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Reversed-phase chromatography of phenylthiocarbamyl amino acid derivatives of physiological amino acids: an evaluation and a comparison with analysis by ion-exchange chromatography

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ABSTRACT

Reversed-phase chromatography of phenylthiocarbamyl (PTC) amino acid derivatives of physiological amino acids was evaluated and compared with the traditional method of ion exchange. The PTC amino acid derivatives were stable for at least 32 h at ambient temperature before injection. The relationship of detector response to concentration for the PTC derivatives was linear from 39 to 1250 pmol. With few exceptions, the within- and between-run precisions of plasma amino acid retention times were less than 0.2 and 0.3%, respectively; the within- and between-run precisions of their concentrations were less than 4.0 and 5.0%, respectively. Twenty-four plasma samples were quantitated by both reversed-phase and ion-exchange chromatography; fifteen of the twenty amino acids determined had correlation coefficients in the range 0.81–1.00. Nine non-standard amino acids and ten therapeutic drugs were added to plasma; D-glucosaminic acid and α-amino-β-guanidinopropionic acid co-eluted with α-aminoadipic acid and threonine, respectively. Of the ten drugs added, only metronidazole and theophylline co-eluted with β-alanine and histidine, respectively. The precision, stability, and sensitivity of the method render it ideal for the quantitation of plasma amino acids.

INTRODUCTION

Traditionally, amino acid analyses of protein hydrolysates and physiological fluids have been performed according to the method of Moore *et al.* [1]. The free amino acids are separated by cation-exchange chromatography and detected photometrically after post-column reaction with ninhydrin. Enhanced sensitivity of post-column detection has been achieved by fluorescence detection of thio-substituted isoindole derivatives formed by the reaction of the amino acids with *o*-phthalaldehyde (OPA) [2–4]. The amino acids proline and hydroxyproline only react with OPA after oxidation with hypochlorite [2–4].

During the last several years, high-performance liquid chromatography (HPLC) has been used for amino acid analysis of both protein hydrolysates and physiological fluids. Many of the methods involve reversed-phase chromatography of amino acid derivatives. One promising derivatization scheme involved the pre-column derivatization of amino acids with OPA [5–11]. The main disadvantages of the method include derivative instability and the inability of OPA to react with proline and hydroxyproline without prior oxidation. Other pre-column derivatization schemes have used dimethylaminoazobenzene isothiocyanate (DABITC) [12–14], 4-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS-Cl) [15–17], 5-dimethylnaphthalene-1-sulfonyl chloride (DNS-Cl) [18,19], 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (FNBOD) [20], and 9-fluorenylmethoxycarbonyl chloride (FMOC) [21–24] to derivatize amino acids before reversed-phase chromatography. The FMOC, DABS-Cl, DABITC, and DNS-Cl derivatizations produce multiple reaction products [13–15,17–19]. The

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FNBOD amino acid derivatives were photosensitive, especially the tyrosine derivative which was completely destroyed [20].

The Edman reagent, phenylisothiocyanate (PITC), has been used to derivatize amino acids before reversed-phase chromatography [25–30]. Depending upon the conditions used, either the phenylthiocarbamyl (PTC) or the phenylthiohydantoin (PTH) derivative can be formed. The PTH derivative has been used to analyze amino acids in plasma and urine [25]. The first application using the PTC derivative involved carboxyl-terminal amino acid analysis of the isozymes of cytochrome P-450 [26]. Subsequently, the PTC amino acid derivatization scheme was used to analyze amino acids of protein hydrolysates [27–29] and plasma [30]. The objective of the present investigation was to evaluate the reversed-phase method [30] as it applies to the amino acid analysis of physiological fluids and, in particular, plasma.

EXPERIMENTAL

Materials

The physiological A/N and B amino acid calibration standards (in 0.1 *M* HCl), phenylisothiocyanate (10 × 1.0 ml), β-mercaptoethanol, Brij 35, 2-aminohexanoic acid, OPA, and glutamine were purchased from Pierce (Rockford, IL, USA). Triethylamine (99+ %, Gold Label) was purchased from Aldrich (Milwaukee, WI, USA). Potassium tetraborate, sodium acetate (HPLC grade, trihydrate), potassium hydroxide, lithium hydroxide, and methanol (HPLC grade) were purchased from Fisher Scientific (Houston, TX, USA). The amino acids D-glucosaminic acid, α-amino-β-guanidinopropionic acid, L-methionine sulfone, 3-nitrotyrosine, S-B-4 pyridylethyl penicillamine, S-B-4 pyridylethyl cysteine, L-kynurenine, L-argininic acid, L-methionine sulfoxide, 7-methyl-DL-tryptophan, and 5-sulfosalicylic acid were purchased from Sigma (St. Louis, MO, USA). Heparin (I.V. grade) was purchased from Invenex Labs. (Melrose Park, IL, USA). Cimetidine · HCl (I.V. grade) was purchased from SK & F Labs. (Carolina, PR, USA). Doxapram · HCl (I.V. grade) was from A. H. Robbins (Richmond, VA, USA). Metronidazole (Flagyl; I.V. grade) was purchased from Searle (Chicago, IL, USA). The amino acid ion-exchange and guard columns, pre-column filter, sample diluent and lithium eluents (buffer A: 0.24 *M* Li^+, pH 2.75; buffer B: 0.64 *M* Li^+, pH 7.50; eluent C: 0.3 *M* Li^+ regenerant) were from Pickering Labs. (Mountain View, CA, USA). The Pico-Tag physiological free amino acid analysis column, pre-column filter, PLGC ultrafiltration membranes, ultrafiltration devices, reaction vials, samples diluent, the pharmaceutical-grade drugs (theophylline, dilantin, phenobarbital, gentamicin, procainamide, N-acetylprocainamide) and the eluents 1 and 2 were provided by Waters Assoc. (Milford, MA, USA).

