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COMPUTERIZED DATA ANALYSIS OF AMINO ACIDS IN PHYSIOLOGIC FLUIDS

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SUMMARY

A Fortran IV computer program is described which identifies, computes, and statistically evaluates amino acid concentrations determined by an automatic amino acid analyzer in physiologic fluids. The program accepts retention time and integrated peak areas from two calibration standards for identification and computational reference. Two internal standards are used to compensate for variations in injector performance and ninhydrin decay. Calibration standard responses are statistically treated to assist in detection of equipment malfunction. The statistical data base is automatically expanded through inclusion of data from normal patients. Amino acid concentrations are printed with appropriate mean and standard deviation values in a format acceptable as a final report.

INTRODUCTION

The use of computers for repetitive calculations such as those required for amino acid analysis has become commonplace. Dedicated computer systems capable of receiving and analyzing data from one or more chromatographs are readily available; alternatively, larger computer systems can be utilized either on-line or through the use of punched cards.

Dedicated and on-line computer systems, of necessity, are matched to the output of the chromatograph and their use may be restricted; nevertheless, a number of papers have been published describing[1–5] the use of such systems. Exss *et al.*[6] experienced difficulty in programming a gradient eluting system for identification and computation because of elution time variations with changing amino acid concentrations. Other considerations complicating identification programs include the occurrence of unusual or unexpected peaks, integrator malfunction, and shifts in elution patterns with buffer decay or column changes. These problems can be easily resolved by visual inspection of the chromatographic tracing, and in physiologic samples such inspections are virtually mandatory to detect drug or other abnormal metabolite interference. For these reasons, some workers[7,8] prefer manual intervention using tape or punched cards which can be modified as necessary before input to a larger more

versatile computer. Fox[7] used such a system to not only identify and compute the concentrations of amino acids in tissues, but to compare concentrations in a number of tissues or perform statistical analyses on the same tissue.

Amino acid analyses in physiologic fluids are usually performed to detect changes in concentrations resulting from some metabolic disturbance whether congenital, dietary, or drug induced. Detection of such changes requires a knowledge of normal variation in the biologic population and the analytical methodology. It is desirable to incorporate statistical analysis as a portion of the computational program to facilitate comparison between groups of individuals and between individuals within a group.

This report describes a Fortran IV program which employs retention time and integrator output from two standard mixtures with two internal standards to identify and compute concentrations of the amino acids in physiologic fluids. Running means and coefficients of variance are printed out for the standards to facilitate detection of equipment malfunction. The computed amino acid concentrations in samples are compared to means and standard deviations for the appropriate age group and sample type (blood, urine, or cerebrospinal fluid). The amino acid concentrations which exceed the mean value, plus two standard deviations, are identified with the factor by which they exceed this value. Samples from normal patients are added to update the statistics and the results are printed in a format acceptable for inclusion in the patient's chart. This program will also compute concentrations of amino acids in protein hydrolysates and is capable of use with multiple amino acid analyzers operating in different modes.

EXPERIMENTAL

The Beckman 121 amino acid analyzer used for the majority of these analyses was operated in an accelerated mode, partially overlapping the basic amino acid output as outlined by Beckman[9]. This analysis program has a 9-h run time using sodium citrate buffers. Twelve-millimeter cuvettes were used to increase sensitivity; effluent optical density was displayed graphically on a Honeywell recorder and quantitatively via an Infotronics CRS 210 integrator with Teletype print out.

Calibration

Physiological amino acid standards (acidic and basic) were obtained from Pierce Chemical Company and diluted to 0.125 μmoles/ml with 0.1 N HCl containing norleucine and α-aminoguanidinopropionic acid to give a final internal standard concentration of 12.5 μg/ml.

Internal standards

Blood serum. Norleucine and α-aminoguanidinopropionic acid were added to sulfosalicylic acid to give a final concentration of 25μg/ml in 6% sulfosalicylic acid.

