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A RAPID CHROMATOGRAPHIC DETERMINATION OF PHENYLALANINE AND TYROSINE FOR PHENYLKETONURIA SCREENING

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SUMMARY

A micromethod is reported for the rapid determination of phenylalanine and tyrosine in plasma or serum samples by employing a new system of automatic amino acid ion-exchange column chromatography previously described, but provided in this case with four resin columns. While two of them are used for analysis on two separate channels, the other two are regenerated. In this way every fifty minutes four analyses are obtained. The resin employed is Aminex A5 operated in a lithium cycle. Peaks are digitized by simply measuring their heights over the base line, as described in the new analytical system. Data processing, including determination of weight and molar phenylalanine/tyrosine ratios, by means of an Olivetti Programma 101 desk-top computer is described and the program instructions for the magnetic card are also reported.

INTRODUCTION

Phenylketonuria is a genetic defect which prevents the patient from properly metabolizing the essential amino acid phenylalanine and causes irreversible damage to the central nervous system, frequently resulting in mental retardation[1]. The disease is characterized by elevated blood levels of phenylalanine and excessive urinary excretion of phenylpyruvic acid; this is caused by a deficiency in the liver enzyme, phenylalanine hydroxylase, which governs the first step of the normal metabolic pathway of phenylalanine, *i.e.*, its conversion to tyrosine[2-5].

It is now generally accepted that if the disease is promptly diagnosed at birth, a low-phenylalanine diet[6] will permit the infant's brain to develop normally. Thus the extreme urgency of a prompt and accurate determination is indicated if mental retardation is to be avoided.

Two types of screening procedure on newborns have been applied to early diagnosis of phenylketonuria. They are based either on the detection of excess phenylpyruvic acid in the urine or elevated phenylalanine in blood.

The ferric chloride test for urinary phenylpyruvic acid is limited in its accuracy in phenylketonuria detection, in that affected newborns may only begin to excrete phenylpyruvic acid after 4–8 weeks[7-9] and in some cases this excretion may not be consistent[10-12]. Blood phenylalanine levels, on the other hand, have been shown to reach toxic values after a few days of life[13,14].

For the determination of blood phenylalanine levels several procedures have been developed, although at present only two techniques are generally accepted. One was devised by GUTHRIE[15,16] for mass screening and it is based on the following principle. β-2-Thienylalanine inhibits the growth of *Bacillus subtilis* in a minimal growth medium. The addition of phenylalanine to the medium reverses the inhibition and allows growth of the organism. The GUTHRIE method is a semi-quantitative screening test and it is not specific for phenylalanine, but positive samples can be repeated with a more quantitative procedure such as the fluorimetric technique devised by MCCAMAN AND ROBINS[17], which is based on the principle that phenylalanine when reacted with ninhydrin in the presence of L-leucyl-L-alanine produces a strongly fluorescent compound.

But these two methods, which are widely employed in phenylketonuria mass screening, and both at the present time based on the small amount of blood that can be collected on the small paper discs of the collection cards[18,19], will only distinguish (with variable precision) individuals presumed positive for phenylketonuria.

As a matter of fact, it has been shown that an elevated phenylalanine blood level in premature infants may be of a transient nature only and not in itself be indicative of phenylketonuria. Actually 90 % of individuals in which the blood phenylalanine level is found to be higher than normal at the time of birth are not phenylketonuric patients[2,20]. They are only newborns in which the metabolic system has not reached a state of maturation at the time of birth and in these cases a high blood tyrosine level can be observed in addition to the high phenylalanine level.

Thus elevated phenylalanine levels in an infant may not necessarily be indicative of phenylketonuria, but may be only a transient elevation due to immaturity. If in this instance a serum tyrosine determination discloses an elevated tyrosine level as well, phenylketonuria may possibly be ruled out.

Consequently the presumed positive individuals for phenylketonuria must additionally be tested for tyrosine.

Another case where both blood phenylalanine and tyrosine levels must be determined is when heterozygosity for phenylketonuria has to be checked[21]. Taking into account that according to PERRY *et al.*[22] possibly 1 in 70 persons may be a heterozygote for phenylketonuria, it is to be hoped that information concerning heterozygosity for phenylketonuria will become widely requested in genetic counselling. Moreover, in genetic counselling of close relatives of phenylketonurics this information will be used to eliminate unnecessary worrying by prospective parents or prevent the birth of phenylketonuric infants.