Methods

Reversed-phase chromatography. The reversed-phase analysis system for physiological amino acids was comprised of a Digital Professional 380 computer, a Digital LA50 printer, a system interface module, two Model 510 pumps, a Model 710B WISP autosampler, a temperature control module, and a Model 440 absorbance detector (all the above instruments were provided for this study by Waters Assoc.).

The PTC amino acid derivatives were separated on a C_{18} reversed-phase column (300 mm × 3.9 mm I.D.). The column temperature was 46°C, and the separation employed a binary gradient at a flow-rate of 1.0 ml/min [30]. The chromatographic system was controlled by Waters TM software. The PTC amino acid derivatives were detected by their absorbance at 254 nm. Internal standard analysis was used as the method of quantitation, which was based on peak heights. The separation required 68 min and an additional 20 min was required for equilibration of the column with eluent 1. Volumes of 10 μl were injected for standards and 20 μl for plasma or urine samples. Both volumes contained 500 pmol of each internal standard.

Ion-exchange chromatography. Samples were injected by a Model 710B WISP autosampler, and the underivatized amino acids were separated on a Pickering analytical amino acid analysis column (150 mm × 3 mm I.D., 5 μm). A temperature control module was used to maintain the column temperature at 46°C. The analytical col-

umn was protected by a pre-column filter and a Pickering amino acid guard column (20 mm × 2 mm I.D., 5 μm). A ternary gradient elution program, provided by Waters, was used to separate the amino acids; a constant flow-rate of 0.3 ml/min was used to effect the separation.

Immediately after the separation, the amino acids went directly into a heated (46°C) reaction coil. OPA and hypochlorite solutions, both in 0.4 *M* sodium borate buffer, pH 10.4, were delivered into the coil (Eldex pumps) at a rate of 0.4 ml/min. The isothioindole amino acid derivatives were detected by fluorescence with a Model 420 fluorescence detector (Waters Assoc.). The 10-μl injection volume used for both calibration standards and samples contained 500 pmol of internal standard. Peak height was used for quantitation.

For the chromatographic comparison experiment, both the reversed-phase and ion-exchange systems were controlled by the Digital Model 380 professional computer. Communication with the two systems was established by two separate system interface modules. The multi-system software required to operate the systems simultaneously was provided by Waters Assoc.

Internal standard preparation

Reversed-phase chromatography. Methionine sulfone was used as the internal standard. The solution to be added to plasma and urine was prepared by adding 160 μl of each internal standard (5.0 μmol/ml in 0.1 *M* HCl) to 3.68 ml of 0.1 *M* HCl. The final concentration of each internal standard was 0.2 μmol/ml.

Ion-exchange chromatography. 2-Aminohexanoic acid, D-glucosaminic acid, and α-amino-β-guanidinopropionic acid were used as internal standards, although only 2-aminohexanoic acid was used to adjust for recovery. The solution used to dilute plasma 1:1 before ultrafiltration was prepared by adding 40 μl of each internal standard solution (5.0 μmol/ml in 0.1 *M* HCl) to 820 μl of 0.1 *M* HCl. The final concentration of each standard was 0.2 μmol/ml.

Calibration standard preparation

Reversed-phase chromatography. Equal parts of the A/N and B standards (in 0.1 *M* HCl) were mixed and 100-μl aliquots were placed in polypropylene tubes with caps and stored in a Revco freezer (Charlotte, NC, USA) at −80°C. The calibration standards were prepared by adding 25 μl of the internal standard solution (5.0 μmol/ml in 0.1 *M* HCl), 25 μl of glutamine (5.0 μmol/ml in water), and 75 μl of water to the 1:1 diluted A/N and B standards in the polypropylene tubes. After mixing well, 10-μl aliquots were placed into reaction vials and stored in the Revco at −80°C until needed.

Ion-exchange chromatography. Equal parts of the A/N and B standards (in 0.1 *M* HCl) were mixed, and 100-μl aliquots were placed in polypropylene tubes with caps and stored in the Revco at −80°C. The ion-exchange calibration standards were prepared by adding 25 μl of each internal standard, 25 μl of glutamine (5.0 μmol/ml in water), and 2.5 ml of 3.75% 5-sulfosalicylic acid (adjusted to pH 2.2 with LiOH) to the 100-μl 1:1 diluted aliquots of the A/N and B standards.

Ultrafiltration

Blood was collected from 24 male volunteers before and after commercially available amino acid supplements were administered. The blood was collected by venipuncture into tubes containing potassium EDTA as the anticoagulant. The plasma was obtained by centrifuging the blood samples at 1500 *g* for 15 min at 4°C. For both reversed-phase and ion-exchange analysis, 100 μl of plasma were diluted 1:1 with the respective 0.2 μmol/l internal standard solution [31]. After mixing, 200 μl of diluted plasma were placed into the ultrafiltration device directly onto the PLGC membrane. The samples were spun in a fixed-angle centrifuge at 1500 *g* for 15 min at ambient temperature. After centrifugation, 25-μl aliquots of the ultrafiltrate were placed into reaction vials. The samples to be analyzed by reversed-phase and ion-exchange analysis were dried and thus prepared for processing. For ion-exchange analysis, the sample was reconstituted in 100 μl of 3.75% sulfosalicylic acid (adjusted to pH 2.20 with LiOH). The reconstituted sample was then filtered with a Millipore HV4 filter (4 mm diameter, 0.45 μm; Bedford, MA, USA). After drying, the samples to undergo reversed-phase analysis were derivatized.

Collection and preparation of urine samples

Human urine was collected from 24 male volunteers for 24 h. Twenty 100-μl aliquots were placed into capped tubes and stored in the Revco at −80°C. The urine samples were diluted 1:1 with the internal standard solution, and 25 μl of the diluted urine were placed into reaction vials. On two consecutive days, ten urine samples were derivatized and separated.