Urine. Norleucine and α-aminoguanidinopropionic acid were added to pH 2.2 citrate buffer to give a final concentration of 12.5 μg/ml.

Citrate buffers. Buffers were prepared from sodium citrate and hydrochloric acid as outlined by Beckman[9], using thiodiglycol as an antioxidant and pentachlorophenol as a mold inhibitor.

Ninhydrin. Ninhydrin was prepared according to the Beckman protocol[9], except that titanous chloride rather than stannous chloride was used as an antioxidant.

Sample preparation

Serum and cerebrospinal fluid were mixed with an equal volume of serum internal standard (sulfosalicylic acid). After centrifugation the supernatant was loaded directly into the sampling coils. Urinary creatinine was measured by the Jaffe reaction and diluted, if necessary, with deionized water to a creatinine concentration of approximately 50 mg/100 ml. Two milliliters of the dilute urine were adjusted to pH 10.0 with sodium hydroxide and lyophilized to remove excess ammonia. The residue was dissolved in two milliliters of urinary internal standard (buffer plus norleucine and α-aminoguanidinopropionic acid) and an aliquot loaded into the sampling coils.

Analysis

Chromatography of the amino acids followed the Beckman protocol[9]. Retention times and integrated peak areas from the integrator and the recorder tracing were inspected to assure proper equipment functioning. The integrator output was

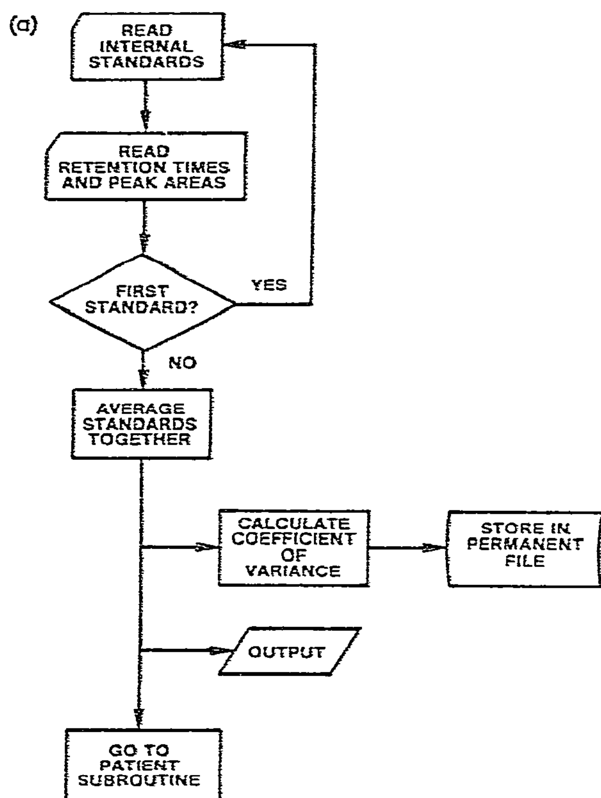

Fig. 1.

(*Continued on p. 296*)

