CHROMBIO. 412

DETERMINATION OF ERYTHROCYTE AMINO ACIDS BY GAS CHROMATOGRAPHY

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SUMMARY

Erythrocyte amino acid levels were determined, by gas chromatography, in a group of 34 normal human adults. No significant sex or age correlations were noted.

A method for the quantitative gas chromatographic analysis of free amino acids in erythrocytes is described. Following hemolysis and deproteinization the amino acids were isolated on a cation-exchange resin. Glutathione was removed from the amino acid mixture by adsorption on an anion-exchange resin. Following conversion to their N-acetyl-*n*-propyl esters, 19 amino acids were separated and quantitated by gas chromatography on a single column in 18 min. Typical reproducibility data indicate that a coefficient of variation of 2—5% is attainable.

INTRODUCTION

Although there is much information regarding the quantities of free amino acids in human plasma or serum in normal and abnormal individuals, little attention has been paid to such concentrations in erythrocytes. Recent studies have documented the importance of the red blood cell (RBC) in the inter-organ transport of free amino acids [1—4] many of which show a significant concentration differential across the erythrocyte cell membrane. The clinical importance of RBC amino acid levels has only been suggested; we have recently reported on a group of manic-depressive patients whose erythrocyte glycine concentration was significantly elevated in comparison to a group of control subjects [5].

The earliest published studies of RBC amino acids in normal adults [6,7] were done without the benefit of advanced analytical techniques, while more recent studies [3,8—11] have reported on small numbers of subjects. There has also been a general absence of specific information as to the sex and age

of the subjects studied, as well as to the time of specimen collection in relation to possible diurnal and nutritional effects, factors which are known to influence plasma amino acid levels [12—15]. It is therefore not surprising to find a wide range of reported values for many of the erythrocyte amino acids (see Table I).

In the past, amino acid analysis has depended on microbiological assay procedures [6] and various paper chromatographic techniques [7]. More

TABLE I

ERYTHROCYTE AMINO ACID LEVELS: SUMMARY OF AVERAGE VALUES REPORTED IN THE LITERATURE

For abbreviations of the amino acids see Table II. Their concentrations are given in μmoles/l.

Reference	6	7	8	3*	9	10	11	This report
Year	1951	1960	1971	1973	1976	1978	1978	1979
n	24	14	6	7	7	6	10	34
Sex: M/F	All M	?	?	All M	?	?	All F	11 M/23 F
Mean age	24	?	?	Adult	Adult	Adult	40	35/33
a.m.—fasting	Yes	Yes	?	Yes	?	Yes	Yes	Yes
Method††	MB	PC	IEC	IEC	IEC	IEC	IEC	GC
ALA		278	250	364	260	259	357	331
2-ABA		16	14	25		34		24
VAL	174	145	137	268	65	155	191	188
GLY		**	264	485	292	350	374	362
ILE	65	***	43	66	14	53	62	58
LEU	118	***	100	134	25	107	115	110
PRO		124		219	86	126	216	175
THR	135	102	154	167	82	113	122	124
4-ABA								12
SER		**	265	217	113	143	198	164
ASN				†	102	102	120	124
ASP			264	388	353		544	
MET	34	20	11	13	1	16	0	24
CYSH	32		7	0	0		0	
PHE	60	38	61	55	10	47	71	58
HYP								11
GLN		391	201	†	315	622	830	468
GLU		243	220		160	294	350	
TYR	58	47	78	69	27	57	75	70
ORN		120	149		105	113	153	179
HIS	70	85	115		55	82	131	
LYS	92	151	143		99	125	179	174
ARG	16	<8	25		5	42	0	29
TRP	14	14			1			

*Calculated from whole blood and plasma values.
**GLY + SER = 616.
***ILE + LEU = 137.
†GLN + ASN = 612.
††MB, microbiological assay; PC, paper chromatography; IEC, ion-exchange chromatography; GC, gas chromatography.

recently, ion-exchange chromatography has been most extensively utilized [3,8—11]. In addition, there have been significant advances in the techniques of gas chromatography (GC), some of which have been applied to amino acid analysis [16]. Advantages of GC include the low cost and greater versatility of the instrument, as well as greater sensitivity and reduced operating time. Of the many techniques which have been developed, the most useful are based on a two-step procedure: esterification of the carboxyl group, followed by acylation of the α-amino and other functional groups.