PITC derivatization

Standards (10 μl), plasma ultrafiltrate (25 μl), and 1:1 diluted urine (25 μl) were placed into reaction vials and dried in the work station. A 10-μl volume of the redry solution (methanol–1 *M* sodium acetate–triethylamine, 2:2:1, v/v), was added to each vial and the contents were mixed well [31]. The samples were dried and 20 μl of the PITC reagent (methanol–water–triethylamine–PITC, 7:1:1:1, v/v) were added, mixed well, and allowed to react for 20 min at ambient temperature [31]. After 20 min, the unreacted PITC was removed by evaporation. When the samples were dry, 100 μl of the sample diluent were added and the contents of the vial mixed [31]. A 10-μl volume of calibration standard and 20 μl of plasma ultrafiltrate and diluted urine were injected; these volumes resulted in the injection of 500 pmol of internal standard.

Concentration versus detector response

A/N and B physiological amino acid standards were mixed 1:1. The 1:1 mixture was then serially diluted (with 0.1 *M* HCl) resulting in dilutions of 1:3, 1:7, 1:15, 1:31 and 1:63. Of each mixture, 10 μl were derivatized and separated. Duplicate injections were performed on each dilution on two separate days ($n = 4$).

Ambient temperature stability of PTC derivatives

The A/N and B calibration standards were prepared as described above. A calibration standard solution (220 μl) was dried, redried, and derivatized. The PTC amino acid derivatives were reconstituted in 2.2 ml of the sample diluent, and 22 100-μl aliquots were placed into sample insert vials. All of the vials were placed into the autosampler. The last sample was separated after being in the autosampler for 32 h at ambient temperature.

Interference studies

Amino acids. The concentration of the stock solutions of amino acids (nine total) to be added to the calibration standards were 5.0 mmol/l (in 0.1 *M* HCl). Before they were added, the stock solutions were diluted 1:9 with 0.1 *M* HCl and each amino acid was derivatized and separated individually to determine retention times.

Once the retention times were identified, a 10-μl aliquot of a different 1:9 dilution was placed into one of ten different reaction vials. Subsequently, 10-μl aliquots of the calibration standard were placed into each vial. The end result was the presence of a single spiked amino acid in the calibration standard. The samples were then dried, redried, and derivatized. A 10-μl injection contained 500 pmol of each spiked amino acid. This experiment was performed on two separate days (ten each day).

Drugs. Before adding the drugs to plasma, each drug was derivatized and separated individually to identify retention times. The final concentration of the 20-μl aliquot of each drug, whether separated individually or after its addition to plasma, represented its therapeutic concentration in plasma.

Stock solutions of 1.12, 1.20, 1.44 and 1.04 g/l (in 0.1 *M* HCl) were made of theophylline, dilantin, phenobarbital, and N-acetylprocainamide, respectively. The dilantin and phenobarbital stock solutions required 5% methanol for dissolution.

The stock solutions were diluted 1:19 with 0.1 *M* HCl. Stock solutions of 6.4 and 5.6 g/l were made of gentamicin and procainamide. The stock solutions were subsequently diluted 1:199 with 0.1 *M* HCl. Concentrations were provided as follows: heparin, 100 000 U/l; cimetidine · HCl, 150 g/l; doxapram · HCl, 20 g/l; and metronidazole, 100 g/l. These solutions were diluted with 0.1 *M* HCl as follows: heparin 1:7.4; cimetidine · HCl, 1:665; doxapram · HCl, 1:249; and metronidazole, 1:1139. Once the diluted stock solutions were prepared, 25 μl of a single drug solution were placed into ten different reaction vials. The individual drugs were derivatized and separated as previously described.

To determine whether any of these drugs interfered with reversed-phase analysis of plasma

amino acids, single drugs were added to ten identical plasma samples. Only one drug was found in each plasma sample. The concentrations of the stock solutions were the same as those used to identify individual retention times. Stock solutions of the drugs were diluted with 0.1 *M* HCl as follows: theophylline, 1:4, dilantin, 1:4, phenobarbital, 1:4; gentamicin, 1:49; procainamide, 1:49; N-acetylprocainamide, 1:4; heparin, 1:1.5; cimetidine · HCl, 1:116; doxapram · HCl, 1:62.5; and metronidazole, 1:284. The concentration of the internal standard solution was 0.4 mmol/l instead of 0.2 mmol/l. A combination internal standard–drug solution was made for each drug by mixing equal amounts of the internal standard solution and the diluted drug solutions. An aliquot (100 μl) of the internal standard–drug solution was added to 100 μl of human plasma (× 10). The final concentrations of the drugs in plasma were the same as those of the drugs used to identify the retention times. The plasma–drug samples were subjected to ultrafiltration and 25-μl aliquots were derivatized, as previously described. A 20-μl aliquot which represented the therapeutic concentration of each drug was injected and separated. This plasma–drug spike experiment was repeated on a different day.

RESULTS

Chromatographic analysis of serial dilutions demonstrated a linear relationship ($r^2 = 1.0$) between the detector response (254 nm) and a 1000-fold difference in the concentration of 36 standard amino acids (data not shown). The data presented in Table I depict the effect of concentration on the within-run and between-run precision of PTC amino acid peak heights. The mean coefficient of variation (C.V.) represents the average error of all of the amino acids quantitated at each dilution. The ambient temperature stability of the PTC amino acid derivatives (data not shown) was evaluated by determining the error (C.V.) for each of 35 amino acids after 22 consecutive runs (0–32 h in WISP). Thirty-two PTC amino acid derivatives had peak-height errors that ranged from 1.09 to 5.45%. The PTC derivatives of phosphoethanolamine, sarcosine, and Val had errors of 8.64, 42.7, and 8.36%, respectively.