By determining, by means of two spectrofluorimetric techniques, the fasting serum phenylalanine level and the fasting phenylalanine/tyrosine ratio, PERRY *et al.*[21] were able to classify accurately about two-thirds of the subjects tested for heterozygosity, although the remainder of the subjects tested fell into the zone of overlap between homozygous normal and heterozygous individuals and hence could not be classified accurately. PERRY *et al.*[22] in a more recent paper reported that this poor degree of precision is partly due to the fact that two fluorimetric techniques had to be used, one for determining the phenylalanine level and the other for the tyrosine level. Tyrosine contributes at least 5 % fluorescence relative

to phenylalanine[23] and thus adversely affects the phenylalanine/tyrosine ratio. For this reason automatic amino acid chromatography was recommended for obtaining this ratio[22], in that it simplifies the test and increases its reliability, in spite of the fact that the analytical technique employed required 6 h for one response.

In this paper a particular application of a new system of automatic ion-exchange amino acid analysis previously described by MONDINO[24,25] is reported. Four short resin columns eluted with a lithium buffer are used and allow four determinations of phenylalanine and tyrosine every fifty minutes.

APPARATUS AND MATERIALS

An apparatus for automatic amino acid analysis described by MONDINO in a previous paper[24] and at the present time manufactured by Optica Co., Milan, was employed. According to the flow diagram in Fig. 1, four resin columns were employed on two separate channels. Each channel consisted of three pumps (one for the buffer employed for eluting the chromatogram, one for the ninhydrin color reagent and one for the buffer employed for the equilibration of the resin), two columns, a flow switch, a reaction coil, a photometer and a recorder and both channels could operate simultaneously.

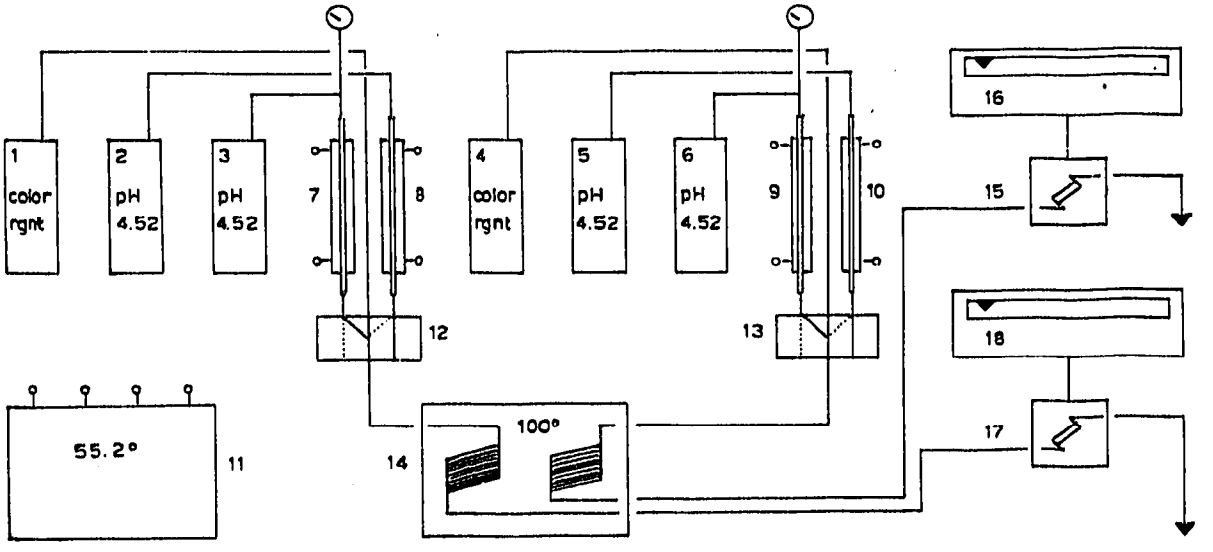

Fig. 1. Flow diagram of the analytical system. (1)(2)(3) Pumps of the first channel; (4)(5)(6) pumps of the second channel; (7)(8) columns of the first channel; (9)(10) columns of the second channel; (11) water circulating bath for column thermostating; (12) flow switch of the first channel; (13) flow switch of the second channel; (14) 100° reaction bath containing the coils of both channels; (15) colorimeter of the first channel; (16) recorder of the first channel; (17) colorimeter of the second channel; (18) recorder of the second channel.

Pumps

The pumps for the eluting buffer are operated at 1 ml/min while the ninhydrin color reagent pumps operate at 0.5 ml/min. The pumps which deliver the equilibrating buffer operate at 1.4 ml/min.

