVs <3.8% except for the lowest concentration of citrulline (4.4%), Tyr (4.5%), and α-aminobutyric acid (7.6%), and for the intermediate concentration of Asp (8.7%). Between-run precision (CV) was <3.1% for 17 AAs and <8.0% for 24 of 25 AAs (CV Asp = 12.0%). For retention times, within-run precision was <0.4% and between-run precision <1.8%. Excellent relations were found between the results from the Hitachi L-8500A and the widely used Beckman 6300 analyzer (0.929≤ *r* ≤0.999). The detection was still linear at 5 μmol/L except for Pro and hydroxyproline (20 μmol/L). The upper limit was at least 2500 μmol/L for 13 AAs and at least 1000 μmol/L for 27 of 29 AAs (anserine = 500, Val = 600 μmol/L). Values from 100 human plasma samples agreed with previously published data. We conclude that the results obtained with the Hitachi L-8500A are satisfactory when compared with those of other AA analyzers utilizing the same method. Furthermore, the Hitachi L-8500A displays several advantages including programming flexibility, microsample capacity, low noise plotting, ammonia filtering, and manual repacking of the analytical column.

INDEXING TERMS: ninhydrin reaction • cation-exchange chromatography • analytical performance evaluation

Several methods are suitable for the determination of amino acids (AAs) in biological fluids, including gas chromatography *[1, 2]*, reversed-phase chromatography with precolumn derivatization with various reagents such as orthophthalaldehyde (OPA) *[3–6]*, 9-fluorenylmethyl-chloroformate *[7]*, phenylisothiocyanate (PITC) *[8, 9]*, dimethylaminonaphthalenesulfonyl chloride *[10]*, dimethylaminoazobenzenesulfonyl chloride *[10]*, 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole *[11]*, and ion-exchange chromatography with postcolumn derivatization utilizing OPA *[12]* or ninhydrin *[13, 14]*.[4] The latter remains the most widely used because of several technical and practical advantages.

The classic ion-exchange separation followed by post-column derivatization with ninhydrin has been considerably improved since its initial inception *[13]*, particularly with availability of modern dedicated AA analyzers *[14, 15]*. However, one remaining problem is the relative instability of the ninhydrin reagent, limiting the use of the ninhydrin/acetate buffer mixture to ~2 weeks. This also probably explains why within-run and between-run precisions are so poor for an automated technique (5–10% or more). The design of the new Hitachi L-8500A is therefore of interest. Although the analytical approach is very conventional, AAs being separated with a cation-exchange resin and their concentration determined with the ninhydrin reaction, the instrument provides for extemporaneous automatic mixing of the detection reagent. The Hitachi L-8500A thus allows very accurate detection and full use of the reagent, regardless of how often the apparatus is used.

The aim of this work was to evaluate the analytical performance of this newly available apparatus.

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[4] Nonstandard abbreviations: AA, amino acid; OPA, orthophthalaldehyde; PITC, phenylisothiocyanate; α-ABA, α-aminobutyric acid; Cit, citrulline; Aclys, acetyllysine; 3MH, 3-methylhistidine; and HyPro, hydroxyproline.

Materials and Methods

APPARATUS

The Hitachi (Tokyo, Japan) L-8500A system is distributed by Sciencetec (Les Ulis, France). Samples were placed in self-sealing vials on the autosampler rack (60 positions) maintained at 4 ± 2 °C. After rinsing the needle, a volume of the sample was aspirated through the sample loop. An accurate volume (20 μL measured with a 0.5-mL precision syringe) was injected via the injection valve onto the analytical column. The chromatographic column was a matrix of a high-molecular-mass organic component, consisting of polystyrene cross-linked by divinylbenzene, with sulfone (SO_3^-) groups as active exchange sites. The column [60 mm × 4.6 mm (i.d.)] was packed with 3-μm particles and equipped with a 40 mm × 4.6 mm (i.d.) guard column (ammonia filter). AAs were separated with four citrate buffers (Table 1). The elution program used (Table 2) was slightly modified from that set by Hitachi to improve the separation of specific AAs. The separation of Asn, Glu, and Gln was improved by decreasing the column temperature by 5 °C at 9 min and by 4 °C at 20 min, that of Gly, Ala, citrulline (Cit), and α-aminobutyric acid (α-ABA) by starting the increased flow of buffer 2 earlier (on at 31.5 min instead of 33.5 min), and that of Val by starting the 100% buffer 2 step later (on at 44.1 min instead of 43.0 min). An improved separation of Trp and ethanolamine was obtained by increasing the concentration of benzyl alcohol in buffer 3. Correct separation of basic AAs was obtained by starting the flow of buffer 4 earlier (on at 74.1 min instead of 77.0 min), adding 10% buffer 2 at 79 min, and decreasing the column temperature by 10 °C at 86 min.

Detection was by spectrophotometry at 570 and 440 nm with the ninhydrin reaction.

Before the next sample injection, the column was equilibrated with 100% buffer 1 for 19 min (total run time 149 min).

