Ion-exchange chromatography for physiological fluid amino acid analysis

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Introduction

In the past 10 years, reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely accepted as a preferred method for the amino acid analysis of protein hydrolysates.[1-3] In contrast, physiological samples are often so varied and complex that application of RP-HPLC to their analysis has always resulted in poor peak resolution.[4-7] Therefore, the reliability of RP-HPLC in the quantitative determination of physiological amino acids remains controversial.[2,8-14]

Analysis of physiological amino acids is traditionally carried out by ion-exchange chromatography (IEC) followed by post-column ninhydrin[15-17] or *o*-phthalaldehyde[18,19] derivatization. Instrumentation required for IEC has had the disadvantage of being more expensive and less versatile than RP-HPLC systems. Recently, with the advances in instrumental design a new generation of IE analyzers (System Gold™ analyzer, Beckman Instruments, San Ramon, USA) has emerged. This system offers the advantage of ease of operation and is highly adaptable for analyses of substances other than amino acids.

Although the manufacturer of the System Gold™ analyzer has made strong claims for its wide applicability in amino acid analysis, there have been no reports fully describing the use of the System Gold™ analyzer for the analysis of free amino acids in physiological fluids. An additional complication to the development of a satisfactory system arises from the manufacturer's unwillingness to disclose the composition of the buffers recommended for use in the system. Thus the user has to purchase commercial buffers at high cost for routine operation.

In this study, we describe the preparation of lithium citrate buffers and their application in physiological amino acid analysis on the System Gold™ analyzer. Quantitative analysis of results obtained for physiological amino acids was examined in terms of accuracy and precision.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals and reagents were analytical grade. A standard mixture of physiological amino acids (0.25 μmol/ml each in

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0.2 N lithium citrate, pH 2.20 with 1.0% thiodiglycol and 0.1% preservative) and ninhydrin (Trione) reagent was purchased from Beckman Instruments. The individual crystalline amino acids were obtained from Sigma (St. Louis, USA). Lithium hydroxide, citric acid, hydrochloric acid, and ethylene-diaminetetraacetic acid disodium salt (EDTA) were purchased from BDH Inc. (Toronto, Canada). Sulfosalicylic acid was obtained from Fisher (Fisher Scientific Ltd., Nepean, Canada). The water used for buffer preparation was purified by a Millipore Milli-Q water system (Millipore Corp., Milford, USA).

Amino acid standard preparation

A standard mixture of physiological amino acids in 0.2 N lithium citrate buffer containing 44 components (see note to *Figure 1*) was mixed with an equal volume of dilution buffer (pH 2.20). This diluted standard (0.125 μmol/ml) was then used for calibration during quantitative analysis. Stock solutions of individual amino acids and biological amines, representative of a physiological sample (2.5 μmol/ml), were prepared in sample dilution buffer (pH 2.20). All standard solutions were kept at −20 C until used.

Plasma sample preparation

Heparinized blood was obtained from rats (Wistar), some of which had received an intraperitoneal injection of TRP, and centrifuged at 2000*g* (Highspeed 18 Refrigerator Model, Measuring & Scientific Ltd., London, UK) for 15 min at 4 C. The plasma was immediately deproteinized with sulfosalicyclic acid (30 mg SSA per ml plasma) and centrifuged at 15,000*g* for 15 min. The protein-free supernatant was filtered through a 0.2 μm acrodisc membrane filter (Gelman Sciences Inc., Ann Arbor, USA). The clear filtrate was collected and stored at −20 C until analysis on the equivalent of 10–25 μl of plasma.

Amino acid analysis

Physiological amino acid analysis of plasma samples was performed by the System Gold™ analyzer. Samples were also analyzed by the Beckman System 6300 amino acid analyzer[17] or by RP-HPLC (Waters Assoc. Inc., Milford, USA) analysis of *o*-phthaldialdehyde (OPA)-derivatives[20] in order to provide a comparison.