TABLE I

EFFECT OF CONCENTRATION ON WITHIN- AND BETWEEN-RUN PRECISION OF PEAK HEIGHTS OF PTC AMINO ACID DERIVATIVES

The data represented in this table were used to determine the within- and between-run precision of the peak-height determination of each amino acid for each dilution. The within-run precision was determined from duplicate injections at each dilution, while the between-run precision was determined from duplicate injections at each dilution on two separate days.

Dilution	Concentration range (pmol)	C.V. (mean ± S.D.) (%)
Within-run precision		
1:63	9.75–39.0	6.09 ± 9.33
1:31	19.5–78.0	4.38 ± 5.47
1:15	39.0–156	3.15 ± 1.67
1:7	78.0–312	1.44 ± 1.37
1:3	156–625	2.24 ± 1.70
1:1	312–1250	1.38 ± 1.68
Between-run precision		
1:63	9.75–39.0	17.9 ± 12.5
1:31	19.5–78.0	9.81 ± 5.56
1:15	39.0–156	6.96 ± 3.00
1:7	78.0–312	5.94 ± 2.67
1:3	156–625	3.09 ± 2.10
1:1	312–1250	4.7 ± 3.50

The within-run precisions of the retention times of standard ($n = 10$, data not shown, 35 amino acids), plasma ($n = 10$, Table II) and urinary amino acids ($n = 10$, Table III) were all under 0.85%. The between-run precisions for standard ($n = 55$, data not shown), plasma ($n = 20$, Table II), and urinary amino acid retention times ($n = 20$, Table III) were all under 2.07%. The within-run ($n = 10$) and between-run precisions ($n = 55$, 35 amino acids, 125–500 pmol) of standard amino acid peak heights were all under 4.90 and 6.02%, respectively (data not shown). The within-run range of precision for plasma amino acid concentrations was 1.59–17.1% ($n = 10$, Table II) and for urinary amino acid concentrations was 3.94–115% ($n = 10$, Table III). The plasma amino acids phosphoserine, Asp, and β-alanine had within-run errors of 9.86, 17.1 and 6.01%, respectively; all of their concentrations were under 10.0 μmol/l. The urinary amino acids demonstrated greater error; Asn, Thr, Ala, Pro, α-amino-*n*-butyric acid, Val, Met, Ile, Phe, Trp, and

TABLE II

WITHIN- AND BETWEEN-RUN PRECISION OF RETENTION TIMES AND CONCENTRATIONS OF PLASMA PTC AMINO ACID DERIVATIVES

Blood was drawn and plasma prepared as described in the Experimental section. Twenty 100-μl aliquots were placed into capped tubes and stored in a Revco at −80°C. On two consecutive days, ten plasmas were deproteinized, derivatized and separated as described in the Experimental section. The mean C.V. depicted represent the average C.V. of all the amino acids measured at each dilution.

Amino acid	Within-run (n = 10)				Between-run (n = 20)			
	Concentration (μmol)		Retention time (min)		Concentration (μmol/l)		Retention time (min)	
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
Pse	3.88	9.8	3.26	0.35	4.30	17.8	3.26	0.30
Asp	4.27	17.1	3.64	0.27	4.31	15.3	3.64	0.32
Glu	47.6	4.58	4.24	0.22	45.1	8.22	4.24	0.22
Hyp	13.9	3.91	7.42	0.18	14.0	4.33	7.44	0.23
Ser	111	2.75	9.47	0.18	109	3.20	9.50	0.26
Asn	64.9	2.39	10.0	0.16	64.3	2.35	10.0	0.23
Gly	242	2.71	10.6	0.17	239	2.59	10.7	0.23
Gln	641	1.67	11.8	0.15	632	2.38	11.8	0.23
βAla	9.93	6.01	13.6	0.00	4.96	103	13.4	1.64
Tau	191	2.42	15.2	0.16	183	5.42	15.2	0.37
His	74.8	2.58	17.4	0.15	74.2	3.14	17.5	0.25
Thr	115	2.66	20.8	0.17	106	11.4	20.9	0.36
Ala	469	3.19	21.9	0.14	460	3.59	22.0	0.20
Arg	204	3.37	25.0	0.19	203	1.97	25.1	0.21
Pro	213	1.90	28.6	0.16	213	1.96	28.7	0.21
Abu	30.2	2.01	36.2	0.12	30.4	3.26	36.2	0.19
Tyr	65.0	3.22	43.5	0.06	64.8	4.61	43.6	0.10
Met	33.0	4.08	48.1	0.07	35.8	10.1	48.2	0.12
CysCys	141	8.70	52.9	0.11	160	14.5	53.0	0.26
Ile	70.5	5.18	54.2	0.07	71.8	5.69	54.3	0.10
Leu	152	2.17	54.9	0.07	151	3.44	55.0	0.11
Phe	42.6	3.41	58.5	0.09	42.6	3.45	58.3	0.51
Trp	59.9	1.86	59.5	0.10	59.6	3.54	59.2	0.67
Orn	72.1	3.09	60.0	0.11	72.8	3.32	59.9	0.31
Lys	273	1.59	65.2	0.16	264	5.59	65.4	0.28

Orn had within-run errors that ranged from 8.12 tot 115%.

The between-run precision ranges of plasma amino acid concentrations were 1.9–103% (n = 20, Table II); those of urinary amino acid concentrations were 5.69–66.1% (n = 20, Table III). The between-run precision of the plasma amino acid concentrations for phosphoserine, Asp, Glu, β-alanine, Thr, and Met had errors that ranged from 8.22 to 103%. The other eighteen plasma amino acids had errors of less than 6.0% (Table II). Of the 22 urinary amino acids quantitated, seven had between-run errors that ranged from 5.69 to 10.0%. The amino acids ILe, Met, Orn, α-amino-n-butyric acid, Thr, and Pro had between-run errors that ranged from 31.6 to 129%. All of these urinary amino acids, however, were present in concentrations lower than 11.0 μmol/l. The urinary amino acids Glu, Asn, and Phe had errors of 16.3, 16.9 and 18.0%, respectively. The amino acids Val, Lys, Leu, and Trp had errors that ranged from 12.5 to 14.9%. Ser and Gly had errors of 10.8 and 11.5%, respectively.