BUFFERS

All buffers can be either purchased (Mitsubishi, Japan) as a whole package or prepared. We prepared the buffers with HPLC-grade water generated with a Milli-Q water purification system (Millipore, Molsheim, France). The chemicals and solvents were of analytical grade. Trilithium citrate tetrahydrate, lithium chloride, citric acid monohydrate, lithium hydroxide monohydrate, and benzyl alcohol were purchased from Merck (Darmstadt, Germany). Pure ethanol was supplied by Farmitalia Carlo Erba (Milano, Italy), thiodiglycol was purchased from Prolabo (Paris, France), and caprylic acid from Sigma-Aldrich (Saint-Quentin Fallavier, France). Chemicals were accurately weighed so that pH measurement was not required. Each buffer was filtered through a 0.22-μm filter (Millipore) before use, and kept under nitrogen in the apparatus. Throughout the elution program the flow rate for buffer solutions was 0.35 mL/min.

NINHYDRIN REAGENT

The ninhydrin reagent, supplied by Wako Pure Chemical Industries (Osaka, Japan), consisted of 1 L of ninhydrin solution (containing ninhydrin, sodium borohydrate, and propylene glycol monomethyl ether) and 1 L of buffer solution (containing dihydrate lithium acetate, glacial acetic acid, and propylene glycol monomethyl ether). During sample running, the delivery pump for the ninhydrin reagent automatically mixes the two solutions kept under nitrogen in the apparatus. The flow rate for ninhydrin solution was 0.30 mL/min.

CALIBRATOR AND SAMPLE PREPARATION

A 100 μmol/L calibrator stock solution containing 37 physiological AAs was prepared with commercial solutions (Sigma-Aldrich). Gln (Sigma-Aldrich) was added at the same concentration and acetyllysine (Aclys; Sigma-Aldrich) was added at 1000 μmol/L.

We evaluated pooled human plasma from hospitalized patients (within-run and between-run precision assays), physiological fluids of various origins such as human or rat plasma, supernatant of homogenate from rat tissue (regression comparison), commercial calibrator solutions (range of linearity), and human plasma from healthy volunteers (determination of physiological range in plasma). In addition, the Bio-Rad urine control solution (Ivry-sur-Seine, France) was used to measure AA concentra-

Table 1. Composition of buffers.

	1	2	3	4	5
Trilithium citrate tetrahydrate, g	5.73	9.80	8.79	9.80	–
Lithium chloride, g	1.24	6.36	26.62	38.15	–
Citric acid monohydrate, g	19.90	12.00	11.27	3.30	–
Lithium hydroxide monohydrate, g	–	–	–	–	8.40
Pure ethanol, mL	30	30	100	–	30
Thiodiglycol, mL	5	5	–	–	–
Benzyl alcohol, mL	–	–	4	–	–
Brij 35, 30%, mL	3.4	3.4	3.4	3.4	3.4
Purified water, quantity for 1 L					
Caprylic acid, μL	100	100	100	100	100
pH (no adjustment required)	2.8	3.7	3.6	4.1	–

Table 2. Elution program for amino acid measurement in biological fluids.

Time, min	Buffer, total 100 %					Column temperature, °C
	1	2	3	4	5	
0.0	100	–	–	–	–	38
9.0		–	–	–	–	27
17.9	100	–	–	–	–	
18.0	80	20	–	–	–	
20.0			–	–	–	56
31.5	70	30	–	–	–	
31.6	10	90	–	–	–	
37.0			–	–	–	45
44.0	10	90	–	–	–	
44.1	–	100	–	–	–	
52.0	–	100	–	–	–	70
52.1	–	–	100	–	–	
68.0	–	–		–	–	45
74.0	–	–	100	–	–	
74.1	–	–	–	100	–	
79.0	–	10	–	90	–	
86.0	–	–	–		–	60
109.0	–	–	–	100	–	70
117.0	–	–	–	100	–	
117.1	–	–	–	–	100	
130.0	–	–	–	–	100	
130.1	100	–	–	–	–	38
149.0	100	–	–	–	–	

tions in urine (within-run and between-run precision assays), especially that of 3-methylhistidine (3MH), an interesting AA to study in both malnutrition and hypercatabolic states, given that its urinary excretion reflects the myofibrillar catabolism in muscle *[16]*.

Our procedures complied with the Helsinki Declaration of 1975 for human subjects, as revised in 1983. Animal care complied with the guidelines of our institution, two of us (C.C.-L. and L.C.) being officially authorized (no. 004963 and no. 005226) by the French Ministry of Agriculture and Forestry for animal experimentation.

Blood was drawn into heparinized tubes after a 5-h fast for rats, and either in the postabsorptive state for hospitalized patients or after an overnight fast for 100 healthy volunteers (36 ± 10 years; 41 men, 59 women). Plasma was immediately separated by cold centrifugation (4 °C, 3500*g*), deproteinized without delay with sulfosalicylic acid (40 g/L), and analyzed or stored at −80 °C until analyzed.

Rat tissues (liver, skeletal muscles) were quickly removed at sacrifice, wiped, weighed, and frozen in liquid nitrogen. Tissue samples were homogenized (4 °C) in 10% trichloroacetic acid (10 mL/g of tissue), containing 0.5 mmol/L EDTA, with an Ultra-Turrax T25 tissue disrupter (Médi Sciences, Saint-Maur-des-Fossés, France). The acid-soluble fraction was separated by cold centrifugation (4 °C, 3500*g*). Free AA concentrations were measured in the supernatant.