Buffer preparation

The composition of the laboratory-prepared lithium citrate buffers used in obtaining a satisfactory separation of amino acids is listed in *Table 1*. Buffer salts were dissolved in HPLC grade water, and concentrated HCl (for pH adjustment) was added prior to final volume adjustment. The pH of the buffer solution was rechecked after final adjustment. The prepared

Table 1 Lithium citrate buffer composition

Component	Buffer number			Regenerant (LiOH)
	1	2	3	
pH at 25 C	2.80	3.55	3.65	
Lithium ion concentration (N)	0.24	0.34	1.00	0.30
Citrate ion concentration (N)	0.20	0.20	0.20	
Lithium hydroxide (hydrous, g)	10.70	14.27	41.96	12.59
Citrate acid monohydrate (crystal, g)	14.01	14.01	14.01	
Concentrated HCl (approx. ml)	8.50	11.00	38.00	
EDTA acid form (g)				0.25
Final volume (ml)	1000	1000	1000	1000

Table 2 Chromatographic program for physiological amino acid analysis

Time (min)	Solvent	Temperature (C)	Remarks
0	Trione	43	Initial condition
0	Buffer #1	43	Initial condition
13.5	Buffer #2	43	Buffer change from #1 to #2
26.0	Relay ON	67	Temperature change from 43 C to 67 C
37.0	Buffer #3	67	Buffer change from #2 to #3
100	Regenerant	67	Buffer change from #3 to regenerant
115	Relay OFF	43	Temperature change from 67 C to 43 C
130	Buffer #1	43	Buffer change from regenerant to #1
160	END	43	Next injection

Note: Flow rate: 0.8 ml/min.; solvent mixture: 50% ninhydrin (Trione), 50% lithium citrate buffer.

buffers were filtered through a 0.45 μm pore size membrane filter (Sartorius, Germany) and stored at 4 C until used.

The detailed composition of the commercial buffers (Beckman Instruments) is unknown, although the information provided indicates the lithium eluent #1 contains lithium ion 0.24 N, pH 2.75; the lithium eluent #2 contains lithium ion 0.34 N, pH 3.60, and the lithium regenerant contains lithium ion 0.3 N with 0.002 N of EDTA. There is no information on the lithium eluent #3 and the ninhydrin (Trione) reagent.

Chromatographic system

The amino acid analyzer System Gold™ (Beckman Instruments) consisted of a system controller (Model NEC), a programmable solvent delivery module (Model 126AA), a post-column reactor (Model 231) set at 130 C, a column heater (Model 235) programmed at 43 C and 67 C, and a variable wavelength detector operating at 500 nm (Model 126). All sample injections were performed with a Rheodyne injection valve (Model 210A), equipped with a 20 μl (or 50 μl) filling loop. Peak areas were recorded and integrated by an integrator (Model C-R601, Shimadzu, USA).

Separation was carried out on a Spherogel™ amino acid column (10 μm, 4.6 × 250 mm, Beckman Instruments), equipped with a guard column of the same material. The flow rate for the buffer was 0.4 ml/min and for the ninhydrin reagent 0.4 ml/min. The latter was flushed continuously with pure nitrogen. The details of the analysis program are shown in *Table 2*.

Results and discussion

The chromatograms of a standard mixture of physiological amino acids are shown in *Figures 1* (commercial buffers) and *2* (laboratory-prepared buffers). *Figure 3* shows the elution profiles of similar physiological amino acid standard obtained with a more sophisticated ion-exchange analyzer (Beckman System 6300AA and commercial buffers). The laboratory-prepared buffers (*Figure 2*) provided comparable peak resolution to that of the commerical buffers (*Figure 1*) at approximately one-third the cost.