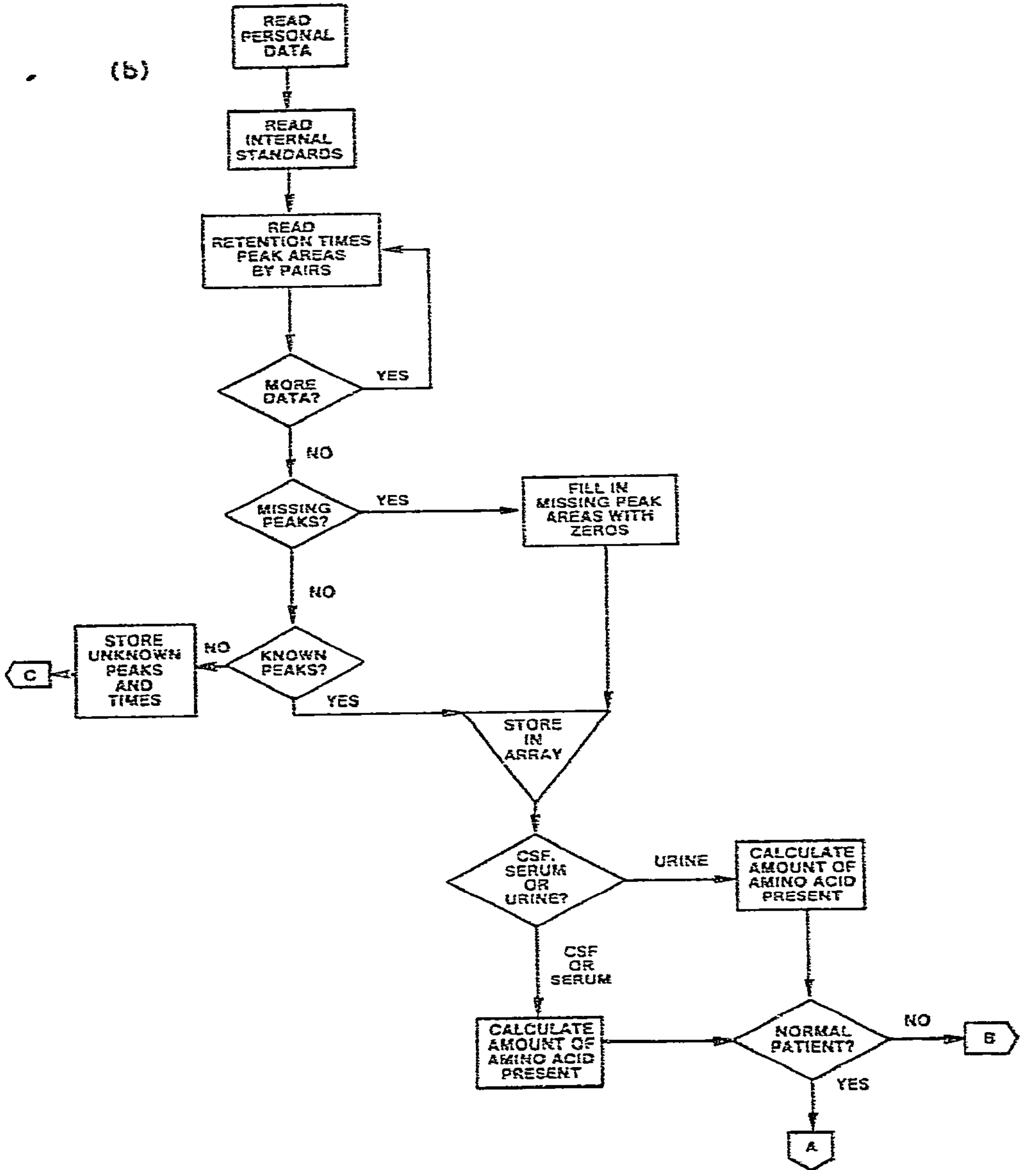

Fig. 1.