Columns

Four Pyrex glass columns of 0.7 cm I.D. were employed. Their total length was 30 cm, while the resin bed was only 15 cm high. While one column of a channel is doing the analysis the other one is washed with lithium hydroxide and equilibrated with the working buffer. The columns are jacketed and thermostated in parallel at 55.2° by means of a thermoregulated circulating water-bath.

Flow switches

Two flow switches specially developed by the Optica Co. were used, one for each pair of columns. These valves allow connection of each column of a pair either to the reaction coil (when the column is under elution), in which case they operate also as mixing devices for the column effluent and the ninhydrin color reagent, or to waste (when the column is under regeneration).

Reaction coils

Two reaction coils made of Teflon tubing of 0.5 mm I.D., each 30 m long and immersed in the same boiling water-bath, were employed. The total flow rate of 1.5 ml/min (1 ml of eluate plus 0.5 ml of color reagent) means that the reaction mixture remains at 100° for 4 min. This reaction time allows precise and accurate results.

Photometers

The system employs two photometers equipped with interchangeable interference filters both reading at 570 nm and containing cylindrical flow-cuvettes with 10-mm light paths. The photosensitive element is a photomultiplier, whose electronic circuit includes a range expander which permits a choice of sensitivity of either 0.1, 0.25, 0.5, 1.0 or 2.0 O.D. units for a full-scale deflection and gives a linearized signal. For this work the sensitivities employed were 1.0 and 0.5 O.D.

Recorders

Two solid-line recorders were employed, one for each channel, which recorded the linearized signal coming from the photometers at 3-in./h chart speed. Other chart speeds are available.

Resin and buffer

Aminex A5 resin purchased from Biorad (München) was employed in a lithium cycle as already described by MONDINO *et al.* in a preceding note[26].

The buffer employed for eluting and equilibrating the resin columns is a lithium citrate buffer used as previously reported[25,26] for the analysis of basic amino acids and related compounds. It has a pH of 4.52 and the following composition: lithium concentration, 0.6 *N*; citrate concentration, 0.2 *M*; lithium citrate tetrahydrate, 56.4 g/l,; concentrated hydrochloric acid, 17.5 ml/l; 10% Brij solution, 2 ml/l; phenol, 1 g/l. The correction to pH 4.52 is made by addition of hydrochloric acid.

Lithium hydroxide

An aqueous solution of 1 *N* lithium hydroxide (23.95 g/l) is employed for washing the columns after each analysis.

Ninhydrin color reagents

This was prepared according to the method described by MONDINO in previous papers[24,25].

Phenylalanine and tyrosine calibrating solution

A solution containing 62.5 μmoles/l of phenylalanine and tyrosine and of all the other acidic, neutral and basic amino acids and related compounds normally found in biological fluids, was made by employing chemicals purchased from Fluka (Buchs F.G., Switzerland)

Human blood plasma

A pool of human blood plasma obtained from the clinical laboratory of the local hospital and blood samples obtained from 30 adult normal healthy and fasting volunteers were employed. For deproteination a 10 % solution of sulfosalicylic acid is added to an equal volume of plasma. The solution is then shaken and centrifuged. 0.4 ml of supernatant, corresponding to 0.2 ml of plasma, are loaded on to the columns. If small centrifuge tubes are employed, 0.3–0.4 ml of plasma is enough for a determination.

METHOD

Suppose that columns 8 and 10, as illustrated in the flow diagram in Fig. 1, are equilibrated and ready for analysis just as columns 7 and 9 have finished a chromatogram. On the drained surface of the resin of columns 7 and 9, 2 ml of 1 *N* lithium hydroxide are carefully introduced, then the columns are filled with pH 4.52 buffer and equilibration is started. On the drained surface of the resin of columns 8 and 10, 0.4 ml of the solution which has to be analyzed and then 0.4 ml of pH 4.52 buffe rare forced in under air pressure. The columns are then filled with buffer and the analyses are started. After 25 min the peaks of tyrosine and phenylalanine are completed and the analyses are finished. At the same time columns 7 and 9 have been washed and equilibrated and are ready for a subsequent analysis. At this point the flow switch of the first channel is operated in order to connect column 7 with the color reagent stream coming from pump 1 and with the reaction coil, and also to send to waste the eluate of column 8. The flow switch of the second channel is simultaneously operated in order to connect column 9 with the color reagent stream coming from pump 4 and with the other reaction coil, and to send to waste the eluate of column 10. For columns 8 and 10 the operation of washing and equilibrating can now take place while a new analysis can be set up on columns 7 and 9.

Operating in this way, every 25 min two analyses can be obtained and a routine daily schedule can give thirty to forty such analyses.