One example of this technique uses the N-trifluoroacetyl-n-butyl esters and requires two GC columns for complete separation [17,18]. Also used are the N-heptafluorobutyryl isobutyl esters which can be separated on a single column in 60 min [19—21]. A third procedure, based on the formation of the N-acetyl-n-propyl amino acid esters, has particular advantages: derivative preparation is simple and rapid, only a single GC column is required, and complete separation is achieved in less than 20 min [22—25]. Many successful applications of this technique have been reported [26—31], including the analysis of plasma and urine, but not of erythrocytes.

When considering the use of this technique for the analysis of erythrocyte amino acids, a potential source of serious error was apparent. Erythrocytes contain a large quantity of the tripeptide glutathione (γ-glutamyl-cysteinylglycine), which would be partially hydrolyzed during the esterification procedure in hot 8 M propanolic HCl. This would, in turn, lead to falsely elevated levels of glutamic acid, cysteine and glycine. Several methods were explored by which the glutathione could be separated from the amino acid mixture [32—36]; these were found to be too complex and unreliable. The procedure adopted for routine use was based on the adsorption of glutathione on an anion-exchange resin (AG 1-X8, 200—400 mesh, acetate form), and elution of the amino acids (except for dicarboxylic acids) with 0.05 N acetic acid [37]. This was shown to be a very dependable procedure as well as being simple and rapid.

The analytical procedure herein described was used to study the erythrocyte free amino acids in a group of normal adults of both sexes. Time of day and nutritional state were controlled by obtaining all blood specimens between 8 and 10 a.m., from subjects who had been fasting since the previous midnight. Informed consent was obtained from all subjects.

MATERIALS

Reagents
    The following reagents were used: cation-exchange resin AG 50W-X8 (H^+), 100—200 mesh, and anion-exchange resin AG 1-X8 (CH_3COO^-), 200—400 mesh (Bio-Rad Labs., Richmond, Calif., U.S.A.); ammonium hydroxide and glacial acetic acid (reagent grade), trichloroacetic acid and sodium chloride (Fisher, Springfield, N.J., U.S.A.); acetic anhydride 99+ % (Aldrich, Milwaukee, Wisc., U.S.A.), ethyl acetate, triethylamine, n-propyl alcohol and acetone, each of highest available purity (Pierce, Rockford, Ill., U.S.A.); dry air, high-purity helium, prepurified hydrogen, electronic grade hydrogen chloride and prepurified nitrogen (Linde, South Plainfield, N.J., U.S.A.); amino acids, A grade

(Calbiochem, San Diego, Calif., U.S.A.); L-norleucine, grade I (for use as internal standard) (Sigma, St. Louis, Mo., U.S.A.); (additional high purity amino acids from Aldrich, and Alltech Assoc., Arlington Heights, Ill., U.S.A.); N-acetyl-*n*-propyl amino acids (Alltech).

Propylation reagent. The propylation reagent, propanolic HCl, was made by passing 27 g of anhydrous electronic grade HCl gas into 100 ml of cooled *n*-propanol. Stored at 4° in a glass-stoppered bottle the reagent lasts several months.

Acylation reagent. A mixture of acetone, triethylamine, and acetic anhydride (5:2:1) was prepared daily.

Resin columns

The columns were made by inserting a small quantity of Pyrex glass wool (Packard, Downer's Grove, Ill., U.S.A.) into a large-volume pasteur pipet (Fisher). The glass wool was gently pushed into the upper portion of the constricted pipet stem, and compacted just enough to prevent loss of resin, while avoiding a significant reduction of flow-rate. A 25-g quantity of each resin was washed with 4 × 100 ml of distilled water, and finally suspended in 100 ml of water for use as needed. A sufficient quantity of this washed resin suspension was applied to a column so that the resulting packed resin bed had a volume of 1.5 ml and measured 0.8 cm × 3.0 cm. The AG 50W-X8 (H$^+$) 100—200-mesh column contains 500 mg of dry resin and provides approximately 2.55 mequiv. of exchange capacity. The AG 1-X8 (CH$_3$COO$^-$) 200—400-mesh column contains 656 mg of dry resin and provides approximately 2.10 mequiv. of exchange capacity. A fresh bed of washed resin was used for each sample.