Analysis of the plasma amino acids from the human subjects is shown in Table IV. Five amino acids (citrulline, Asp, 3-methyl-L-histidine, Met and His) had correlation coefficients (r) that ranged from of 0.09 to 0.70. Of the twenty amino

TABLE III

WITHIN- AND BETWEEN-RUN PRECISION OF RETENTION TIMES AND CONCENTRATIONS OF URINARY PTC-AMINO ACID DERIVATIVES

Amino acid	Within-run (n = 10)				Between-run (n = 20)			
	Concentration (μmol/l)		Retention time (min)		Concentration (μmol/l)		Retention time (min)	
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
Aad	41.5	5.57	6.32	0.23	40.8	8.16	6.24	1.28
Ser	84.7	6.27	9.85	0.15	78.3	10.8	9.70	1.55
Asn	41.5	12.0	10.4	0.85	37.9	16.9	10.3	1.57
Gly	340	5.08	11.1	0.13	313	11.5	10.9	1.44
Gln	117	4.70	12.3	0.18	106	16.3	12.1	1.67
Tau	283	4.84	15.9	0.19	268	8.61	15.6	1.97
His	227	5.08	18.2	0.26	221	6.44	17.9	2.09
Thr	20.6	8.12	21.8	0.18	10.3	103	21.4	1.58
Ala	48.6	8.50	22.8	0.20	50.9	8.43	22.5	1.35
Pro	3.06	56.3	29.0	0.64	1.53	129	28.9	0.66
1MHis	74.0	4.03	30.3	0.16	72.3	5.69	30.0	1.19
3MHis	110	3.96	31.2	0.13	109	5.95	30.9	0.93
Abu	7.54	9.21	37.0	0.12	3.77	103	36.7	1.00
Tyr	14.0	7.90	44.1	0.06	14.4	7.61	43.9	0.53
Val	11.2	14.6	47.1	0.05	10.7	12.5	46.9	0.47
Met	4.56	91.0	48.6	0.08	5.36	61.4	48.3	0.78
AlaHcy	96.3	4.20	51.7	0.09	111	15.1	51.2	0.93
Ile	11.1	10.0	54.7	0.07	8.73	31.6	54.2	0.92
Leu	16.9	7.26	55.4	0.07	15.1	14.2	55.2	0.37
Phe	13.7	10.1	59.2	0.08	11.9	18.0	58.9	0.54
Trp	8.48	18.6	60.2	0.09	9.15	14.9	59.9	0.60
Orn	2.98	115	60.8	0.09	3.93	66.1	60.4	0.68
Lys	38.6	5.95	66.3	0.18	35.7	13.6	65.8	0.85

acids quantitated by both methods, 75% had values greater than 0.81. Seven (35%) of the amino acids compared had values greater than 0.93.

The data depicted in Table V represent the mean (± S.D.) concentrations of the amino acids described in Table IV. The mean (± S.D.) concentration (n = 24) as determined by both methods were similar and compared well (r = 0.996; graph not shown). To assess interferences, nine non-calibration standard amino acids and ten therapeutic drugs were analyzed. The data in Table VI show the retention times of the nine amino acids. When run individually, the amino acids L-argininic acid, kynurenine, and 7-methyl-DL-tryptophan were not seen on chromatograms. The only amino acids that interfered were D-glucosaminic acid and α-amino-β-guanidinopropionic acid which co-eluted with α-aminoadipic acid and Thr, respectively (Table VI, Fig. 1). The other four amino acids were observed on chromatograms, but did not co-elute with any of the standard amino acids. Ten drugs were separated individually and subsequently added separately to plasma samples. Dilantin, gentamicin, cimetidine, and doxapram were not observed on the chromatogram when run individually. Metronidazole and theophylline co-eluted with the plasma amino acids β-alanine and His, respectively. The other four drugs, N-acetylprocainamide, heparin, phenobarbital, and procainamide, were observed on the chromatograms but did not co-elute with any plasma amino acids (Table VII, Fig. 2).

DISCUSSION

The reversed-phase chromatographic method enabled sensitive, rapid, and precise quantitation

TABLE IV

CORRELATION OF REVERSED-PHASE AND ION-EXCHANGE ANALYSIS OF PLASMA AMINO ACIDS

Amino acid	Range (μmol/l)	Regression equation	r
Cit	20–100	$y = 30.6 - 0.02x$	0.09
Asp	3–10	$y = 4.81 - 0.15x$	0.14
3MHis	3–10	$y = 10.8 + 2.91x$	0.24
Met	10–60	$y = 16.0 + 0.4x$	0.58
His	40–130	$y = 29.0 + 0.43x$	0.70
Phe	15–90	$y = 18.0 + 0.71x$	0.81
Tyr	20–150	$y = 18.4 + 0.91x$	0.81
Asn	45–60	$y = 12.4 + 0.84x$	0.86
Abu	8–50	$y = 0.73 + 1.19x$	0.86
Gly	120–130	$y = 39.5 + 0.85x$	0.87
βAla	3–20	$y = 3.87 + 0.79x$	0.87
1MHis	2–35	$y = 0.16 + 1.17x$	0.88
Gln	400–750	$y = 112 + 0.84x$	0.88
Lys	110–450	$y = 82.1 + 0.58x$	0.93
Ala	220–900	$y = 100 + 0.74x$	0.93
Val	110–400	$y = 7.49 + 0.98x$	0.97
Ile	25–200	$y = 3.67 + 1.06x$	0.98
Leu	50–250	$y = 4.05 + 1.09x$	0.98
Arg	80–300	$y = 0.19 + 1.05x$	0.99
Tau	40–250	$y = 3.02 + 1.01x$	1.00