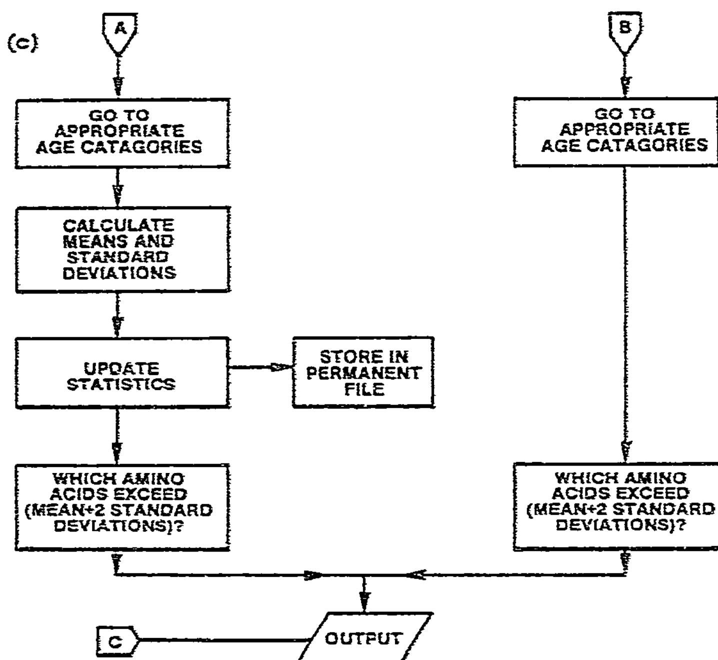

Fig. 1. (a) "Standard" subroutine. (b) "Patient" subroutine. (c) "Patient" subroutine.

keypunched along with appropriate patient identification data, then presented to the computer for data reduction.

The computer analysis of the output from the automatic amino acid analyzer was designed as a set of two programs and one permanent data file. The programs are used independently, but each uses the permanent data file. This set of programs could be adapted for use on any relatively large computer with capability for permanently storing data on either disks or tapes.

The first program, and the larger of the two, was actually a statistical and bookkeeping package composed of four main subroutines called Initialize, Store, Standard, and Patient; all of them need not be executed each time. This program was designed to create and update the data file. The subroutine "Initialize" erased all storage locations used by the permanent data file. The subroutine "Store" filed information needed by the programs such as names of amino acids and concentration of each amino acid in the standard solution. Subroutine "Standard" using information derived during calibration of the instrument, averaged retention times and integrated values (independently) for two standard chromatograms. These averages were stored for use in identification and quantification of amino acid concentrations in the samples. A running average of these values and their coefficient of variance was computed as an aid in detecting changes in instrumental performance. Fig. 1a is a diagram of this subroutine.

Running average was computed as

$$<X>_{n+1} = (n <X>_n + X_{n+1})/(n + 1)$$

where X was either the retention time or integrated value of the peaks and n was the number of samples.

Standard deviation was computed as:

$$\sigma_{n+1} = \sqrt{(\langle X^2\rangle_{n+1} - \langle X\rangle^2_{n+1})}$$

where

$$\langle X^2\rangle_{n+1} = (n\,\langle X^2\rangle_n + X^2_{n+1})/(n+1)$$

Coefficient of variance was computed as:

$$V = \frac{\sigma_{n+1}}{X_{n+1}}$$

The fourth part, called subroutine "Patient", considered the sample analysis and is diagrammed in Figs. 1b and 1c. It required entry of information desired for the final report, including patients name, sex, age, hospital number, location (hospital or town), physicians name, sample type (serum, urine, or other), creatinine concentration (if urine), laboratory number, date of receipt, date of analysis, and dilution factor if necessary. These data were followed by retention times and integrator responses for all the peaks in the chromatogram. This subroutine searched through the retention times of the sample and compared them to the known average retention times obtained from subroutine "Standard". Amino acids were identified on the basis of the peak retention times of the sample that closely approximated the known average from the calibration standard. Extra unidentifiable peaks were sorted out and missing amino acids (if any) were identified.

Having identified which amino acids were present, the subroutine computed the quantity of each present in the sample using the following appropriate equation:

$$X_1 = \text{SAMA}\cdot\frac{\text{CON}}{\text{AVEA}}\cdot\frac{\text{INST1}}{\text{INS1}}\cdot\frac{\text{DIL}}{\text{DILST}}\cdot\text{K}$$

or

$$X_2 = \text{SAMA}\cdot\frac{\text{CON}}{\text{AVEA}}\cdot\frac{\text{INST2}}{\text{INS2}}\cdot\frac{\text{DIL}}{\text{DILST}}\cdot\text{K}$$

where

X = amount of amino acid present in sample; 1 = acidic or neutral, 2 = basic amino acid