The ninhydrin color reagent contained in the pumps of both channels is 500 ml in volume; considering that the reagent consumption per analysis is less than 15 ml, one charge will last a day.

The chromatograms obtained are evaluated by simply measuring in millimeters the heights of the peaks over the base line. This is the most interesting feature of our analytical system and, as previously discussed[24,25,27], is due to the exact repeatibility which can be obtained from chromatogram to chromatogram; as

has been demonstrated[27], in our system this method of peak digitizing gives greater precision than that of the method of height × width integration, and moreover is much faster.

RESULTS AND DISCUSSION

Thirty-two chromatograms of 0.4 ml of the calibration mixture containing a 25 nmoles of tyrosine and phenylalanine were run on the four columns. The results are reported in Table I. The mean values, the standard deviations and the coefficients of variation are also given for evaluating the degree of precision. It can also be seen that there is no appreciable difference from column to column.

In order to evaluate the degree of accuracy and the linearity of response of

TABLE I

REPEATED DETERMINATIONS OF 25 nMOLES OF TYROSINE AND PHENYLALANINE

The results are expressed as peak heights in mm over the base line.

	Column							
	1		*2*		*3*		*4*	
	Tyr	*Phe*	*Tyr*	*Phe*	*Tyr*	*Phe*	*Tyr*	*Phe*
	69.8	62.2	68.2	61.1	70.0	62.4	70.0	62.5
	69.0	61.3	69.1	61.6	69.6	61.9	70.2	61.9
	69.2	62.0	69.0	61.3	69.0	61.4	70.0	62.0
	69.2	62.1	68.9	61.9	69.2	61.4	68.9	61.9
	69.0	61.7	68.9	61.4	69.1	61.5	68.6	61.8
	70.0	61.0	69.5	61.2	68.6	61.0	69.2	61.3
	69.0	61.9	69.0	61.0	69.0	61.3	69.2	61.0
	69.5	62.5	70.0	62.0	70.1	62.2	68.9	61.2
Mean	69.3	61.8	69.1	61.6	69.3	61.6	69.4	61.7
S.D.	0.39	0.48	0.50	0.34	0.51	0.47	0.60	0.48
Coeff. of variation	0.56	0.77	0.72	0.56	0.73	0.76	0.84	0.77

the analytical system and at the same time the efficiency of the deproteination method, the following experiment was performed.

Eight chromatograms were run on eight aliquots of the pooled plasma, which were processed as follows. To 0.5 ml of plasma, 0.5 ml of 10 % sulfosalicylic acid solution was added. After centrifugation, 0.4 ml of the supernatant was loaded on to the columns.

Eight chromatograms of 0.4 ml of a standard solution containing 25 nmoles of tyrosine and phenylalanine in 0.4 ml of 5 % sulfosalicylic acid solution were also run. This standard solution was prepared by diluting with an equal quantity of water a solution containing 50 nmoles of the two amino acids in 0.4 ml of 10 % sulfosalicylic acid.

Finally, eight chromatograms of eight aliquots of the pooled plasma, which were deproteinated by addition of an equal volume of the 10 % sulfosalicylic acid solution containing 50 nmoles of tyrosine and phenylalanine per 0.4 ml, were run. In this way, to the 0.4 ml of the supernatant loaded on to the columns, 25 nmoles of the two amino acids were added. The heights of the peaks obtained were expected

TABLE II

RECOVERY TEST

The results are expressed as peak heights in mm.

	Calibration mixture (25 nmoles)		*Pooled plasma*		*Calibration mixture (25 nmoles) plus pooled plasma*	
	Tyr	*Phe*	*Tyr*	*Phe*	*Tyr*	*Phe*
	70.0	62.0	49.9	27.8	117.5	88.7
	70.0	62.0	49.2	28.0	118.2	89.2
	68.5	61.0	48.4	27.5	119.0	89.0
	68.8	60.5	49.2	28.2	116.5	87.5
	68.8	61.0	49.2	28.0	116.0	87.4
	68.5	61.2	48.4	27.5	117.5	88.9
	69.0	61.5	49.0	28.2	117.2	89.6
	69.0	61.1	48.5	28.0	118.2	88.6
Mean	69.1	61.3	49.0	27.9	117.5	88.6
S.D.	0.60	0.52	0.51	0.26	0.97	0.78
Coeff. variation	0.87	0.85	1.04	0.93	0.85	0.90
Expected values					118.1	89.2
Recoveries					99.5%	99.3%