Standards

Stock solutions of selected individual amino acids, including norleucine (NLE), to be used as the primary internal standard, were made up in aqueous 0.1 N HCl at a level of 5 μmoles/ml. A solution of "secondary standards" was made up to contain 2-aminoadipic acid (2-AAA) and 2-aminoisobutyric acid (2-AIBA). A single "calibration standard" solution was likewise prepared which contained equimolar amounts of the following amino acids: 2-aminobutyric acid, alanine, valine, glycine, isoleucine, leucine, proline, threonine, 4-aminobutyric acid, serine, asparagine, methionine, cysteine, phenylalanine, hydroxyproline, tyrosine, ornithine, histidine, lysine, arginine and tryptophan. These solutions are stable for several months when stored in glass-stoppered bottles at 4°. Glutamine, however, is not stable in solution and must be freshly prepared at frequent intervals.

Glassware

Glassware included 13 mm × 100 mm and 16 mm × 125 mm disposable glass culture tubes (Fisher), 16 mm × 125 mm glass culture tubes with screw-cap (PTFE-lined) closures (Kimax No. 45066-A), 15-ml graduated Corex conical centrifuge tubes (Corning No. 8080-A), 40-ml graduated heavy-duty conical centrifuge tubes with screw-cap (PTFE-lined) closures (Corning No. 8142), 15 mm × 45 mm glass vials with screw-cap (PTFE-lined) closures

(Supelco, Bellefonte, Pa., U.S.A.), 145-mm and 230-mm disposable glass pasteur pipets and "large-volume" glass pasteur pipets (Fisher).

Equipment

The following equipment was used: "Temp-blok" module heaters (Scientific Products); Sorvall GLC-1 centrifuge; "Evapo-Mix" vortex evaporator (Buchler Instruments); "Vortex-Genie" mixer; Eppendorf pushbutton pipets.

Gas chromatograph

A Packard Model A7400 gas chromatograph with flame ionization detector and linear temperature programming was used together with a Honeywell Model 551 1-mV recorder. Injection was carried out using a Hamilton 701 RN syringe with a 26 S needle.

GC column

The glass column (76 cm × 6 mm O.D. × 2 mm I.D.) was terminated with a septum well at each end. This was packed with a mixed polar packing, consisting of 0.31% Carbowax 20 M, 0.28% Silar 5CP and 0.06% Lexan on Chromosorb W AW, 120—140 mesh (Alltech). The column ends were sealed with special high-temperature 1½-hole cylindrical septa (Supelco). Before connecting the outlet to the detector, the column was conditioned with a helium flow of 12 ml/min at 260° for 2 h, then at 220° for 16 h.

METHODS

Collection of blood

Blood specimens were obtained between 8 and 10 a.m. from subjects who had been fasting since midnight. A "vacutainer" apparatus was used to draw 20 ml, by antecubital venipuncture, into a rubber-stoppered tube containing 357 USP units of sodium heparin. Complete mixing of anticoagulant with blood was accomplished by repeated and gentle inversion of the tube. All specimens were promptly transported to the laboratory where immediate separation of erythrocytes from plasma was performed using 15-ml graduated "Corex" centrifuge tubes, which were spun for 30 min at 5000 rpm (3200 g) at room temperature.

Initial preparation of erythrocytes

The plasma was removed from the tube and a large-size pasteur pipet was used to carefully aspirate the buffy coat along with a portion of the packed red cells so that exactly 3 ml of the latter remained in the tube. To this were added 6 ml (2 parts) of distilled water, followed by thorough mixing. Four milliliters of this diluted RBC solution were transferred to a graduated, heavy-duty, 40-ml screw-top centrifuge tube, and a further dilution was made by adding 19 ml of distilled water. A PTFE-lined screw-cap was used to seal the tube, which was then subjected to quite vigorous agitation on a Vortex mixer for about 30 sec. Complete hemolysis was indicated by a change in appearance of the RBC solution: from cloudy and dark maroon in color to quite clear and bright cherry red. To this dilute hemolysate was added 1 ml of 50%

trichloroacetic acid solution, followed by 30 sec more of vigorous agitation. The precipitated hemoglobin and other proteins were removed by centrifugation at 2500 rpm (1200 g) for 30 min. The clear and colorless supernatant was passed through a folded cone of dry Whatman No. 2 filter paper to remove occasional floating clumps of precipitate. Approximately 20 ml of protein-free filtrate were recovered; this was placed in a tightly sealed plastic vial and stored at −70° for future analysis. Nine milliliters of this dilute preparation represent 0.5 ml of packed erythrocytes.