CON = concentration of amino acid calibration standard (μmoles/ml)
AVEA = the average integrated response value for the calibration standards
INST1 = the average integrated response for the first internal standard
INST2 = the average integrated response for the second internal standard
DILST = the dilution factor of the calibration standard solutions
SAMA = the integrated response for the sample
DIL = dilution factor of the sample
INS1 = the integrated response for the first internal standard in the sample

INS2 = the integrated response for the second internal standard in the sample
K = appropriate units for the type of output needed

This program was intended for serum or urine samples obtained from four different age groups and it maintained a running average of the values from all the normal patients to establish a mean or "usual" value for age and type of sample. It also kept an average of all patients including pathological cases as a matter of interest to compare with only normal patient values. The subroutine then computed the standard deviation of the normal values from the mean.

QUANTITATIVE AMINO ACID ANALYSIS
ANALYZED BY THE LABORATORY OF BIOCHEMICAL GENETICS
DEPARTMENT OF PEDIATRICS
UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER AT HOUSTON
MEDICAL SCHOOL
HOUSTON, TEXAS

PATIENT NAME: HOSPITAL NO. 751-05634
SEX: FEMALE
AGE: 2 YEARS 0 MONTHS LAB NO. 9436F
PHYSICIAN: DR. HOWELL
LOCATION: HERMANN
SPECIMAN TYPE: URINE CREATININE CONC. 40.0 MG PERCENT
DATE RECEIVED: 05-26-75 DILUTION FACTOR 1.00
DATE ANALYZED: 06-03-75 TOTAL NO. OF PEAKS 40

AMINO ACID	PATIENT RESULT MICRO M/ G CREAT.	USUAL VALUE FOR AGE	STANDARD DEVIATION	PEAK NO.	RET TIME	PEAK AREA
PHOSPHOSERINE	118.8	170.4	86.2	1	28.0	47521.0
PHOSPHOETHANOLAMINE	42.7	381.8	178.5	2	39.0	18648.0
TAURINE	326.3	488.5	420.7	3	45.0	149496.0
ASPARTIC ACID	36.7	27.8	25.8	4	85.0	22397.0
THREONINE	243.6	249.7	105.2	5	92.0	153171.0
SERINE	593.6	583.3	267.1	6	99.0	409662.0
ASPARAGINE	1309.9	502.3	168.2	7	104.0	943032.0
PROLINE	243.0	9.3	29.5	8	129.0	25088.0
GLUTAMIC ACID	47.3	56.8	55.3	9	135.0	31284.0
CITRULLINE	0.0	.2	.8	10	146.0	0.0
GLYCINE	1932.8	1342.8	461.5	11	156.0	1281749.0
ALANINE	646.0	517.6	273.0	12	164.0	498523.0
A-AMINOADIPIC ACID	52.9	122.7	159.6	13	175.0	45461.0
A-AMINO-N-BUTYRIC A	602.1	54.2	66.2	14	186.0	457470.0
VALINE	23.3	47.6	50.0	15	209.0	17079.0
CYSTINE	29.7	2.5	5.5	16	223.0	27465.0
METHIONINE	49.3	89.4	100.9	17	265.0	29852.0
ISOLEUCINE	40.1	216.0	89.6	18	269.0	27915.0
LEUCINE	0.0	75.4	62.9	19	275.5	0.0
TYROSINE	147.3	235.1	134.6	20	309.0	102536.0
PHENYLALANINE	74.3	176.8	84.7	21	317.0	47661.0
BETA-ALANINE	0.0	3.6	4.0	22	330.5	0.0
B-AMINOISOBUTYRIC A	433.5	949.2	525.6	23	345.0	238437.0
ORNITHINE	251.9	566.6	263.4	24	392.0	221138.0
1-METHYL HISTIDINE	220.8	246.0	186.9	25	416.0	167691.0
LYSINE	156.5	82.7	67.4	26	425.0	174258.0
HISTIDINE	682.0	1164.3	300.0	27	436.0	782524.0
3-METHYL HISTIDINE	149.7	187.7	106.6	28	448.0	194970.0
ARGININE	93.9	68.3	279.3	29	560.0	76519.0

COMMENTS:

Fig. 2. Computer output: Patient copy.