A complete analysis of the 44 components in the standard took about 120 min in each case, a somewhat shorter time than reported in the literature for other dedicated systems.[16-18,21] With the present conditions, most amino acids were satisfactorily resolved, the exceptions being TRP and HYLYS; 1-MEHIS and HIS; and 3-MEHIS and ANS (*Figure 2*). This is in contrast to the results obtained with the Beckman System 6300AA using commercial buffers, where TYR and NORLEU, and TRP and HYLYS were coeluted.[17] In our comparative studies on the Beckman System Gold™, System 6300AA and RP-HPLC-OPA (Waters), only TRP and HIS

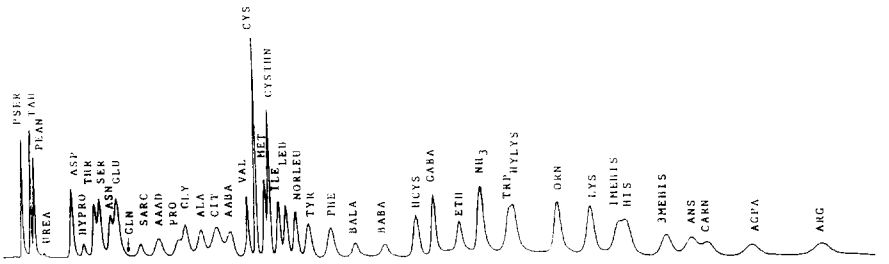

Figure 1 Chromatogram of a standard mixture as determined by the System Gold™ analyzer with commercial lithium citrate buffers. Each peak corresponds to 2.5 nmol except HCYS (1.18 nmol) and ORN (1.95 nmol). (*Note:* PSER: phosphoserine, TAU: taurine, PEAN: phosphoethanolamine, UREA: urea, ASP: aspartic acid, HYPRO: hydroxyproline, THR: threonine, SER: serine, ASN: asparagine, GLU: glutamic acid, GLN: glutamine (not included), SARC: sarcosine, AAAD: α-amino-adipic acid, PRO: proline, GLY: glycine, ALA: alanine, CIT: citrulline, AABA: α-amino butyric acid, VAL: valine, CYS: cystine, MET: methionine, CYSTHN: cystathionine, ILE: isoleucine, LEU: leucine, NORLEU: norleucine, TYR: tyrosine, PHE: phenylalanine, BALA: β-alanine, BABA: β-amino butyric acid, HCYS: homocystine, GABA: γ-amino butyric acid, ETH: ethanolamine, NH_3: ammonia, TRP: tryptophan, HYLYS: hydroxylysine, S_2-AEC: S_2-aminoethyl-cysteine, ORN: ornithine, LYS: lysine, 1MEHIS: 1-methylhistidine, HIS: histidine, 3MEHIS: 3-methylhistidine, ANS: anserine, CARN: carnosine, AGPA: α-amino-β-guanidinopropionic acid, ARG: arginine. Creatinine does not appear on the chromatogram, though it is present in the standard mixture.)

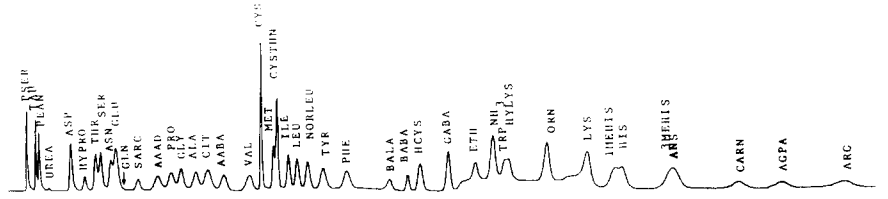

Figure 2 Chromatogram of a standard mixture as determined by the System Gold™ analyzer with laboratory-prepared lithium citrate buffers. Each peak corresponds to 1.25 nmol except HCYS (590 pmol) and ORN (950 pmol).