Pretreatment by ion-exchange chromatography

Nine milliliters of the dilute lysed protein-free erythrocyte solution were transferred to a 16 mm × 125 mm tube. To this were added 25 μl of 5 μmoles/ml norleucine internal standard solution. Using a pasteur pipet, the mixture was transferred to the first resin column (AG 50W-X8) and allowed to pass through the column at about 1 drop per 3 sec. Care was taken not to allow the column to run dry. The eluate was discarded. The sample tube was washed with 2 ml of distilled water and the washings transferred to the column. This was immediately followed with another 2 ml of distilled water and the eluate was discarded. The resin was eluted using 4 ml of 2 N NH$_4$OH followed by 2 ml of distilled water. After collecting the eluate in a 16 mm × 125 mm screw-top culture tube, it was concentrated to a small volume (about 0.5 ml) using the Evapo-Mix vortex evaporator with the water-bath at 40° (a short length of Tygon tubing was used for connecting the tube to the vortex evaporator). One milliliter of 0.05 N acetic acid was added to the sample, which was mixed well and transferred to the second resin column (AG 1-X8) [37]. The mixture was allowed to pass through the column at about 1 drop per sec, and the eluate was collected in a 16 mm × 125 mm screw-top culture tube. The sample tube was washed with 3 ml of 0.05 N acetic acid and the washings were transferred to the column. This was followed immediately with another 3 ml of 0.05 N acetic acid. Twenty-five microliters of the "secondary standards" solution, containing 2-AAA and 2-AIBA (5 μmoles/ml) were added to the combined column eluates. This solution, which was clear and colorless, was then evaporated to dryness on the vortex evaporator.

Derivatization

To insure complete dryness of the eluate residue, a stream of dry nitrogen was passed through the tube for 10 min. One milliliter of propylating reagent was added and the tube was flushed with nitrogen and capped firmly (using a PTFE-lined screw-cap). The tube was placed in a heating block at 110° for 20 min. (Note: Care was taken to use a tube which was free of chips or nicks around the tube lip — this prevented loss of pressure and evaporation of reagent during the propylation step.) The tube was cooled for 10 min before opening, and was then evaporated to dryness at 110° using a current of dry nitrogen. After cooling the tube briefly, 1 ml of freshly made acylating reagent was added, the tube was flushed with nitrogen and sealed tightly using a clean PTFE-lined screw-cap. The tube was placed in a heating block at 60° for 20 min. The cap was removed and the solution was carefully evaporated just

to dryness, using a gentle stream of dry nitrogen. (Care was taken not to exceed 60°, or 100 ml/min of gas flow per tube. Derivatized material at this point was relatively volatile and care was taken to prevent losses, particularly of alanine and valine.) The residue was redissolved in 2 ml of ethyl acetate. To this was added 1 ml of saturated sodium chloride solution. The tube was then capped and shaken vigorously. After centrifugation for 5 min at 700 g, the ethyl acetate layer was carefully transferred to a 15 mm × 45 mm glass vial. Again using gentle heat and nitrogen gas flow (as above) the sample was evaporated just to dryness. The residue was redissolved in 50 μl of ethyl acetate, mixed well, and the vial was sealed with a PTFE-lined screw-cap. The derivatized material, except for methionine and histidine, was stable for several weeks when stored at 4°. GC analysis was done using a 2-μl aliquot of this solution.

Gas chromatography

The following instrument settings were used: the helium carrier gas flow-rate was 25 ml/min at an inlet pressure of 30 p.s.i. The hydrogen and the air for the flame ionization detector were set at flow-rates of 30 and 300 ml/min, respectively. The inlet temperature was 250° and the detector temperature was 275°. The column oven was temperature programmed as follows: held at 100° for 1 min following injection, then increased at 10°/min to 215°, then ballasted to 250° and held for 10 min. Attenuation was set at 32 × 10^{-11} with a suppression current ranging from 0.5 × 10^{-10} to 0.2 × 10^{-10}, depending on the age of the column. A needle guide was used to prevent bending of the syringe needle and to prolong septum life by allowing multiple injections through a single hole; the depth of penetration by the needle was also thereby controlled and limited to a consistent 8 mm beyond the septum.