The second program was devised in the interest of economy, to protect the statistical data from errors, and to assist in proof reading of data. It is an abbreviated version of the first program using identical techniques for sample analysis, but performing no statistical bookkeeping. It uses the information stored on the permanent data file when needed. The second program produces a very specialized output suitable for inclusion on a patient's chart by a physician.

RESULTS

Fig. 2 represents the computer-generated report. Since it is more rapid and economical to print multiple reports for laboratory and physicians' files than make copies, four copies of the report were printed, followed by the data shown in Fig. 3. The size of the normal population, with which the patient was compared, was listed

PATIENT NAME: LAB NO. 9436F

THIS PATIENT WAS CONSIDERED ABNORMAL

11 NO. OF NORMAL PATIENTS ACCUMULATED IN THIS AGE GROUP.

THE FOLLOWING WERE IDENTIFIED AS EXTRA PEAKS OR INTERNAL STANDARDS.

RET. TIME	PEAK AREA	
51.0	4269288.0	UREA
67.0	206744.0	
71.0	522638.0	
171.0	9701.0	
195.0	2449.0	
231.0	4721.0	
260.0	762313.0	FIRST INTERNAL STANDARD
371.0	26478.0	BUFFER CHANGE
383.0	3921.0	
399.0	2659275.0	
461.0	313265.0	
456.0	77676.0	
477.0	146657.0	SECOND INTERNAL STANDARD
487.0	148535.0	
498:0	113496.0	
511.0	169780.0	
549.0	61194.0	

THE FOLLOWING PEAKS EXCEED (MEAN + 2 STD DEVIATIONS)

AMINO ACID	PATIENT RESULT MICRO M/ G CREAT.	USUAL VALUE FOR AGE	STANDARD DEVIATION	PEAK NO.	RATIO PAT/ (M+2S)
ASPARAGINE	1309.9	582.3	168.2	7	1.6
PROLINE	243.0	9.3	29.5	8	3.6
A-AMINO-N-BUTYRIC A	602.1	54.2	66.2	14	3.2
CYSTINE	29.7	2.5	5.5	16	2.2

Fig. 3. Computer output: Evaluation of sample (laboratory only).

STATISTICAL INFORMATION FOR URINE SAMPLES

NUMBER OF NORMAL PATIENTS ACCUMULATED IN EACH AGE GROUP.