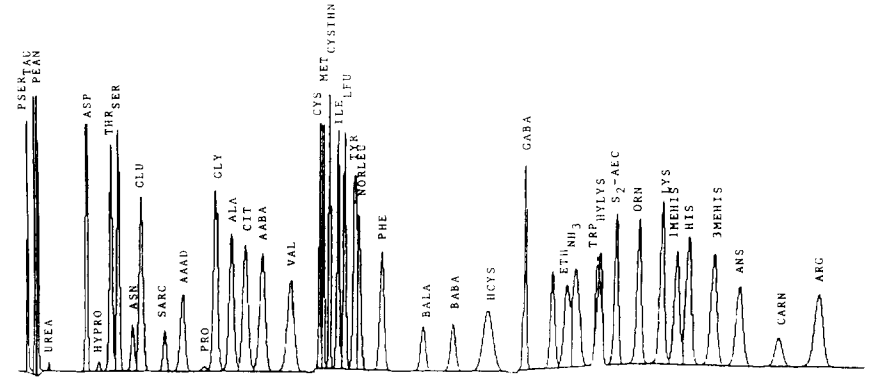

Figure 3 Chromatogram of a standard mixture as determined by the Beckman System 6300AA analyzer with commercial lithium citrate buffers. Each peak corresponds to 6.25 nmol except HCYS (2.95 nmol) and ORN (4.875 nmol).

are present at detectable concentrations in physiological fluids (*Tables 3* and *4* and *Figures 4* and *5*). However, if the separation of these compounds from HYLYS and 1-MEHIS, respectively, is of interest (e.g., urine), changes could easily be made in the chromatographic conditions.[22-25] Since the reaction of ninhydrin with creatinine is poorly developed in the post-column reactor, it does not appear on the chromatogram. Buffer change peaks were not observed between buffers. The baseline remains stable

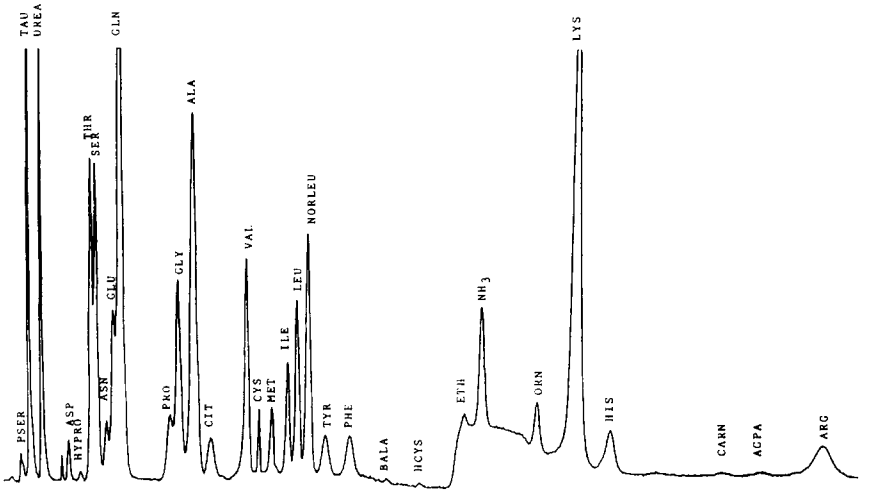

Figure 4 A typical chromatogram of rat plasma obtained by the System Gold™ analyzer with laboratory-prepared lithium citrate buffers.

throughout elution without the plateau around the ammonia peak, which often arises from buffer contamination.[21,26]

In *Figure 4*, a typical chromatogram for plasma is given, showing that 32 ninhydrin reactive components present in this physiological fluid can be satisfactorily separated, identified, and subsequently quantified with the aid of an external standard by the System Gold™.

A comparsion between the results of analysis of plasma obtained by the System Gold™ analyzer, by the System 6300AA analyzer using commercial lithium citrate buffers and ninhydrin (Beckman Instruments), and by the RP-HPLC-OPA method is shown in *Tables 3* and *4*. The free amino acid concentrations in plasma, as determined by the System Gold™ with laboratory-prepared buffers in the present work, are in good agreement with results obtained using the System 6300AA analyzer and commercial buffers (analyzed by the Laboratory of Clinical Biochemistry, Hospital for Sick Children, Toronto). Compared to the RP-HPLC-OPA method, the levels for most of the amino acids were higher when analyzed by the System Gold™ analyzer, except for ASP, ANS, GLU, and HIS. The lower values obtained by the RP-HPLC-OPA method compared with post-column ninhydrin derivatization are similar to those reported previously.[6,27] This effect is reportedly a result of instability of the OPA–amino acid derivatives.[28,29]