Before analysis, the control and samples were diluted (1:1 by vol) in buffer 1 containing 1000 μmol/L Aclys as internal calibrator.

The within-run precision was studied by performing 10 consecutive runs of the plasma pool from hospitalized patients and of the Bio-Rad urine control solution. To have a wide range of values for plasma, three concentrations were studied: low (L, plasma pool diluted with buffer 1; 1:2 by vol), medium (M, plasma pool), and high (H, plasma pool supplemented with the AA calibrator solution supplied by Sigma; 1:1 by vol). The within-run precision of retention times was studied by 10 consecutive injections of the M concentration plasma pool and that of the urine control solution.

Between-run precision assays were carried out with the M concentration of the plasma pool and the Bio-Rad urine control solution measured in 10 different series. Series were defined by new calibration procedures when the ninhydrin reagent and (or) one or more buffers had to be changed.

A regression comparison was performed by analyzing 77 different physiological fluids [human or rat plasma, supernatant of homogenate from rat tissue (liver and skeletal muscles)] with the Hitachi L-8500A and the Beckman 6300 (Palo Alto, CA), a widely used system for AA analysis.

Linearity and detection limit assays were performed by serial dilutions of the calibration solutions in buffer 1. A Gln solution was diluted in the same manner. The concentrations studied ranged from 5 to 2500 μmol/L.

Sample carryover was analyzed as recommended by the French Society for Clinical Biology *[17]*. Two plasma pools were used: AA at L and H concentrations. We measured the initial concentration for each AA in each specimen (L_1 and H_1), then the H concentration twice (H_2, H_3), and then L concentration twice (L_2, L_3). This sequence (H_2, H_3, L_2, L_3) was assayed six times. For each AA we used the Student's paired *t*-test to compare the mean value obtained for L_2 with that obtained for L_1, and the mean value of L_3 with that of L_2.

STATISTICAL ANALYSIS

Results were expressed as mean ± SD.

Student's paired *t*-test and regression were applied with the PCSM (Programme Conversationnel de Statistiques pour les Sciences et le Marketing) Deltasoft program (Grenoble-Meylan, France). Differences were considered significant when $P < 0.05$.

The Kolmogorov–Smirnov test showed that human plasma AA concentrations were normally distributed, in agreement with the literature *[18]*.

Results and Discussion

TYPICAL CHROMATOGRAM

With a constant flow rate of 0.35 mL/min for buffers, the AAs were eluted in 115 min with the program described in Table 2. Under these conditions, the Hitachi L-8500A

apparatus separated 38 AAs (including Aclys as internal calibrator) during the elution program.

Typical chromatograms for the calibration solution and for human plasma are shown in Fig. 1. AA separation is satisfactory except for that of Asn and Glu, a major concern of AA separation by ion-exchange chromatography.

PERFORMANCES

The results of the within-run precision assays for concentrations and retention times performed on human plasma pools are presented in Table 3. For concentrations, the CVs were <4.5% for L values of 24 of 25 AAs (CV α-ABA = 7.6%). CVs were <3.8% for M values, except for Asp (8.7%), and <1.9% for H values for all the AAs studied. Retention times were very stable, with CVs from 0.0% to 0.3%. These CVs appeared better than those shown by other HPLC systems with the same *[14]* or other *[8, 9, 12]* methods. The CVs of the concentrations in the urine samples (data not shown) were about twice those observed with serum, <4.0% for most of the AAs (20 of 23). Three AAs had high CVs: α-ABA (6.9%), Pro (14.4%), and Cit (19.8%). This may be explained by the difficulty in measuring AAs at low concentrations in urine samples (23, 25, and 12 μmol/L, respectively). Values for 3MH were 108 ± 3 μmol/L (CV = 2.7%). The results of the within-run precision assay for the retention time of AAs from the urine sample were similar to those obtained with serum.

The results of between-run precision assays for concentrations and retention times performed on a human plasma pool are presented in Table 4. Concerning between-run precision of the peak area, most of the AAs studied (19 of 25) had CVs <5.2%. The CVs of 5 AAs (Glu, Cit, α-ABA, Trp, and Arg) were between 5.6% and 7.9%. The comparatively poor result for Asp (CV = 12.0%) may have been due to its low concentration (7 μmol/L) in the sample used.

Concerning the between-run precision of peak position, CVs of 21 AAs were <1.0%. The CVs of four AAs (Ser, Asn, Glu, and Gln), with retention times between 17.62 and 23.36 min, were between 1.1% and 1.8%. These poorer results could have occurred because two buffers were programmed simultaneously in different proportions. Hence small changes in composition occurring during preparation could have caused additional varia-