The GC performance was tested for resolution and sensitivity by injecting 2 μl of a mixture of pure derivatized amino acids in ethyl acetate containing 2 nmoles/μl of each derivative. The relative molar response factor (RMR_{NLE}) of each amino acid was calculated with respect to NLE by peak height measurement. Although peak area measurements are commonly used in GC analysis, they require the use of an additional (expensive) integrating device or, alternatively, more tedious manual methods. In the interest of simplicity, the direct manual measurement of peak height was used as the basis of all quantitative calculations. This technique did not involve any sacrifice in accuracy [38—40] since the internal standard method was used, all operating parameters were strictly controlled and carefully reproduced, and a standard amino acid mixture was chromatographed with every series of unknown samples so that RMR values were determined under identical GC conditions.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the N-acetyl-*n*-propyl esters of a mixture of 23 pure amino acids which were taken through the entire procedure, as described above. The secondary standards, 2-AAA and 2-AIBA, were added following resin column pretreatment. Each peak represents 5 nmoles of amino acid in the original mixture. Table II summarizes the retention temperatures,
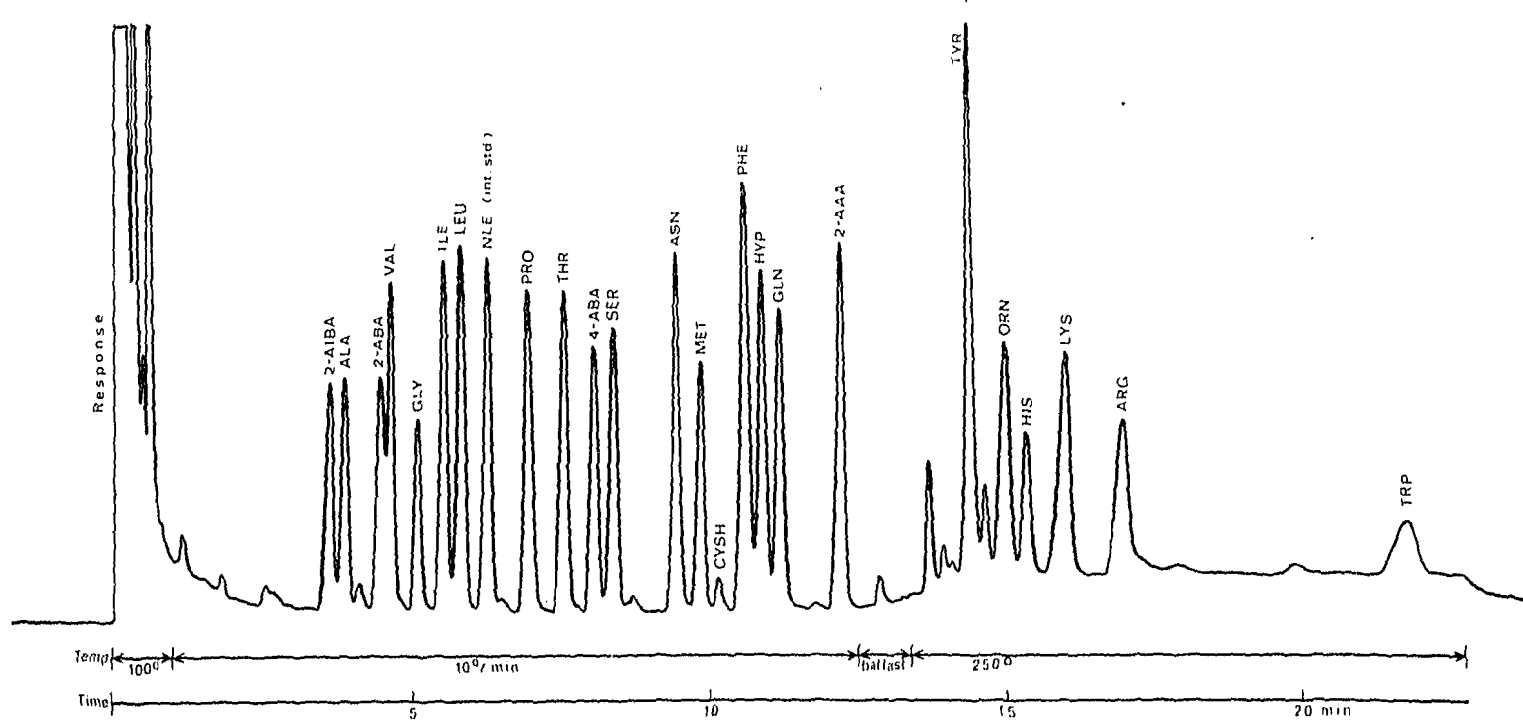
Fig. 1. GC separation of the N-acetyl-n-propyl esters of a mixture of 23 pure amino acids (for abbreviations see Table II) subjected to the complete procedure as described in Methods. The secondary standards 2-AAA and 2-AIBA were added after the resin columns. Temperature program: 1 min isothermal at 100°, followed by 10°/min to 215°, then ballasted to 250° and held for 10 min. Each peak represents 5 nmoles of amino acid derivative.