In order to examine the linearity of response of the present method, a standard mixture of physiological amino acids, varying from 0.625 nmol to 3.125 nmol per amino acid, was run at least twice, and the average peak areas were calculated and plotted (*Figure 6*). Almost all components commonly found in plasma and brain show good linearity over the concentration range examined except GABA, NH_3 and ORN. A possible reason for this is baseline shift.

To examine the reproducibility of the present chromatographic method and different batches of buffers, repeated runs on the physiological amino acid standard and a single plasma sample were performed. The coefficients of variation (% CV) for within-day and between-day runs for the 27 ninhydrin reactive components of the plasma are presented in *Table 4*. As expected, the coefficient of variation of individual amino acids for within-day assays was lower than for between-day assays. The variability for most of the amino acids of interest is below 5%, in agreement to that for the physiological amino acid standard (data not shown). Overall, the variability obtained using the physiological standard was very similar to that reported by Lee and Slocum for the Beckman System 6300AA.[17] Aside from ETH

Table 3 Rat plasma free amino acid concentrations (determined by System Gold™ analyzer and RP-HPLC)

Amino acid	System Gold™ (IEC)	Waters System (RP-HPLC)
TAU	192 ± 9	144 ± 3
UREA	4432 ± 302	N.D.*
ASP	23 ± 1	41 ± 3
THR	530 ± 16	N.D.
SER	431 ± 15	329 ± 4
ASN	70 ± 4	152 ± 4
GLU	102 ± 4	213 ± 3
GLN	757 ± 33	570 ± 8
PRO	342 ± 28	N.D.
GLY	326 ± 13	N.D.
ALA	571 ± 14	456 ± 7
CIT	94 ± 7	N.D.
AABA	16 ± 2	N.D.
VAL	203 ± 9	204 ± 3
CYS	9 ± 3	N.D.
MET	57 ± 2	58 ± 2
ILE	76 ± 2	114 ± 5
LEU	127 ± 4	132 ± 2
TYR	86 ± 4	91 ± 1
PHE	58 ± 3	65 ± 3
ETH	19 ± 1	N.D.
NH_3	61 ± 5	N.D.
TRP	442 ± 45	442 ± 7
ORN	78 ± 4	64 ± 2
LYS	420 ± 36	326 ± 5
HIS	47 ± 1	105 ± 5
ARG	93 ± 5	56 ± 1

Note: Plasma from rat receiving intraperitoneal injection of TRP (100 mg/kg). Values (μmol/L, mean ± SD) are means of three analyses.
* N.D. = Not determined.

Table 4 Rat plasma free amino acid concentrations (determined by System 6300AA analyzer and System Gold™ analyzer)

Amino acid	System 6300AA (n = 1)	System Gold™* Mean ± SD (n = 7)	System Gold™* Within-day % CV (n = 3)	System Gold™* Between-day % CV (n = 4)
PSER	4	7 ± 1	21.5	15.4
TAU	244	299 ± 15	1.1	2.4
UREA	5971	5472 ± 396	1.9	2.5
ASP	22	23 ± 1	2.9	8.5
HYPRO	41	33 ± 3	5.2	10.5
THR	220	252 ± 7	4.5	2.1
SER	194	230 ± 7	2.4	1.4
ASN	N.D.**	57 ± 2	4.2	5.0
GLU	126	121 ± 3	2.2	0.8
GLN	N.D.	638 ± 22	4.9	1.3
PRO	185	230 ± 13	0.5	3.7
GLY	220	261 ± 7	2.4	2.4
ALA	448	515 ± 15	1.4	3.1
AABA	13	12 ± 3	12.7	20.2
VAL	185	226 ± 7	1.8	1.1
CYS	13	22 ± 2	5.9	7.2
MET	41	58 ± 8	4.5	8.2
ILE	82	102 ± 10	1.1	4.2
LEU	141	158 ± 5	0.5	1.8
TYR	56	63 ± 3	3.4	1.0
PHE	61	69 ± 8	1.4	3.7
ETH	162	84 ± 14	17.0	17.7
NH_3	61	91 ± 12	3.0	18.0
ORN	35	38 ± 3	4.6	9.0
LYS	444	430 ± 19	7.0	5.9
HIS	65	57 ± 5	8.3	7.2
ARG	102	104 ± 10	3.3	8.7