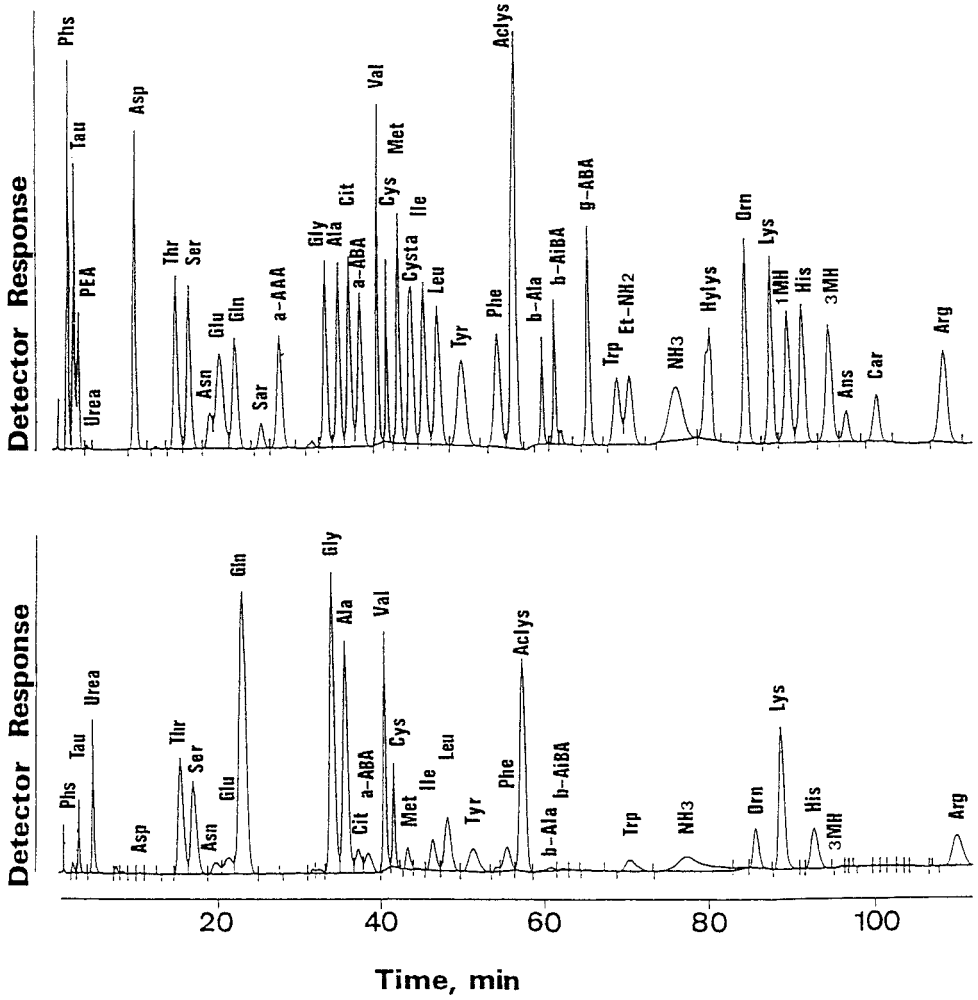

Fig. 1. Profile of elution of a 2.0 nmol/20 μL synthetic physiological mixture of AAs supplemented with Gln (*top*) and of a human deproteinized plasma sample from one healthy subject (*bottom*) assayed with Hitachi L-8500A.

Phs, phosphoserine; Tau, taurine; PEA, phosphoethanolamine; Sar, sarcosine; a-AAA, α-aminoadipic acid; Cysta, cystathionine; Et-NH_2, ethanolamine; Hylys, hydroxylysine; Orn, ornithine; Ans, anserine; Car, carnosine.

Table 3. Within-run precision assays for amino acid concentrations and retention times.[a]