of physiologic amino acids. Although precision studies were conducted on plasma and urinary amino acid retention times and concentrations, the present study addresses primarily the quantitation of plasma amino acids. The detector response to differences greater than 100-fold in amino acid concentrations was linear. Nevertheless, the data depicted in Table I demonstrate the effect of concentration on precision. As one would expect, both within-run and between-run errors of peak height decreased as the concentration increased. An extemely important feature of the reversed-phase method is the stability of PTC amino acid derivatives at ambient temperature. With the exception of sarcosine, Val, and phosphoethanolamine, all of the PTC amino acid derivatives were stable for up to 32 h at room temperature. In contrast, pre-column OPA-derived amino acids were unstable: derivatives must be analyzed within 2–5 min after they are formed [2–5]. Furthermore, as opposed to FNBOD derivatives, the PTC amino acids were not photosensitive.

The effectiveness of the reversed-phase method was demonstrated by the precision of both within-run and between-run retention times and concentrations. Good precision was found for within-run retention times of standard, plasma, and urinary amino acids. With the exception of urinary Asn and Pro (Table III), the error for the remaining amino acids was less than 0.26%. In addition, the error of plasma L-anserine and Pro retention times was 0.16%. With the exception of plasma β-alanine, the between-run precision of plasma amino acid retention times was much better than that for urinary amino acids.

The within- and between-run precisions for plasma amino acid concentrations were good. The point illustrated in Table I, *i.e.*, the dependence of error on amino acid concentration, was further demonstrated by the within- and between-run precisions of plasma and urinary amino acid concentrations. In regard to the within-run error of plasma amino acids, all except phosphoserine, Asp, and β-alanine had errors between 1.59 and 5.18%. Phosphoserine, Asp, and β-alanine had errors of 9.86, 17.1, and 6.01%, respectively; most importantly, however, all their concentrations were lower than 10.0 μmol/l. The between-run error of the plasma amino acid concentrations reflected the results obtained for within-run precision. As observed in Table I, the error was greater for between- than within-run precision.

The precision data obtained for urinary amino acids demonstrated greater error. This result may have been the consequence of the sample matrix or of the concentrations of the urinary amino acids, most of which were lower than those of their plasma counterparts. The urinary amino acids Orn, Pro, and Met had within-run errors of 115, 56.3 and 91.0%, respectively; they were present in concentrations less than 5.0 μmol/l. Of the 22 urinary amino acids measured, eleven had within-run errors less then 8.0%. Only seven urinary amino acids had between-run precision errors less than 10%. Six of the 22 amino acids (Ile, Met, Orn, α-amino-*n*-butyric acid, Thr and Pro) had errors from 31.6 to 129%. Concomitantly, their concentrations were less than 10.0 μmol/l.

The data depicted in Tables IV and V demonstrate the excellent correlations obtained between reversed-phase and ion-exchange quantitation of

TABLE V

DETERMINATION OF PLASMA AMINO ACIDS BY REVERSED-PHASE AND ION-EXCHANGE METHODS

Twenty-four human plasma samples were prepared (in duplicate) for and quantitated by reversed-phase and ion-exchange analysis as described in the Experimental section. The data represent the mean concentration of each amino acid for all the samples quantitated. Under "*n*", the first number represents the number of samples used to calculate the mean concentrations, and the second number represents the number of injections.

Amino acid	Reversed-phase		Ion-exchange	
	n	Concentration (mean ± S.D.) (μmol/l)	*n*	Concentration (mean ± S.D.) (μmol/l)
Cit	23–46	28.3 ± 11.2	24–46	17.6 ± 29.7
Asp	24–48	3.47 ± 1.78	24–46	7.76 ± 2.88
3MHis	24–48	2.56 ± 3.94	24–46	7.65 ± 12.3
Met	24–48	27.6 ± 11.0	24–45	29.7 ± 14.7
His	24–48	62.0 ± 12.3	24–46	81.3 ± 24.2
Phe	21–42	47.6 ± 18.0	24–46	43.1 ± 17.8
Tyr	24–48	54.6 ± 26.2	24–45	39.0 ± 23.1
Asn	24–48	59.1 ± 16.6	6–10	54.2 ± 3.89
Abu	24–48	21.4 ± 26.2	24–46	15.1 ± 8.05
Gly	24–48	221 ± 39.0	24–46	215 ± 37.7
βAla	24–47	9.73 ± 4.02	24–46	6.68 ± 4.45
1MHis	24–48	9.73 ± 9.54	24–46	8.20 ± 7.19
Gln	24–48	582 ± 94.1	24–46	549 ± 95.4
Lys	24–48	201 ± 47.0	24–46	205 ± 76.5
Ala	24–48	467 ± 123	24–46	492 ± 147
Val	24–48	229 ± 74.0	24–46	218 ± 68.1
Ile	24–48	69.7 ± 35.2	24–45	68.1 ± 33.3
Leu	24–48	145 ± 59.4	24–45	132 ± 48.9
Arg	24–48	146 ± 56.4	24–44	143 ± 50.8
Tau	24–48	104 ± 60.2	24–46	102 ± 57.2

TABLE VI

RETENTION TIMES OF NON-CALIBRATION STANDARD AMINO ACIDS AS DETERMINED BY THE REVERSED-PHASE METHOD

The amino acids listed were added to the calibration standards, derivatized, and separated as described in the Experimental section. Abbreviations: DGAA = D-glucosaminic acid; AAGPA = α-amino-β-guanidinopropionic acid; S-B(4EP)cys = S-B-4 pyridylethyl cysteine; 3NO2Tyr = 3-nitrotyrosine; Ahx = 2-aminohexanoic acid; S-B(4PE)PA = S-B-4 pyridylethyl penicillamine; LAA = L-argininic acid; Kyn = kyrnurenine; 7CH3Trp = 7-methyltryptophan. The symbol – indicates that the cited amino acid was not observed on the chromatogram when analyzed individually.