Note: Values are in μmol per liter plasma.
* Coefficient of variation (% CV) of plasma amino acid concentrations measured by System Gold™ analyzer for within- and between-day assays.
** N.D. = Not determined.

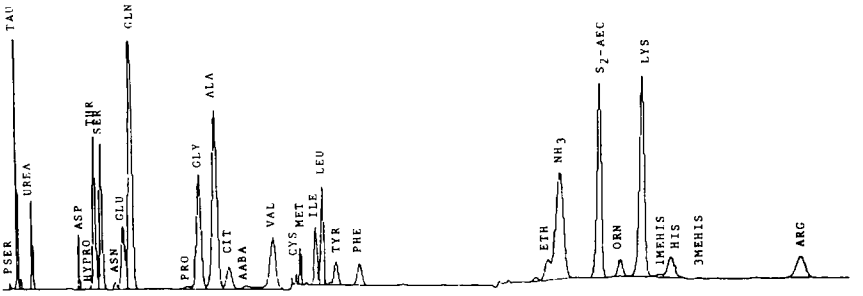

Figure 5 A typical chromatogram of rat plasma obtained by the Beckman System 6300AA analyzer with commercial lithium citrate buffers.

Table 5 Reproducibility of retention times of rat plasma amino acids. (determined by System Gold™ analyzer)

	Within-day (n = 3)			Between-day (n = 4)		
Amino acid	Mean	SD	% CV	Mean	SD	% CV
PSER	3.0	0.012	0.41	3.0	0.012	0.47
TAU	4.3	0.001	0.01	4.3	0.013	0.30
UREA	6.1	0.007	0.12	6.1	0.015	0.25
ASP	9.1	0.020	0.22	9.1	0.030	0.22
HYPRO	10.8	0.032	0.30	10.8	0.041	0.38
THR	12.2	0.025	0.21	12.2	0.008	0.07
SER	12.8	0.031	0.24	12.9	0.010	0.08
ASN	14.2	0.026	0.19	14.3	0.026	0.19
GLU	14.9	0.080	0.54	15.0	0.072	0.48
GLN	16.0	0.051	0.32	16.0	0.042	0.26
PRO	22.1	0.064	0.29	22.1	0.024	0.11
GLY	23.4	0.075	0.32	23.5	0.045	0.19
ALA	25.4	0.081	0.32	25.4	0.050	0.20
AABA	29.0	0.198	0.68	29.2	0.155	0.53
VAL	32.8	0.097	0.30	32.8	0.051	0.16
CYS	37.4	0.119	0.32	37.4	0.034	0.09
MET	39.3	0.056	0.14	39.3	0.064	0.16
ILE	41.7	0.112	0.27	41.6	0.032	0.08
LEU	43.1	0.105	0.24	43.1	0.054	0.12
TYR	47.4	0.156	0.33	47.5	0.160	0.34
PHE	51.0	0.172	0.34	51.1	0.193	0.38
ETH	68.2	0.132	0.19	68.1	0.230	0.34
NH_3	70.4	0.041	0.06	70.4	0.140	0.20
ORN	79.3	0.047	0.06	79.1	0.200	0.25
LYS	85.0	0.090	0.11	84.9	0.289	0.34
HIS	90.0	0.165	0.18	89.9	0.370	0.41
ARG	122.3	0.710	0.58	122.2	1.360	1.11

and NH_3, the largest variations were found for amino acids in very low concentrations (< 250 pmol), i.e., PSER and AABA in 10 μl of plasma.