Amino acid	Conc.	Concentration Mean ± SD, μmol/L	Concentration CV, %	Retention time Mean ± SD, min	Retention time CV, %	Amino acid	Conc.	Concentration Mean ± SD, μmol/L	Concentration CV, %	Retention time Mean ± SD, min	Retention time CV, %
Phs	L	5.1 ± 0.1	0.7			Val	L	74.7 ± 0.3	0.4		
	M	14.8 ± 0.2	1.3	1.90 ± 0.01	0.3		M	221.8 ± 2.2	1.0	41.85 ± 0.04	0.1
	H	141.2 ± 0.7	0.5				H	353.8 ± 3.6	1.0		
Tau	L	26.0 ± 0.1	0.3			Cys	L	17.4 ± 0.3	1.6		
	M	78.2 ± 0.8	1.0	2.56 ± 0.01	0.3		M	56.1 ± 0.6	1.1	42.95 ± 0.04	0.1
	H	207.5 ± 0.4	0.2				H	117.4 ± 1.0	0.8		
Asp	L	2.3 ± 0.0	0.0			Met	L	8.6 ± 0.0	0.0		
	M	6.8 ± 0.6	8.7	10.62 ± 0.01	0.1		M	25.0 ± 0.3	1.1	44.38 ± 0.04	0.1
	H	135.0 ± 0.7	0.5				H	151.5 ± 1.3	0.9		
Thr	L	44.2 ± 0.0	0.0			Ile	L	19.6 ± 0.6	3.1		
	M	129.0 ± 2.0	1.5	15.67 ± 0.02	0.1		M	56.9 ± 0.6	1.0	47.70 ± 0.04	0.1
	H	254.9 ± 2.0	0.8				H	185.2 ± 1.4	0.8		
Ser	L	37.2 ± 0.6	1.7			Leu	L	40.0 ± 0.0	0.0		
	M	106.3 ± 1.1	1.1	17.35 ± 0.02	0.1		M	119.2 ± 1.2	1.0	49.31 ± 0.04	0.1
	H	233.7 ± 0.9	0.4				H	247.3 ± 1.5	0.6		
Asn	L	23.8 ± 0.0	0.0			Tyr	L	22.0 ± 1.0	4.5		
	M	65.8 ± 0.6	0.9	19.99 ± 0.03	0.2		M	72.5 ± 0.9	1.2	51.43 ± 0.06	0.1
	H	201.1 ± 3.2	1.6				H	185.9 ± 2.8	1.5		
Glu	L	72.7 ± 0.7	1.0			Phe	L	31.4 ± 0.0	0.0		
	M	161.3 ± 4.7	2.9	21.45 ± 0.05	0.2		M	96.1 ± 1.0	1.0	55.48 ± 0.04	0.1
	H	308.8 ± 3.7	1.2				H	219.0 ± 1.5	0.7		
Gln	L	154.7 ± 1.1	0.7			Trp	L	14.8 ± 0.0	0.0		
	M	532.3 ± 6.3	1.2	23.13 ± 0.03	0.1		M	40.9 ± 0.9	2.2	69.64 ± 0.05	0.1
	H	616.4 ± 3.8	0.6				H	176.2 ± 3.3	1.9		
Pro	L	70.0 ± 0.0	0.0			Orn	L	32.3 ± 0.6	1.7		
	M	191.6 ± 7.3	3.8	33.19 ± 0.02	0.1		M	95.3 ± 1.0	1.1	87.82 ± 0.02	0.0
	H	341.1 ± 3.3	1.0				H	223.8 ± 1.5	0.7		
Gly	L	82.2 ± 0.6	0.7			Lys	L	64.4 ± 0.0	0.0		
	M	236.7 ± 2.2	0.9	34.87 ± 0.02	0.1		M	190.9 ± 1.8	0.9	90.81 ± 0.03	0.1
	H	370.2 ± 2.4	0.6				H	321.0 ± 1.6	0.5		
Ala	L	117.2 ± 1.0	0.8			His	L	25.7 ± 0.8	3.3		
	M	337.1 ± 3.2	1.0	36.68 ± 0.02	0.1		M	73.9 ± 0.9	1.2	94.90 ± 0.03	0.1
	H	481.8 ± 2.2	0.5				H	200.0 ± 0.0	0.0		
Cit	L	11.1 ± 0.5	4.4			Arg	L	19.5 ± 0.0	0.0		
	M	29.2 ± 0.4	1.3	38.21 ± 0.04	0.1		M	53.3 ± 2.0	3.8	110.50 ± 0.08	0.1
	H	169.0 ± 2.2	1.3				H	183.8 ± 3.5	1.9		
α-ABA	L	4.7 ± 0.4	7.6								
	M	21.4 ± 0.6	2.7	39.72 ± 0.03	0.1						
	H	143.2 ± 1.4	1.0								

[a] The within-run precision assays (10 consecutive runs) were performed on human plasma pool. Amino acids are ranked according to their elution time. Phs, phosphoserine; Tau, taurine; Orn, ornithine.

tions on the chromatographic profile observed. CVs of the concentrations of the urine samples (data not shown) were higher than those observed with serum, <10% for 17 of 24 AAs. The particularly poor results for α-ABA (15.3%), Asp (19.7%), and Glu (23.1%) were probably due to their low concentration—20, 12, and 26 μmol/L, respectively. Values for 3MH were 108 ± 6 μmol/L (CV = 5.6%). The results of the between-run precision assay for the retention time of AAs from the urine sample were similar to those obtained with serum (CV <1.6%).

Collectively, the results of the between-run precision assays were satisfactory and close to or even better than those obtained by classic ion-exchange chromatography *[14, 19]*, other HPLC techniques *[8–10, 12]*, or gas chromatography *[20]*.

Data for the relation between the results of the two systems used (Hitachi L-8500A, Beckman 6300) are presented in Table 5. The n values were different for each AA because when they were analyzed with the Beckman 6300, the samples were assayed under routine conditions for the apparatus (diluted 1:6 by vol vs 1:1 by vol with the Hitachi L-8500A); thus concentrations of some AAs were

Table 4. Between-run precision assays for amino acid concentrations and retention times.[a]