Amino acid	Retention time (mean ± S.D.) (min)	Peak height (mean ± S.D.)	Elution position
DGAA	5.41 ± 0.01	38 727 ± 1975	Elutes with αAAd
AAGPA	19.7 ± 0.18	17 142 ± 1362	Elutes with Thr
S-B(4PE)cys	54.4 ± 0.00	55 189 ± 5665	Between Ile and Leu
3NO2Tyr	55.5 ± 0.01	87 889 ± 1560	After Leu
Ahx	56.0 ± 0.00	28 577 ± 1001	After 3NO2Tyr
S-B(4PE)PA	63.1 ± 0.14	14 470 ± 255	Between Orn and Lys
LAA	–	–	–
Kyn	–	–	–
7CH3Trp	–	–	–

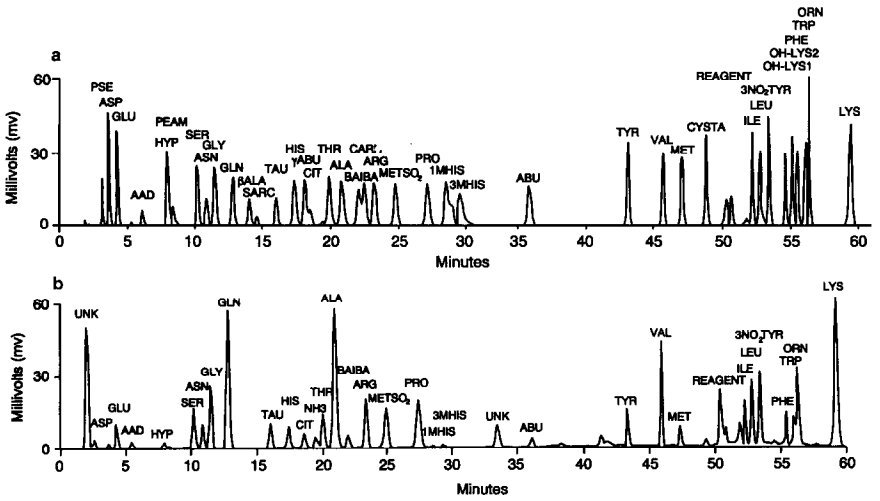

Fig. 1. (a) Reversed-phase separation of standard PTC amino acids; (b) reversed-phase separation of plasma PTC amino acid derivatives. Both standards and plasma samples were prepared, derivatized, and separated as described in the Experimental section.

physiological amino acids. All of the samples quantitated were prepared by ultrafiltration, which enabled a comparison of the chromatography only. Twenty amino acids were quantitated by both methods; 75% had correlation coefficients greater than 0.81 (Table IV), and 35% had coefficients greater than 0.93.

The 25% of the amino acids that had correlations below 0.81 included citrulline (r = 0.09), Asp (r = 0.14), 3-methyl-L-histidine (3MHis) (r = 0.24), and Met (r = 0.58). In the reversed-phase separation, citrulline and γ-aminobutyric acid eluted as a doublet peak (Fig. 1a); in the ion-exchange separation, citrulline and α-amino-n-butyric acid (Abu) eluted as a doublet peak (Fig. 2a). It is possible that the decreased resolution of the doublet peaks in both the reversed-phase (Fig. 1b) and ion-exchange (Fig. 2b) separations resulted from the low concentrations of citrulline, which could therefore have been re-

TABLE VII

RETENTION TIMES OF THERAPEUTIC DRUGS AS DETERMINED BY THE REVERSED-PHASE METHOD

The drugs listed were added to plasma samples, ultrafiltered, derivatized, and separated as described in the Experimental section. The – symbol indicates that the drug cited was not observed on the chromatogram when the drug was analyzed individually. The drugs metronidazole and theophylline co-eluted with the amino acids βAla and His, respectively. The asterisk denotes the calculated concentration of the drugs that co-eluted with the amino acids. In plasma alone, the concentrations of βAla and His were 5.0 and 65 μmol/l, respectively.

Drug	Retention time (mean ± S.D.) (min)	Peak height (mean ± S.D.)	Elution position
Metronidazole	12.4 ± 0.00	24.0* ± 2.00	Elutes with βAla
Theophylline	16.5 ± 0.01	290* ± 9.00	Elutes with His
N-Acetylprocainamide	43.6 ± 0.06	19 808 ± 329	Between Tyr and Val
Heparin	48.5 ± 0.08	17 023 ± 1430	Between Met and cystathionine
Phenobarbital	51.1 ± 0.35	5623 ± 0.00	Tailing shoulder of reagent peak
Procainamide	61.6 ± 0.26	4542 ± 1138	After Orn, before Lys
Dilantin	–	–	
Gentamicin	–	–	
Cimetidine	–	–	
Doxapram	–	–	