TABLE III

RESULTS OF PLASMA FROM 15 NORMALLY HEALTHY FASTING ADULTS

Sex	*Peak height (H mm)*		*mg/100 ml*		*Phe/Tyr by wt.*	*Phe/Tyr molar*
	Tyr	*Phe*	*Tyr*	*Phe*		
M	42.0	24.5	1.37	0.83	0.60	0.66
	43.5	26.0	1.42	0.88	0.62	0.68
M	51.0	23.0	1.67	0.78	0.47	0.51
	51.0	23.5	1.68	0.80	0.48	0.52
M	54.2	32.0	1.79	1.09	0.61	0.67
	55.5	32.0	1.83	1.09	0.60	0.66
F	42.0	22.0	1.38	0.75	0.54	0.59
	43.0	22.5	1.42	0.77	0.54	0.59
M	52.5	30.0	1.73	1.02	0.59	0.64
	52.5	30.0	1.73	1.02	0.59	0.64
M	48.0	31.5	1.58	1.07	0.68	0.74
	49.0	32.0	1.61	1.09	0.67	0.73
F	48.5	27.2	1.60	0.93	0.58	0.63
	49.0	26.0	1.61	0.89	0.55	0.60
M	55.0	28.0	1.81	0.95	0.53	0.58
	54.0	27.0	1.78	0.92	0.52	0.57
M	48.5	32.8	1.60	1.12	0.70	0.77
	51.2	34.2	1.69	1.16	0.69	0.75
M	45.5	24.8	1.48	0.83	0.56	0.61
	45.5	25.0	1.48	0.84	0.56	0.61
F	52.5	25.5	1.71	0.86	0.50	0.55
	53.5	25.2	1.71	0.85	0.48	0.52
M	42.5	28.0	1.39	0.94	0.67	0.73
	42.0	27.5	1.37	0.92	0.67	0.73
M	48.5	30.0	1.58	1.01	0.63	0.69
	48.3	30.0	1.57	1.01	0.64	0.70
M	50.2	24.8	1.64	0.83	0.50	0.55
	48.9	23.7	1.59	0.79	0.49	0.53
M	54.8	34.0	1.79	1.14	0.63	0.69
	54.7	33.8	1.78	1.14	0.64	0.70

to be equal to the sum of the heights of the peaks of plasma alone plus the heights of the peaks of the standard solution.

The results obtained, reported in Table II, can be considered very satisfactory in that the values found for tyrosine are 99.5 % and those of phenylalanine are 99.3 % of the calculated values.

In Table III the results for 15 blood plasma samples, collected from 15 adult healthy fasting volunteers found in our laboratory, are reported. The blood samples were centrifuged and deproteinated immediately after collection; the analyses were performed in duplicate. It should be noted that the phenylalanine/tyrosine ratios are rather lower than the mean value reported by PERRY *et al.*[22] when employing a chromatographic method of analysis. It would be advisable that a screening of very large populations from different countries or regions were carried out in order to ascertain if different life conditions or genetic factors may have some influence on this ratio.

It must be stressed that the determination of these two amino acids must be carried out on plasma samples processed immediately after collection from the patient if true values are to be obtained[23].

As far as the resin is concerned, a crushed Amberlite IR-120 prepared as previously described by MONDINO[24,25] was also tried and eluted with sodium and lithium buffers. Sodium buffers were also tried for the elution of Aminex A5 resin. The best results in the shortest time were obtained by employing the Aminex A5 resin in a lithium cycle, according to the conditions previously indicated. In this way a very heavy working schedule giving satisfactory repeatability is feasible, even if the operating conditions, as far as resin equilibration is concerned, do not rigorously fulfil the conditioning requirements. As a matter of fact the working schedule reported here, which permits 4 analyses every 50 min, allows only 20 min for washing the resin with lithium hydroxide and equilibrating it with the working buffer. These operations are carried out by introducing 2 ml of 1 *N* lithium hydroxide into the column over the drained surface of the resin bed and pouring over them the 0.6 *N* buffer of pH 4.52, which afterwards is pumped through the resin at 1.4 ml/min. The amount of hydroxide is the least that permits the release from the resin of the amino acids, polypeptides and compounds contained in the sample, but the quantity of working buffer fed to the resin and particularly the time of conditioning are not enough for a thorough equilibration of the resin column to occur. In spite of this, the Aminex A5 resin gives perfect reproducibility as far as the required determination of tyrosine and phenylalanine is concerned. But it must be emphasized that, if the chromatogram were continued after the phenylalanine separation, separation of subsequent amino acids and related compounds would not be obtained, owing to the incomplete equilibration of the resin bed.