Amino acid	Concentration		Retention time	
	Mean ± SD, μmol/L	CV, %	Mean ± SD, min	CV, %
Phs	15.0 ± 0.4	2.4	1.90 ± 0.00	0.0
Tau	78.6 ± 1.2	1.5	2.56 ± 0.02	0.1
Asp	7.0 ± 0.9	12.0	10.68 ± 0.06	0.5
Thr	126.9 ± 3.9	3.1	15.79 ± 0.13	0.9
Ser	105.2 ± 2.5	2.3	17.62 ± 0.32	1.8
Asn	72.1 ± 3.7	5.1	20.24 ± 0.26	1.3
Glu	189.3 ± 15.0	7.9	21.71 ± 0.33	1.5
Gln	475.8 ± 22.4	4.7	23.36 ± 0.27	1.1
Pro	201.0 ± 3.0	1.5	33.34 ± 0.17	0.5
Gly	243.2 ± 4.5	1.8	35.02 ± 0.18	0.5
Ala	344.4 ± 9.6	2.8	36.84 ± 0.17	0.5
Cit	26.7 ± 2.0	7.5	38.34 ± 0.16	0.4
α-ABA	20.4 ± 1.5	7.3	39.89 ± 0.19	0.5
Val	222.8 ± 3.9	1.8	41.89 ± 0.07	0.2
Cys	53.5 ± 1.1	2.1	42.96 ± 0.05	0.1
Met	25.1 ± 0.4	1.4	44.42 ± 0.09	0.2
Ile	56.8 ± 0.8	1.5	47.73 ± 0.10	0.2
Leu	119.4 ± 1.7	1.4	49.34 ± 0.12	0.2
Tyr	72.3 ± 1.0	1.4	51.47 ± 0.14	0.3
Phe	96.2 ± 1.8	1.9	55.52 ± 0.10	0.2
Trp	41.5 ± 2.3	5.6	69.65 ± 0.12	0.2
Orn	94.7 ± 1.3	1.3	87.82 ± 0.05	0.1
Lys	193.2 ± 3.5	1.8	90.80 ± 0.07	0.1
His	72.4 ± 2.2	3.0	94.92 ± 0.09	0.1
Arg	48.8 ± 3.6	7.3	110.53 ± 0.13	0.1

[a] The between-run precision assays (10 different series) were performed on human plasma pool (M conc.).
Abbreviations as in Table 3.

Table 5. Linear regressions of the relation between amino acid concentrations determined with Hitachi L-8500A and Beckman 6300.[a]

Amino acid	Slope	y-intercept[b]	r	$S_{y|x}$[c]	n
Asn	0.736	+4.280	0.929	9.007	23
Trp	1.351	−14.958	0.961	6.162	21
Glu	0.872	+8.800	0.967	17.011	74
Cys	1.123	−14.083	0.968	3.636	28
Orn	0.921	+2.855	0.971	4.154	34
Phe	0.953	+2.820	0.976	4.853	50
His	1.045	+2.462	0.982	4.401	47
Tyr	0.941	+0.430	0.986	3.836	54
Ile	0.947	+0.581	0.990	4.176	57
Gln	1.073	+18.196	0.993	38.289	77
Pro	0.950	+5.762	0.993	9.645	73
Cit	1.108	+0.814	0.993	6.149	65
Arg	0.921	+1.841	0.993	6.204	72
Val	0.951	+4.508	0.995	6.975	72
Met	0.892	+1.115	0.995	2.111	61
Leu	0.952	+0.657	0.995	4.799	69
Gly	0.987	+4.654	0.996	14.133	73
Thr	0.952	+1.034	0.998	5.393	70
Ser	0.941	+4.322	0.998	6.766	75
Ala	0.983	+7.522	0.998	12.510	73
Lys	0.935	+9.145	0.999	10.077	59

[a] The assay was performed by analyzing human or rat plasma and supernatant of homogenate from rat tissue.
[b] y = Hitachi L-8500A.
[c] $S_{y|x}$ = residual standard deviation.
Abbreviations as in Table 3. Amino acids are ranked according to the r value.

less than the lower detection limit of the Beckman 6300. Also, for some samples, some AAs were not measurable with either apparatus. A fair relation was found for all the AAs analyzed (21), *r* values being >0.929. Furthermore, for a large majority of the AAs studied (15 of 21), *r* was >0.980. Asp was not included in this analysis; its measurement on the Beckman 6300 was not accurate because of its coelution with reduced glutathione when analyzing rat tissues (Coudray-Lucas et al., unpublished data).

The results of the linearity limit assays showed that the reaction was linear up to a concentration of at least 2500 μmol/L for 13 AAs: Asp, Thr, Ser, Glu, Gln, Pro, Gly, Ala, α-ABA, Cys, Ile, Leu, and Tyr. The reaction was linear up to 2000 μmol/L for Arg; 1500 μmol/L for phosphoserine, taurine, cystathionine, and Phe; 1250 μmol/L for Met and 1-methylhistidine; and 1000 μmol/L for Asn, hydroxyproline (HyPro), ornithine, Lys, His, 3MH, and carnosine. The upper limits of linearity for Val and anserine were 600 and 500 μmol/L, respectively. However, limit values were always higher than usual plasma concentrations for all AAs. The lower limit of detection was <5 μmol/L for all the AAs except for Pro and HyPro (20 μmol/L). However, we did not measure concentrations between 5 and 20 μmol/L. Further studies are therefore required for more accurate determination of the lower detection limit for these last two AAs.

Concerning sample carryover analysis, the comparison for each AA between its mean concentration in the plasma pool of low concentration, assayed after that of high concentration (L2) and (*a*) its initial concentration (L1) and (*b*) its mean concentration determined after analyzing L2 (i.e., L3), showed no statistical differences (data not shown), indicating no contamination between samples.