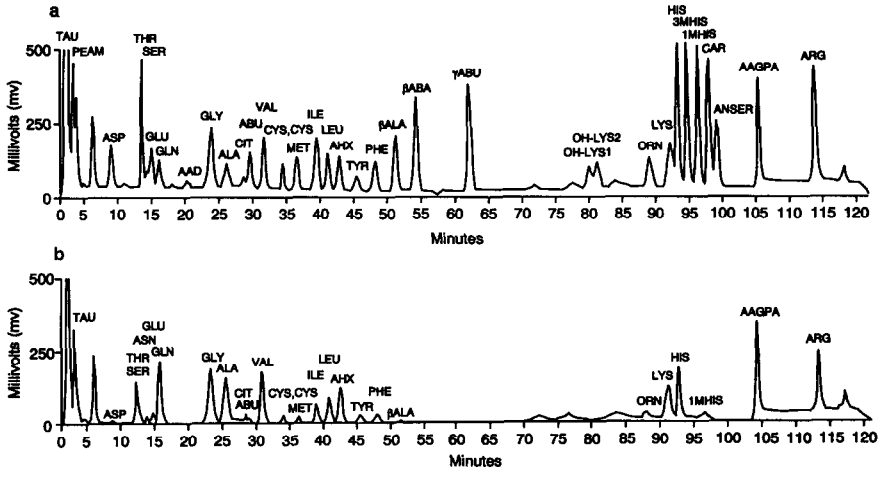

Fig. 2. Ion-exchange separations of (a) standard amino acids and (b) plasma amino acids. Standards and plasma samples were prepared and separated as described in the Experimental section.

sponsible for the low correlation coefficient between the two methods. The mean concentration of Asp was 3.47 μmol/l for the reversed-phase separations and 7.76 μmol/l for the ion-exchange separations (Table V); the mean concentration of 3MHis was 2.56 μmol/l for the reversed-phase and 7.65 μmol/l for the ion-exchange separations (Table V). These concentrations correspond to injections of 6.25–18.7 pmol. The poor correlation between the two separations of Asp and 3MHis is probably the result of the high analytical error that can occur at such low concentrations (Table I; Figs. 1b and 2b). In the case of Met, 64–74 pmol were injected for both separations and although this amount was greater than the amount of Asp and 3MHis injected, it was still at the lower end of the concentration–detector response curve. The error was less than that for Asp and 3MHis (Table V), and the correlation coefficient was correspondingly higher.

It should be noted that when the mean concentration of each amino acid for all 24 subjects was determined for each method (Table V), the correlation between methods was more apparent. The mean concentrations and concentration ranges determined by each method were similar. The correlation coefficient for the mean concentrations, determined by both methods, was 0.996.

In this comparison of reversed-phase and ion-exchange chromatography, the reversed-phase method was found to be sensitive, reliable, rapid, and precise. These qualities make it an excellent alternative to the classic ion-exchange method for the quantitation of plasma amino acids.

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REFERENCES

1 S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
2 M. Roth, *Anal. Chem.*, 43 (1971) 143.
3 Y. Ishida, T. Fujita and K. Asai, *J. Chromatogr.*, 204 (1981) 143.
4 P. A. St. John, *Aminco Lab. News*, 31 (1975) 1.
5 D. L. Hogan, K. L. Draemer and J. I. Isenberg, *Anal. Biochem.*, 127 (1982) 17.
6 M. Griffin, S. J. Price and T. Palmer, *Clin. Chim. Acta*, 125 (1982) 89.
7 D. Hill, L. Burnworth, W. Shea and R. Pfeifer, *J. Liq. Chromatogr.*, 5 (1982) 2369.
8 M. O. Fleury and D. A. Ashley, *Anal. Biochem.*, 133 (1983) 330.

9 B. N. Jones and J. P. Gilligan, *J. Chromatogr.*, 266 (1983) 471.
10 M. H. Joseph and P. Davies, *J. Chromatogr.*, 277 (1983) 125.
11 T. A. Graser, H. G. Godel, S. Albers, P. Foldi and P. Furst, *Anal. Biochem.*, 151 (1985) 142.
12 J.-Y. Chang, *Biochem. J.*, 199 (1981) 537.
13 C.-Y. Yang and S. J. Wakil, *Anal. Biochem.*, 137 (1084) 54.
14 J.-Y. Chang, E. H. Creaser and K. W. Bentley, *Biochem. J.*, 153 (1976) 607.
15 J.-K. Lin and C.-H. Wong, *Clin. Chem.*, 26 (1980) 579.
16 J.-Y. Chang, R. Knecht and D. G. Braun, *Biochem. J.*, 199 (1981) 547.
17 J.-K. Lin and J.-Y. Chang, *Anal. Chem.*, 47 (1975) 1634.
18 V. T. Wiedmeier, S. P. Posterfield and C. E. Hendrick, *J. Chromatogr.*, 231 (1982) 410.
19 C. De Jong, G. J. Hughes, E. van Wieringen and K. J. Wilson, *J. Chromatogr.*, 241 (1982) 345.
20 Y. Watanabe and K. Imai, *J. Chromatogr.*, 309 (1984) 279.
21 S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, 282 (1983) 609.
22 S. Einarsson, *J. Chromatogr.*, 348 (1985) 213.
23 I. Betner and P. Foldi, *Chromatographia*, 22 (1986) 381.
24 I. Betner and P. Foldi, *LC · GC*, 6 (1988) 812.
25 H. G. Biggs and L. J. Gentilcore, *Clin. Chem.*, 30 (1984) 851.
26 D. R. Koop, E. T. Morgan, G. E. Tarr and M. J. Coon, *J. Biol. Chem.*, 257 (1982) 8472.
27 B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
28 R. L. Heinrikson and S. C. Meridith, *Anal. Biochem.*, 136 (1984) 65.
29 J. Scholze, *J. Chromatogr.*, 350 (1985) 453.
30 S. A. Cohen, B. A. Bidlingmeyer and T. L. Tarvin, *Nature*, 320 (1986) 769.
31 S. A. Cohen, M. Meys and T. L. Tarwin, *The Pico Tag® Method — A Manual of Advanced Techniques for Amino Acid Analysis*, Millipore, Bedford, MA, WM02, 1989, Rev. 1, pp. 1–123.