Furthermore, it must be mentioned that in this case a spherical resin having a narrow classification degree, such as Aminex A5, is to be preferred to a crushed one as far as the column packing is concerned. If one considers that this system is capable of performing more than 200 analyses weekly, it is advisable that at least once a week the resin be washed on a suction funnel with strong hydrochloric and nitric acids and with boiling hydroxide[24,25]. Consequently once a week a new packing of the four columns is recommended. Using this spherical resin a perfect packing can be easily obtained even if a slurry in an equal volume of a

pH 4.52 buffer is employed. This is not possible with a crushed resin[20]. Since the resin bed employed has a volume no higher than half that of the glass columns used, the packing of the resin can be obtained by filling the glass column with the 1:1 resin slurry and then by pumping the buffer, which will settle the resin bed at half the height of the column. This operation does not last more than 30 min. Consequently, if the operator does have an extra resin batch available, clean and suspended in pH 4.52 buffer, changing the resin in all the columns will not seriously interfere with his working schedule.

The use of a lithium buffer is justified because for the elution of these amino acids from the resin a Li^+ molarity of 0.6 is required. This molarity is not very far from the molarity of the hydroxide required for satisfactory washing of the resin. If sodium buffer were employed this difference would be greater and consequently the equilibration of the resin in the time available would be less complete. As a matter of fact, even if sodium buffer is employed the molarity of the sodium hydroxide cannot be less than 1 and the quantity to be employed cannot be less than 2 ml; on the other hand the sodium working buffer cannot have a molar strength as high as that of the lithium buffer for obtaining the tyrosine and phenylalanine chromatography.

In Fig. 2 chromatograms of the calibration mixture and of some plasma samples are shown. Concerning the evaluation of the peaks, it must be stressed that because it can be done by simply measuring their heights over the base line,

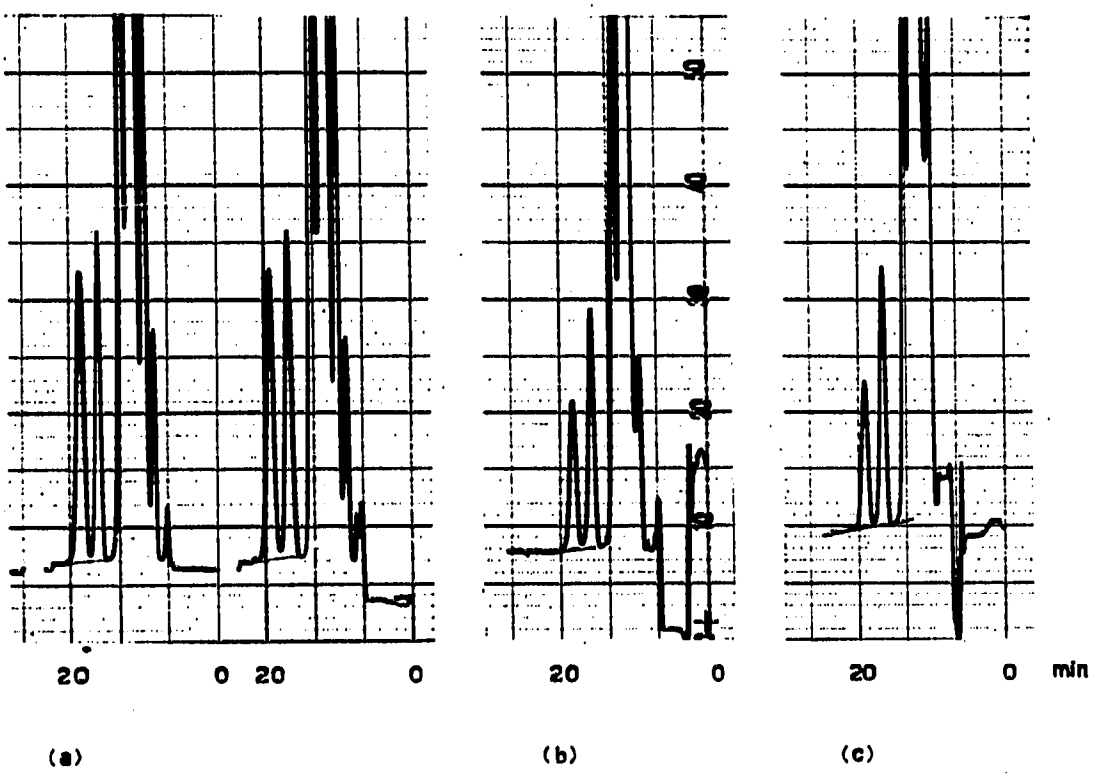

Fig. 2. (a) Examples of chromatograms obtained with a calibration solution containing all the amino acids. Tyrosine and phenylalanine, whose peaks emerge just after the acidic and neutral amino acids break through, have a concentration of 25 nmoles. (b), (c) Examples of chromatograms obtained from 0.2 ml of normally healthy plasma.

the time required for digitizing so many results is reduced to a few minutes per day and the necessity of employing expensive and complicated automatic integrators, which would also affect to some extent the accuracy of the results, is avoided. Possibilities of clerical errors are also reduced, for the evaluation becomes much more objective.