PHYSIOLOGICAL RANGE FOR AA CONCENTRATIONS IN HUMAN PLASMA

The results of the analysis of plasma samples from 100 healthy volunteers are presented in Table 6. These results were very close to those obtained by other HPLC systems with the same *[1, 2, 21]* or other *[4, 5, 9]* methods. However, the results for Asn and Glu concentrations obtained in the present study were moderately lower than those in the literature. This may partly be explained by the difficulty in obtaining a correct separation of these two AAs with the ion-exchange chromatography system. Furthermore, in our study, the mean concentration for Glu was specifically low, but the total of Glu plus Gln was in agreement with other studies *[4, 5, 21]*. High Glu concen-

Table 6. Amino acid concentrations in human plasma determined by HPLC.

	Ion-exchange chromatography					Reversed-phase chromatography	
	Ninhydrin				OPA	OPA	PITC
Amino acid	Present study[a] (n = 100)	Lewis et al. [1] (n = 7)	Labadarios et al. [2] (n = 10)	Cynober et al. [21] (n = 70)	Fekkes et al. [5] (n = 44)	Ziegler et al. [4] (n = 27)	Feste [9] (n = 24)
Tau	55 ± 13	–	–	90 ± 39	50 ± 11	70 ± 11	104 ± 60
Asp	3 ± 1	–	–	12 ± 5	5 ± 3	–	3 ± 2
HyPro	13 ± 10	–	–	–	–	–	–
Thr	140 ± 33	120 ± 20	125 ± 31	150 ± 33	133 ± 33	121 ± 22	–
Ser	114 ± 19	119 ± 21	130 ± 24	124 ± 29	99 ± 20	118 ± 15	–
Asn	41 ± 10	–	–	67 ± 19	58 ± 14	46 ± 7	59 ± 17
Glu	24 ± 15	–	–	72 ± 22	36 ± 12	86 ± 74	–
Gln	586 ± 84	–	–	475 ± 68	576 ± 74	493 ± 95	582 ± 94
Pro	168 ± 60	246 ± 65	141 ± 21	221 ± 61	–	–	–
Gly	230 ± 52	271 ± 39	164 ± 41	227 ± 61	202 ± 48	221 ± 41	221 ± 39
Ala	333 ± 74	452 ± 91	309 ± 82	367 ± 81	399 ± 71	340 ± 50	467 ± 123
Cit	38 ± 8	–	–	–	31 ± 7	24 ± 5	28 ± 11
α-ABA	23 ± 8	–	–	–	–	22 ± 6	–
Val	233 ± 43	239 ± 48	177 ± 37	236 ± 50	251 ± 56	232 ± 40	229 ± 74
Cys	52 ± 11	–	–	56 ± 17	–	–	–
Met	25 ± 4	–	32 ± 4	31 ± 6	27 ± 7	31 ± 5	28 ± 11
Ile	62 ± 14	66 ± 12	68 ± 18	69 ± 21	71 ± 14	65 ± 12	70 ± 35
Leu	123 ± 25	119 ± 26	138 ± 35	132 ± 34	135 ± 24	124 ± 21	145 ± 59
Tyr	59 ± 12	56 ± 11	64 ± 14	68 ± 16	63 ± 19	57 ± 13	55 ± 26
Phe	57 ± 9	56 ± 7	53 ± 7	62 ± 13	55 ± 7	54 ± 7	48 ± 18
Trp	44 ± 7	–	–	–	55 ± 10	38 ± 5	–
Orn	55 ± 16	–	–	112 ± 37	72 ± 19	50 ± 10	–
Lys	188 ± 32	–	163 ± 42	212 ± 43	171 ± 26	150 ± 27	201 ± 47
His	82 ± 10	–	52 ± 10	112 ± 20	92 ± 13	109 ± 14	62 ± 12
3MH	3 ± 2	–	–	–	7 ± 5	–	3 ± 4
Arg	80 ± 20	–	71 ± 16	87 ± 30	79 ± 24	82 ± 20	146 ± 56

[a] This study was performed with plasma samples collected from healthy volunteers after an overnight fast. Results are expressed in μmol/L, as mean ± SD. Abbreviations as in Table 3. Amino acids are ranked according to their elution time.

trations, as reported in other publications, may be due to the partial conversion of Gln, which is unstable in solution, to Glu during storage of the samples. In our study, we analyzed the samples very promptly after blood collection, so that, as expected, the concentrations of Gln we measured were high and those of Glu low.

In conclusion, the Hitachi L-8500A affords accurate results for physiological AA analysis.

This apparatus displays some further advantages:

- During the analysis program, buffer solution switching times and proportions, together with column temperature (from 30 to 70 °C in 1 °C steps), can be modified as required. Similarly, the setting up of new programs is very simple.
- The possibility of analyzing small quantities of samples with microvials. In this case, only 32 μL are necessary when using the recommended 20-μL injection option (it is possible to inject 10 μL, reducing the volume required to 22 μL).
- A refrigerated (4 ± 2 °C) sample rack (60 samples), allowing the conservation of samples in appropriate conditions.
- Two plungers for each pump (two pump heads), reducing noise and giving a very linear stable baseline, allowing an average limit of sensitivity of 10 pmol (for a signal-to-noise ratio of 3).
- A loop that leads the selected buffer to the required temperature before its run onto the analytical column, also reducing noise.
- An ammonia filter column.
- The possibility to manually treat and pack the analytical resin. After resin treatment (acetone, ethanol, 2 mol/L hydrochloric acid, and 2 mol/L lithium hydroxide), the column is automatically filled (1 h, 2×10^7 Pa). This procedure is required about every 800 runs. This prevents the exchange of the column, allowing the same accurate determination with time.
- Failures are infrequent, provided the apparatus is regularly maintained. Special attention should be paid to cleaning the column filters and changing pump seals on a monthly basis. During our 28-month experimental period, the apparatus was idle for ~42 days because of equipment failures or having to wait for materials, and 25 days were spent on maintenance. Allowing for this, ~2000 samples were analyzed per year at a cost of about $15 (US)

per analysis (excluding labor costs), similar to the cost calculated for the Beckman 6300.