	0 TO 1 MO.		1 MO. TO 6 MO.		6 MO. TO 5 YRS.		5 YRS. TO ADULT	
	4.0		1.0		1.0		5.0	
AMINO ACID	USUAL VALUE	STD DEV	USUAL VALUE	STD DEV	USUAL VALUE	STD DEV	USUAL VALUE	STD DEV
PHOSPHOSERINE	126.0	49.6	17.0	0.0	69.5	0.0	85.1	29.0
PHOSPHOETHANOLAMINE	139.7	46.1	41.2	0.0	328.0	0.0	288.0	155.6
TAURINE	1116.7	835.1	31.7	0.0	1555.9	0.0	1076.1	1110.0
ASPARTIC ACID	21.3	17.1	8.2	0.0	80.3	0.0	13.8	27.6
THREONINE	156.3	68.6	39.4	0.0	270.5	0.0	212.5	161.4
SERINE	462.2	96.3	129.1	0.0	635.9	0.0	874.8	355.0
ASPARAGINE	230.0	56.4	195.4	0.0	820.0	0.0	384.6	387.6
PROLINE	40.3	61.9	0.0	0.0	0.0	0.0	0.0	0.0
GLUTAMIC ACID	87.6	77.1	15.7	0.0	81.5	0.0	102.2	69.7
CITRULLINE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GLYCINE	1572.0	315.1	249.9	0.0	1511.1	0.0	1153.9	1263.0
ALANINE	203.2	75.0	65.7	0.0	627.9	0.0	210.6	159.4
A-AMINOADIPIC ACID	0.0	0.0	74.7	0.0	42.1	0.0	35.4	43.3
A-AMINO-N-BUTYRIC A	0.0	0.0	16.0	0.0	0.0	0.0	1.9	3.0
VALINE	11.0	21.4	19.2	0.0	18.1	0.0	30.3	44.0
CYSTINE	40.1	30.6	29.4	0.0	0.0	0.0	24.0	32.6
METHIONINE	21.9	33.5	13.7	0.0	134.4	0.0	72.0	82.2
ISOLEUCINE	45.2	51.6	26.0	0.0	157.2	0.0	57.6	47.5
LEUCINE	42.5	23.0	0.0	0.0	69.2	0.0	46.5	48.5
TYROSINE	82.9	52.3	22.9	0.0	204.3	0.0	179.3	121.5
PHENYLALANINE	54.2	61.3	15.8	0.0	114.1	0.0	147.6	82.3
BETA-ALANINE	1.7	3.9	0.0	0.0	0.0	0.0	0.0	0.0
B-AMINOISOBUTYRIC A	133.1	110.0	70.3	0.0	169.6	0.0	124.2	85.6
ORNITHINE	533.7	140.3	603.5	0.0	316.2	0.0	307.2	234.5
1-METHYL HISTIDINE	33.1	43.2	234.9	0.0	127.4	0.0	108.1	52.4
LYSINE	280.8	62.2	1228.1	0.0	108.0	0.0	110.5	87.2
HISTIDINE	205.1	90.7	2698.3	0.0	1477.3	0.0	690.8	634.0
3-METHYL HISTIDINE	161.6	36.2	364.7	0.0	326.1	0.0	306.2	107.7
ARGININE	4.7	10.5	58.5	0.0	0.0	0.0	50.8	95.6

USUAL VALUE FOR THE DIFFERENT AGE CATEGORIES
EXPRESSED IN MICRO MOLES/GRAM CREATININE

Fig. 4. Computer output: Sample statistics.

INFORMATION FROM SUBROUTINES INITIALIZE AND STANDARD.