TABLE II

RETENTION TIME, RETENTION TEMPERATURE AND RELATIVE MOLAR RESPONSE
OF N-ACETYL-n-PROPYL ESTERS OF AMINO ACIDS

Standard solution of 21 amino acids, plus GLN and NLE (as internal standard, I.S.) taken
through complete procedure; 2-AAA and 2-AIBA added after resin columns. Data obtained
from chromatogram shown in Fig. 1.

Amino acid	Abbreviation	Time (min)	Temperature (°C)	RMR_{NLE}
2-Aminoisobutyric acid	2-AIBA	3.6	125	0.66
Alanine	ALA	3.9	130	0.68
2-Aminobutyric acid	2-ABA	4.4	133	0.67
Valine	VAL	4.6	135	0.94
Glycine	GLY	5.0	139	0.55
Isoleucine	ILE	5.5	144	1.00
Leucine	LEU	5.9	148	1.04
Norleucine	NLE	6.2	151	1.00 (I.S.)
Proline	PRO	6.9	158	0.91
Threonine	THR	7.5	164	0.90
4-Aminobutyric acid	4-ABA	8.0	169	0.74
Serine	SER	8.3 ·	172	0.79
Asparagine	ASN	9.4	183	1.01
Methionine	MET	9.8	187	0.69
Cysteine	CYSH	10.1	190	0.08*
Phenylalanine	PHE	10.6	195	1.19
Hydroxyproline	HYP	10.8	197	0.95
Glutamine	GLN	11.1	200	0.84
2-Aminoadipic acid	2-AAA	12.1	210	1.04
Tyrosine	TYR	14.4	250	1.64
Ornithine	ORN	14.9	250	0.69
Histidine	HIS	15.3	250	0.43*
Lysine	LYS	15.9	250	0.67
Arginine	ARG	16.8	250	0.44
Tryptophan	TRP	21.6	250	0.16*

*When resin columns are omitted, RMR_{NLE} value for cysteine is 0.38, for histidine is
0.58 and for tryptophan is 0.36.

retention times and relative molar response factors (RMR_{NLE}) which were
obtained from the chromatogram in Fig. 1. The RMR_{NLE} value of an amino
acid is defined as its peak height relative to the peak height of an equimolar
amount of the primary standard NLE.

The secondary standard, 2-AAA, added after resin column clean-up and
glutathione removal, served as a convenient monitor of recovery from the
two resin columns. Comparison of the RMR_{2-AAA} values for each amino acid
in the "calibration standard" solution, as obtained with and without resin
column treatment, showed that recovery was virtually quantitative (92—107%)
for 19 of the amino acids as well as for the primary standard NLE. (During
analysis of an unknown, the NLE peak height, relative to that of 2-AAA, was
a clear indication of column elution efficiency.)