The average between-day coefficient of variation (0.29%) in retention time for plasma amino acids was comparable to that of the within-day variation (0.27%). The coefficient of variation of individual amino acids was less than 0.68% for within-day assays and for the between-day assays, with the exception of ARG (CV = 1.11%), the variation was less than 0.53% (*Table 5*). This further shows that the present system is highly reproducible.

The idea of using a single wavelength for amino acid quantification in IEC was first reported by Ellis and Garcia.[30] Detection at wavelength 405 nm, however, resulted in interference of unknown ninhydrin-positive compounds other than amino acids and amines commonly found in the physiological fluids (Beckman Instruments, personal communication). In the present system, the manufacturer recommended the use of wavelength 500 nm to detect all physiological amino acids and amines in one run. As evidenced by the linearity of response (*Figure 6*) and data shown in *Tables*

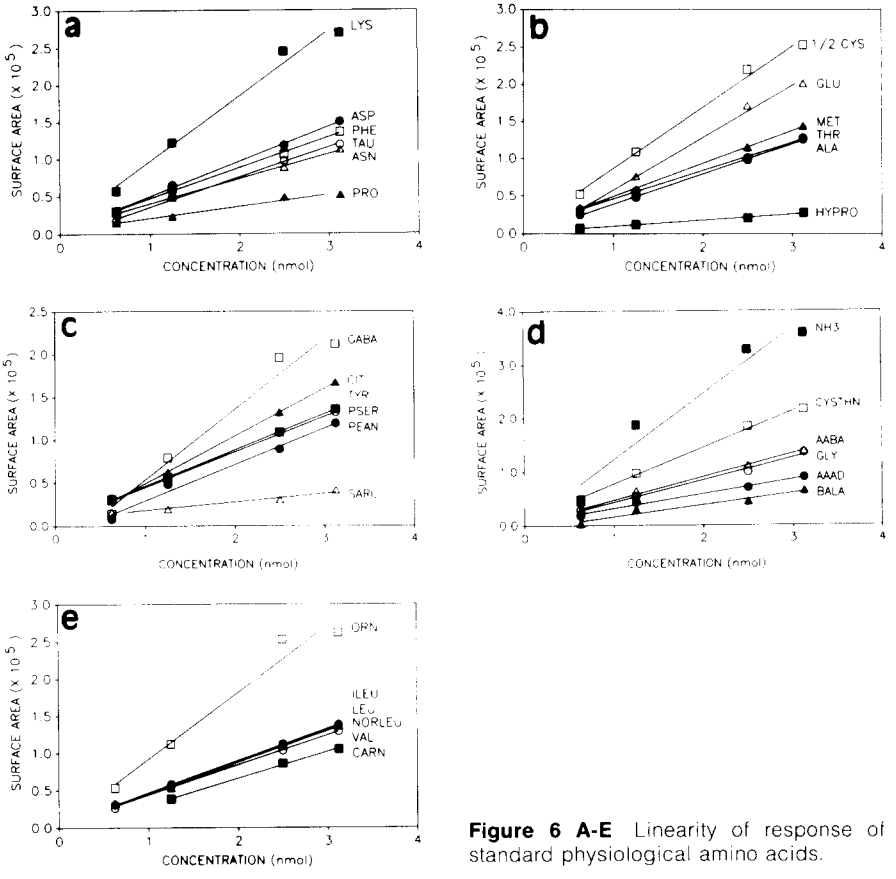

Figure 6 A-E Linearity of response of standard physiological amino acids.

3, *4* and *5*, the separation and quantification of plasma amino acids indicated the validity of the present chromatographic system and single wavelength detection.

In conclusion, the system reported herein, compared to other complete and dedicated IE analyzer systems, provides equivalent analytical strengths but also has the advantage of cost saving based on component equipment and laboratory-prepared buffers. In addition, when not in use for IE amino acid analysis, the components can be easily used for other laboratory applications.

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