RANGE +OR- 2.5 DILUTION FACTOR 20.0 NUMBER OF STANDARDS 4.0

AMINO ACID	PEAK NO.	CON	FIRST STANDARD RET TIME	FIRST STANDARD PEAK AREA	SECOND STANDARD RET TIME	SECOND STANDARD PEAK AREA	AVERAGE RET TIME	COEF. OF VARIANCE	AVERAGE PEAK AREA	COEF. OF VARIANCE
PHOSPHOSERINE	1	1.16	29	113311	29	127509	29.0	.014	120410.0	.130
PHOSPHOETHANOLAMINE	2	1.16	48	111599	40	137902	40.0	.016	124750.5	.131
TAURINE	3	1.16	47	121818	48	141964	47.5	.012	131491.0	.131
ASPARTIC ACID	4	2.32	90	250233	92	317707	91.0	.012	200000.0	.153
THREONINE	5	2.32	96	236105	100	265913	98.0	.019	251049.0	.122
SERINE	6	2.32	103	253071	107	306940	105.0	.016	279915.5	.136
ASPARAGINE	7	1.16	109	111516	114	151113	111.5	.010	131314.5	.214
PROLINE	8	2.32	130	59922	130	46763	130.0	.009	53342.5	.087
GLUTAMIC ACID	9	2.32	141	234093	149	309766	145.0	.010	271920.5	.105
CITRULLINE	10	.90	150	63100	150	130947	154.0	.017	97023.5	.208
GLYCINE	11	2.32	163	258623	170	333310	166.5	.017	295966.5	.143
ALANINE	12	2.32	171	250338	179	331971	175.0	.010	291154.5	.160
A-AMINOADIPIC ACID	13	.50	181	66644	191	120463	186.0	.015	93553.5	.324
A-AMINO-N-BUTYRIC A	14	2.32	193	270907	203	343265	198.0	.017	307086.0	.190
VALINE	15	2.32	216	235034	227	369024	221.5	.017	302029.0	.176
CYSTINE	16	1.16	226	96567	246	225390	236.0	.027	160978.5	.331
METHIONINE	17	2.32	266	315552	266	301182	266.0	.003	340367.0	.122
ISOLEUCINE	18	2.32	270	292551	272	342569	271.0	.002	317560.0	.116
LEUCINE	19	2.32	277	274401	278	335068	277.5	.002	305171.5	.124
TYROSINE	20	2.32	311	251542	313	368369	312.0	.003	310055.5	.192
PHENYLALANINE	21	2.32	319	236944	321	353427	320.0	.003	295185.5	.235
BETA-ALANINE	22	1.16	333	70469	334	175507	333.5	.000	123008.0	.453
B-AMINOISOBUTYRIC A	23	2.32	348	181108	349	309247	348.5	.009	245177.5	.299
ORNITHINE	24	2.32	390	294529	390	345614	390.0	.003	320071.5	.120
1-METHYL HISTIDINE	25	2.32	414	239496	415	308876	414.5	.003	274186.0	.082
LYSINE	26	2.32	425	314344	425	369510	425.0	.005	341931.0	.078
HISTIDINE	27	2.32	433	288355	434	338966	433.5	.004	313660.5	.079
3-METHYL HISTIDINE	28	2.32	445	287371	446	338573	445.5	.004	312972.0	.069
ARGININE	29	2.32	556	333988	558	462018	557.0	.003	398003.0	.221
1ST INTERNAL STANDARD			282	222060	284	310232				
2ND INTERNAL STANDARD			475	139302	475	186164				

Fig. 5. Computer output: Calibration statistics.

with retention times and peak areas of unidentified ninhydrin-positive components. Abnormal amino acid concentrations were identified and the factor by which they exceeded the mean plus two standard deviations was printed.

Other statistical information recovered included the number of normal patients accumulated in each age group with the mean values, and the total number of patients in each age group, with the mean concentrations for amino acid, as shown in Fig. 4. In addition, mean retention times and integrator responses for calibration standards (Fig. 5) were filed with their coefficient of variance for comparison with current standards.

DISCUSSION

Computer identification of amino acids eluted from a column is a formidable task, particularly when physiologic fluids are the source. This task is more reliably performed where technical interaction with the computer is possible. Our solution to this problem was the simplest approach consistent with reliable performance. Retention times were determined with each new buffer and ninhydrin preparation and the standard mixtures were analyzed again just before the buffer supply was exhausted. It was assumed that buffer (and ninhydrin) decay is a linear phenomenon and that averaging these standard retention times (and responses) would closely approximate the true values over a one-week period. A small variation in retention time, determined by the operator, was allowed. If an amino acid had a greater variation in retention time it would not be identified by the computer but would be printed out as an unidentified peak. The operator would, at his discretion, assign the proper retention time by manipulation of data while punching cards.

Another problem associated with amino acid analysis in an automated system is that of nonreproducible responses, whether through variations in sample application or ninhydrin reactivity. The use of internal standards was an effort to compensate for these effects, and since we used a two-column system, two internal standards were desirable. This program normalized the response of the unknown amino acids with that of the calibration standard since both had the same concentration of internal standard.

Although other programs, some of which are quite elaborate, have been published for identification and computation of amino acids, none analyze and present physiologic data in a format acceptable as a final report. It is our opinion that this is a desirable option which should be available to those who have access to computers of requisite capacity. Modification of the program by limiting the number of populations and thus the statistical data file could diminish this requirement, though not sufficiently for use with desk top calculators. Use of programmable calculators in amino acid analysis will be the subject of a future report.

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