Significant and variable losses following the resin column procedure were
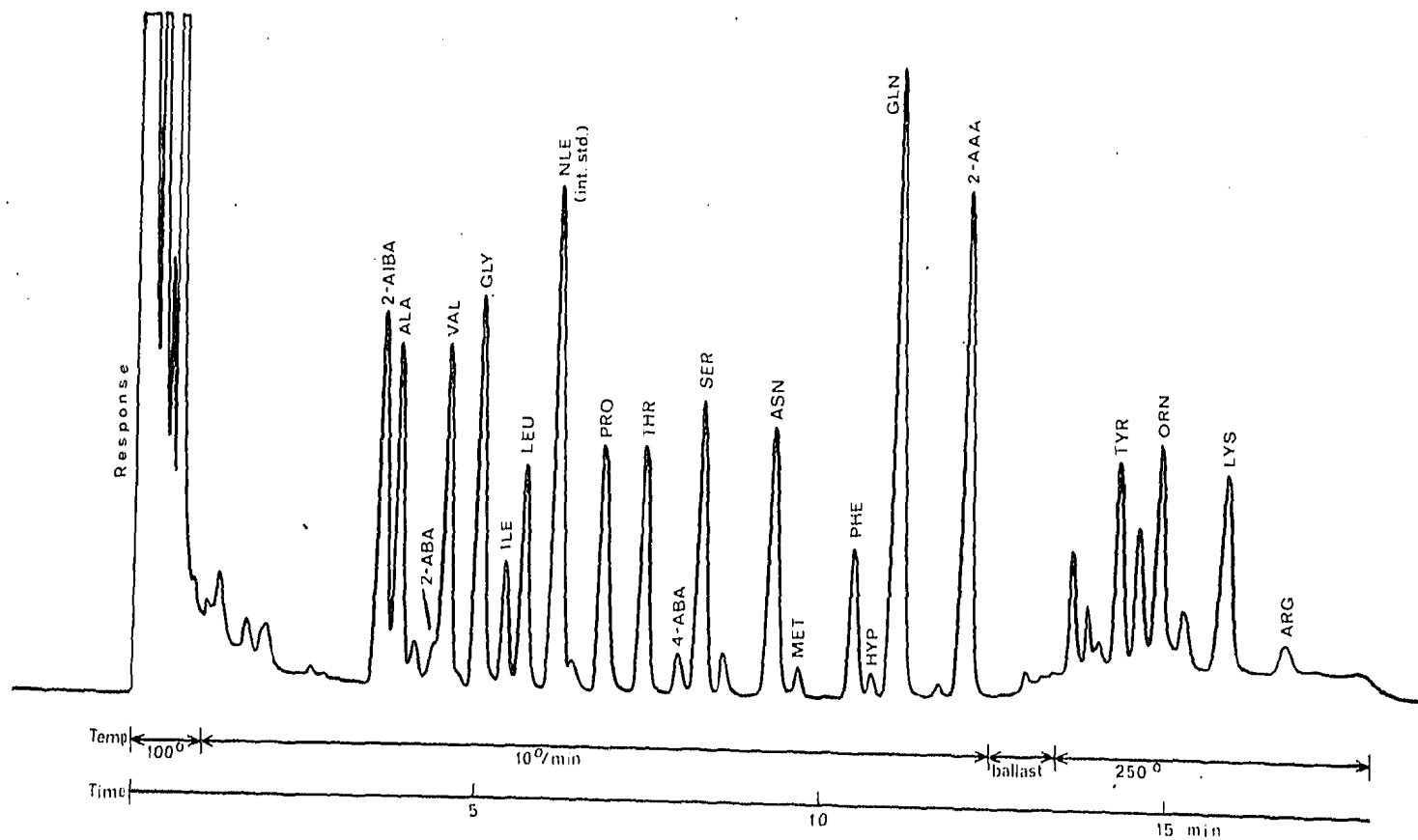
Fig. 2. Gas chromatogram of normal erythrocyte specimen subjected to the complete procedure. Temperature program as in Fig. 1. The three standard peaks represent 5 nmoles each of NLE, 2-AAA and 2-AIBA.

noted only for cysteine, histidine and tryptophan; their quantitation was therefore not reliable. It should be noted here that determination of erythrocyte cysteine and tryptophan is also not feasible by ion-exchange chromatography [10]. The dicarboxylic acids, aspartic and glutamic, were adsorbed on the anion-exchange column and were therefore not determined. This did, however, allow for the accurate quantitation of asparagine and glutamine which, under the conditions for esterification, were converted to the respective di-n-propyl esters of the parent acids. Reproducibility data for the 19 amino acids taken through the complete procedure show a coefficient of variation (C.V.) not exceeding 6%.

The secondary standard 2-AIBA forms the most volatile of all the derivatives studied here, and is therefore the first peak to elute from the GC column. A significant diminution in the size of this peak, relative to 2-AAA, was a warning that solvent evaporation, following acylation, had been excessive, and that some losses of alanine, 2-ABA, valine and possibly glycine had probably occurred. This problem, however, was seldom encountered in the course of many routine analyses.

Results of the application of this GC method of analysis to an erythrocyte sample from a normal adult is shown in Fig. 2. Clear and well-defined peaks can be identified for 19 amino acids, in addition to the NLE internal standard peak and the two secondary standard peaks, 2-AAA and 2-AIBA. A few minor unidentified peaks are present which do not interfere with the analysis.