The following equations will be applied in obtaining from the digitized values of the chromatograms either the milligrams of amino acid per 100 ml of plasma or the micromoles per liter:

$$\text{mg/100 ml of plasma} = \frac{H \cdot \text{n}M \cdot \text{MW}}{H_S \cdot V \cdot 10^4} \quad (1)$$

$$\mu\text{moles/l} = \frac{H \cdot \text{n}M}{H_S \cdot V} \quad (2)$$

where H = peak height in mm of the amino acid on the sample chromatogram; nM = nmoles of the amino acid, which has given H_S on the standard chromatogram; MW = molecular weight; H_S = peak height (mm) of the amino acid on the standard chromatogram; V = volume of plasma loaded on to the column (ml), calculated by dividing the volume of the deproteinated plasma solution applied to the column by the dilution factor.

An Olivetti Programma 101 desk-top electronic computer can be used with advantage for doing these calculations; the program instructions for the magnetic card are reported later in this paper. As will be shown, the operator has only to insert into the computer the heights in mm of the tyrosine and phenylalanine peaks and the computer will print the plasma concentrations in mg/100 ml and in μmoles/l of both amino acids and also the phenylalanine/tyrosine ratios, both by weight and molar.

The high number of daily analyses which can be obtained, the ease of digitizing peaks and the small volume of sample required suggest that this system could be used with advantage in centers for newborn phenylketonuria screening, where it could be employed in all those cases for which the methods for mass screening give a presumed positive response and consequently for which a more precise determination of phenylalanine and tyrosine blood levels is required.

Moreover, it could be used with advantage in centers for eugenic counselling, as far as the phenylketonuric heterozygous "depistage" is concerned, for which PERRY *et al.*[22] have already stated that amino acid chromatography is preferred since the phenylalanine/tyrosine ratio must be determined. As a matter of fact the tedious use of two different spectrofluorimetric assay methods for the two amino acids, requiring in this case absolute precision and accuracy, can be avoided. Since the ratio of these two amino acids, rather than their absolute values, is utilized in the test, small undetected technical errors in sample size are not as important as they would be in two separate spectrofluorimetric analyses, and moreover, little changes in the ninhydrin reagent or some faulty column charging operation should affect both tyrosine and phenylalanine peaks equally, since these amino acids are eluted very close together, giving a practically unaltered ratio.

Furthermore, this test could also be applied to the parents of newborns presumed positive for phenylketonuria in which only moderately elevated con-

centrations of blood phenylalanine occur. If one or both parents give a phenylalanine/tyrosine ratio not showing heterozygosity for phenylketonuria, a diagnosis of transient hyperphenylalaninemia only will be given for the child.

Olivetti · programma 101

PROGRAMME INSTRUCTIONS CARD. N. .....

REGISTER 1		REGISTER 2		REGISTER F		REGISTER E		REGISTER D	
1	A V	25	E ×	49	F/$	73		97	
2	B/*	26	F/$	50	D :	74		98	
3	S	27	S	51	A ◊	75		99	
4	C/↑	28	↓	52	B $	76		100	
5	S	29	D/×	53	F/↓	77		101	
6	C ↑	30	E/×	54	E/:	78		102	
7	↓	31	F/:	55	A ◊	79		103	
8	A W	32	F/$	56	B :	80		104	
9	/◊	33	B ↓	57	A ◊	81		105	
10	A :	34	A ◊	58	/◊	82		106	
11	B/+	35	F/↓	59	W	83		107	
12	B/◊	36	A ◊	60		84		108	
13	B/◊	37	B :	61		85		109	
14	/◊	38	A ◊	62		86		110	
15	C/↓	39	A/↑	63		87		111	
16	E ×	40	R/S	64		88		112	
17	B $	41	R S	65		89		113	
18	S	42	R S	66		90		114	
19	↓	43	R S	67		91		115	
20	D/×	44	D ↓	68		92		116	
21	D ×	45	B ↓	69		93		117	
22	B :	46	×	70		94		118	
23	B $	47	F/$	71		95		119	
24	C ↓	48	×	72		96		120	

CONTENTS OF REGISTERS	
M	Operational
A	Operational
R	Operational
b	Counter
B	Storage
c	H_g Tyrosine
C	H_g Phenylalanine
d	nM
D	MW Tyrosine
e	MW Phenylalanine
E	V × 10^4
f	Storage
F	Instructions

CONSTANTS ON CARD		CONSTANTS ON CARD	
nM	D/ ↑	V × 10^4	E ↑
MW Tyrosine	D ↑		↑
MW Phenylalanine	E/ ↑		↑

NOTES

Fig. 3. Program instructions for the Olivetti Programma 101 desk-top electronic computer.