References

1. Lewis AM, Waterhouse C, Jacobs LS. Whole-blood and plasma amino acid analysis: gas–liquid and cation-exchange chromatography compared. Clin Chem 1980;26:271–6.
2. Labadarios D, Shephard GS, Botha E, Jackson L, Moodie IM, Burger JA. Determination of plasma amino acids by gas-chromatography. J Chromatogr 1986;383:281–95.
3. Van Eijk HMH, Van der Heijden MAH, Van Berlo CLH, Soeters PB. Fully automated liquid-chromatographic determination of amino acids. Clin Chem 1988;34:2510–3.
4. Ziegler F, Le Boucher J, Coudray-Lucas C, Cynober L. Plasma amino acid determinations by reversed-phase HPLC: improvement of the orthophthalaldehyde method and comparison with ion-exchange chromatography. J Automat Chem 1992;14:145–9.
5. Fekkes D, Van Dalen A, Edelman M, Voskuilen A. Validation of the determination of amino acids in plasma by high-performance liquid chromatography using automated precolumn derivatization with *o*-phthaldialdehyde. J Chromatogr B 1995;669:177–86.
6. Dorresteijn RC, Berwald LG, Zomer G, de Gooijer CD, Wieten G, Beuvery EC. Determination of amino acids using *o*-phthalaldehyde-2-mercaptoethanol derivatization. Effect of reaction conditions. J Chromatogr A 1996;724:159–67.
7. Einarsson S, Josefsson B, Lagerkvist S. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. J Chromatogr 1983;282: 609–18.
8. Biggs HG, Gentilcore LJ. Liquid-chromatographic measurement of amino acids in biological samples after formation of phenylthiohydantoin derivatives. Clin Chem 1984;30:851–5.
9. Feste AS. Reversed-phase chromatography of phenylthiocarbamyl amino acid derivatives of physiological amino acids: an evaluation and comparison with analysis by ion-exchange chromatography. J Chromatogr 1992;574:23–34.
10. MacClung G, Frankenberger WT Jr. Comparison of reverse-phase high-performance liquid chromatographic methods for precolumn-derivatized amino acids. J Liq Chromatogr 1988;11:613–46.
11. Kotaniguchi H, Kawakatsu M. Automatic amino acid analysis utilizing 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole. J Chromatogr 1987;420:141–5.
12. Moller SE. Quantification of physiological amino acids by gradient ion-exchange high-performance liquid chromatography. J Chromatogr 1993;613:223–30.
13. Moore S, Spackman DH, Stein WH. Chromatography of amino acids on sulfonated polystyrene resins. Anal Chem 1958;30: 1185–90.
14. Cynober L, Coudray-Lucas C, Ziegler F, Giboudeau J. High performance ion-exchange chromatography of amino-acids in biological fluids using Chromakon 500—performance of the apparatus. J Automat Chem 1985;7:201–3.
15. Lee PLY, Slocum RH. A high-resolution method for amino acid analysis of physiological fluids containing mixed disulfides. Clin Chem 1988;34:719–23.
16. Grecos GP, Abbott WC, Schiller WR, Long CL, Birkhan RH, Blakemore WS. The effect of major thermal injury and carbohydrate-free intake on serum triglycerides, insulin and 3-methylhistidine excretion. Ann Surg 1984;200:632–7.
17. Naudin C, Vassault A, Truchard A. Protocole d'étude de la contamination dans les analyseurs biochimiques. Inf Sci Biol 1984;10:373–5.
18. Cynober L, Blondé F, Nguyen Dinh F, Gerbet D, Giboudeau J. Mesure des acides aminés plasmatiques et urinaires par chromatographie en phase gazeuse chez des sujets sains. Variations en fonction de l'age et du sexe. Ann Biol Clin 1983;41:33–8.
19. Castets JC, Parvy P, Huang Y. Identification et dosage rapide des amino acides dans les liquides biologiques et les tissus. Ann Biol Clin 1981;39:45–7.
20. Yamamoto S, Kiyama S, Watanabe Y, Makita M. Practical gas-liquid chromatographic method for the determination of amino acids in human serum. J Chromatogr 1982;233:39–50.
21. Cynober L, Ziegler F, Coudray-Lucas C, Chauffert M, Giboudeau J. Comparaison des méthodes de dosage d'acides aminés par chromatographie en phase gazeuse et par chromatographie d'échange d'ions. Ann Biol Clin 1987;45:537–40.