The C.V. for simultaneous quadruplicate analysis of a single specimen was 8% for arginine, 6% for asparagine, and 1—4% for the other amino acids. When repeat analyses of a single specimen were done on different days, the C.V. values were slightly higher. Analysis of duplicate aliquots of a typical specimen of lysed erythrocytes, with and without a known amount of added glycine, indicated 100% recovery. When the glycine was added to the protein-free filtrate (following treatment with trichloroacetic acid) the recovery was 97%. Aliquots of selected erythrocyte specimens were sent to another laboratory for analysis by conventional automated ion-exchange chromatography. Values thereby obtained were in substantial agreement with the corresponding data from the GC analysis.

Table III summarizes the erythrocyte free amino acid concentrations in morning, fasting blood specimens obtained from 34 healthy adult volunteers. There were 23 female subjects, ranging in age from 21 to 60 years, and 11 male subjects whose age range was comparable (19—63). The mean, standard deviation, and range are shown for each of the 19 amino acids in both sex groups. (The mean concentration values for the combined group of 34 subjects are shown in Table I, for comparison with values published elsewhere.) Analysis of the data showed the absence of a significant correlation (r values in Table III) between subject age and the level of any amino acid in either sex group. The "two-tailed t test" was used to determine if there was a significant difference (P values in Table III) in the mean concentrations of any of the amino acids between the male and female groups. No sex group differences (NS) were seen in 17 of 19 erythrocyte amino acids. Differences of possible significance were indicated only for methionine ($P < 0.05$) and arginine ($P < 0.01$), both of which were found in very small amounts.

TABLE III

FREE AMINO ACID CONCENTRATIONS IN THE ERYTHROCYTES OF NORMAL ADULTS

For abbreviations, see Table II. Concentrations given in μmoles/l.

Amino acid	Males (n = 11)			Females (n = 23)			P* (M vs. F)
	Mean ± S.D.	Range	r (age)	Mean ± S.D.	Range	r (age)	
ALA	342 ± 76	213—441	+0.46	326 ± 67	228—438	+0.33	NS
2-ABA	26 ± 7	19— 39	+0.23	23 ± 7	13— 41	+0.03	NS
VAL	200 ± 30	166—275	+0.29	182 ± 31	123—232	+0.05	NS
GLY	351 ± 76	258—549	+0.75	368 ± 87	267—580	+0.06	NS
ILE	63 ± 13	44— 84	+0.03	56 ± 13	40—104	—0.05	NS
LEU	116 ± 17	89—137	—0.20	107 ± 19	78—172	—0.09	NS
PRO	190 ± 84	97—403	+0.31	168 ± 73	93—400	—0.24	NS
THR	123 ± 18	102—162	+0.28	124 ± 33	67—196	—0.12	NS
4-ABA	11 ± 5	6— 19	+0.06	12 ± 6	4— 27	+0.27	NS
SER	157 ± 14	135—179	+0.09	167 ± 26	123—219	—0.26	NS
ASN	120 ± 13	99—141	+0.16	126 ± 19	94—158	—0.18	NS
MET	26 ± 5	20— 35	—0.28	23 ± 3	18— 29	—0.18	<0.05
PHE	59 ± 12	48— 86	+0.21	58 ± 11	40— 85	+0.15	NS
HYP	14 ± 13	6— 51	+0.47	10 ± 6	4— 26	+0.19	NS
GLN	481 ± 31	420—519	+0.18	461 ± 78	310—589	—0.02	NS
TYR	72 ± 24	46—132	+0.14	69 ± 15	35—110	—0.07	NS
ORN	181 ± 28	152—230	+0.83	177 ± 37	112—249	+0.03	NS
LYS	181 ± 28	149—239	+0.68	170 ± 21	121—216	—0.02	NS
ARG	36 ± 13	17— 60	+0.76	26 ± 8	14— 45	—0.42	<0.01
Subject age (years)	35 ± 13	19— 63	1.00	33 ± 10	21— 60	1.00	

*Probability of the difference being due to chance, based on the "two-tailed test". NS, not significant.

Studies in progress have indicated the importance of controlling specimen collection time in regard to time of day and to food intake. These factors are related to quite significant changes in the levels of many erythrocyte amino acids.

CONCLUSION

We have described in this report a simple, rapid and reliable technique for the determination of free amino acids in erythrocytes. Interfering substances, principally glutathione, were removed by cation- and anion-exchange resins, and the amino acids were converted to their N-acetyl-n-propyl esters, separated and quantitated by gas chromatography. The comparable accuracy and precision, as well as reduced analysis time and equipment cost of the GC method make it a practical alternative to classical ion-exchange chromatography. As such, it seems well suited to facilitate further research in the field of amino acid metabolism in health and disease.

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