We would also like to mention that in our laboratory the possibility of submitting to this kind of analysis blood samples collected on filter paper is under investigation. Preliminary results obtained by employing an ultramicro method of amino acid extraction from the card and of deproteination, along with an amplification of the sensitivity of the photometric system, suggest that the chromatographic determination of tyrosine and phenylalanine could be routinely possible even from a single blood drop[30].

PROGRAM INSTRUCTIONS FOR OLIVETTI PROGRAMMA 101 COMPUTER

The desk-top Olivetti Programma 101 electronic computer receives program instructions by the insertion of a magnetic card on which the instructions, given in Fig. 3, have been stored previously by means of the computer itself.

```
        V
69•25   S
 61•2   S

 1•00  b 0

   42   S
 24•5   S
 1•37  A 0
 0•82  A 0
 0•59  A 0
75•60  A 0
49•63  A 0
 0•65  A 0


 2•00  b 0

   51   S
   23   S
 1•66  A 0
 0•77  A 0
 0•46  A 0
91•61  A 0
46•61  A 0
 0•50  A 0


 3•00  b 0

 54•2   S
   32   S
 1•77  A 0
 1•07  A 0
 0•60  A 0
97•68  A 0
64•76  A 0
 0•66  A 0
```

Fig. 4. The first two numbers after V indicated with S are the peak heights (mm) of tyrosine and phenylalanine from the standard chromatogram and are introduced into the computer by the keyboard before beginning the computation. With b ◇ is indicated the sample identification number which the computer prints by itself in that a counter is included in the computation program. The first two data following the sample identification number and indicated with an S are the peak heights (mm) of tyrosine and phenylalanine, which are introduced into the computer for each sample. The following data indicated with A ◇ are automatically printed out by the computer and in order are: mg/100 ml of tyrosine, mg/100 ml of phenylalanine, phenylalanine/tyrosine ratio by weight, μmoles/l of tyrosine, μmoles/l of phenylalanine and phenylalanine/tyrosine molar ratio.

The following data are memorized on the card as variable constants: (a) nmoles of tyrosine and phenylalanine, which have given H_S on the standard chromatogram, on register D/; (b) the molecular weight of tyrosine on register D; (c) the molecular weight of phenylalanine on register E/; (d) the volume of plasma (ml) loaded on to the column, multiplied by 10^4, on register E.

In this way, if necessary, a rapid substitution in the program of the values of (a) and (d) is possible before beginning the computation of a daily set of analyses. The operating procedures are as follows: (1) set decimal wheel on 2; (2) introduce the magnetic card into the computer; (3) press V; (4) introduce the height in mm (H_S) of tyrosine, obtained from the standard chromatograms, by typing it on the computer keyboard and then pressing S; (5) introduce the height in mm (H_S) of phenylalanine, as above; (6) the computer will print out 1.00 as the counter for sample identification starts to operate (the subsequent data then relate to sample 1); (7) introduce the height in mm (H) corresponding to the tyrosine peak on the chromatogram of sample no. 1; (8) introduce the height in millimeters (H) corresponding to the phenylalanine peak on the chromatogram of sample no. 1; (9) the computer will print the mg/100 ml of plasma of tyrosine; (10) the computer will print the mg/100 ml plasma of phenylalanine; (11) the computer will print the phenylalanine/tyrosine ratio by weight; (12) the computer will print the μmoles/l of plasma of tyrosine; (13) the computer will print the μmoles/l of plasma of phenylalanine; (14) the computer will print the phenylalanine/tyrosine molar ratio; (15) the computer will print the number 2.00 (counter) and subsequent data on the strip chart will refer to sample no. 2; (16) for the second and subsequent samples loop to (7).

In Fig. 4 a computer printout example of some calculations is given.

Since it is advisable, before starting to analyse a daily amount of samples, to run one standard chromatogram on each column, the H_S values of tyrosine and phenylalanine indicated at points (4) and (5), respectively, can be the mean values of four determinations.

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