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A Shortened Method for the Separation and Estimation of Plasma Phenylalanine and the Establishment of the Clinical Norm for Ceylonese Subjects

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Abstract : A method is described by which phenylalanine in blood can be separated and quantitatively estimated with reliability using thin layer chromatography. Silica Gel-G was used as the adsorbent and n-butanol/acetic acid/water (4 : 1 : 1) as the developing solvent. The method was sensitive enough to detect quantities as low as 0.5 μ; the volume of serum required was 4 μ (0.004 ml) and the time taken for the entire estimation was less than 3 h. The clinical norm for Ceylonese children, determined by using this method, is reported. The values of serum phenylalanine range from 0 to 16 mg/100 ml with a mean of 7.07 $\pm$ 4.86. Two positive cases of phenylketonuria, belonging to the members of a single family are also reported.

1. Introduction

Phenylketonuria is an inborn error of metabolism characterised by the failure to metabolise the amino acid, phenylalanine. The serum phenylalanine rises and neurotoxic metabolites are formed which cause mental deficiency. Phenylketonuria is treated by a low phenylalanine diet which should be started at the age of a few weeks. This condition is detected by testing of the urine for phenylpyruvic acid and finally confirmed by the testing of the serum for phenylalanine levels.

The basic screening test is the detection of excess of phenylpyruvic acid in urine with ferric chloride.[9] This test should be carried out with fresh urine since atmospheric oxygen oxidizes the acid. Further, as phenylpyruvate only appears in the urine 2 or 3 weeks after birth, the test is done up to 4 to 6 weeks. Therefore, an increase in the blood phenylalanine gives an earlier indication of the deficiency and is used in the case of new born siblings of identified cases.

The methods available at present are the agar diffusion microbiological method,[4] thin layer chromatography on cellulose layers impregnated with cyclohexylamine,[7] paper chromatographic method,[9] Colorimetric method,[1] the fluorimetric method,[2] and the enzymic method.[6]

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There is a need for a rapid method suitable for very small quantities of serum/plasma to separate and estimate quantitatively phenylalanine without deprotenizing or desalting. This paper describes the successful application of the thin layer chromatographic technique in a new solvent system and its value for the separation of phenylalanine in particular and its probable value for many other amino acids.

Some of the findings have been previously reported.[8]

2. Materials and Methods

2.1 Chromatoplates

The chromatographic plates used for thin layer were of glass (20 cm × 20 cm) with Kiesel Gel G (Nach Stahl) as the adsorbent. The Gel (30 g) was made into a slurry using a mixture of ethanol (72 ml) and water (8 ml). With this quantity we were able to obtain 5 plates of 0.2 mm thick gel layer ; the slurry was spread on the glass plates using a Desaga Spreader.[5] The plates were then dried at room temperature in the open, for 30 min and subsequently dried at 110°C for another $\frac{1}{2}$ h before storing in a Desaga drying cabinet. A sharp boundary of 2 mm of each edge of the layer was made possible by wiping the edges with a grooved cork ; a line 10 cm from the edge was also marked.

2.2. Application of sample

Standard solutions of the amino acids were prepared in double distilled water (500μg/ml); the insoluble amino acids were dissolved with the aid of dilute Na_2CO_3 solution and subsequently neutralised with dilute HCl. The chromatoplate was divided into 9 equal strips using a fine needle and then the standard solution of the amino acid was applied on each strip using a 10μl pipette to deliver the sample. With phenylalanine varying amounts ranging from 0.5 to 2.0 μg were applied with care so that the diameter of the spot was less than 4 mm and the spots were in one horizontal line. The plates were irrigated with n-butanol/acetic acid/H_2O (4 : 1 : 1) for about 1 h during which period the solvent front moved 10 cm from the starting line ; the tank was equilibrated with the same solvent system over-night before use. The plates were dried and the amino acid detected by spraying with ninhydrin (BDH ninhydrin spray can) and drying at 120°C for 10 min. The spots appeared reddish on the white background. The intensity of the colours of the spots was found directly proportional to the amount of phenylalanine. However, with more than 3μg (6μl) of the amino acid it was difficult to estimate the quantity by the intensity of the colour produced by the spot.

Among various types of adsorbent and different solvent systems tried, Kiesel-Gel G adsorbent with n-butanol/acetic acid/water in the proportion of 4 : 1 : 1 was found to give the best separation for this particular purpose, because most amino acids present in the serum of human blood either had very low or very high R_F values, whereas phenylalanine had an R_F value in the region of 0.65 to 0.68.

2.3. Quantitative estimation of phenylalanine in human blood

In the 4 strips of the chromatoplate were placed phenylalanine standards, viz 1, 2, 3 and 4 μl of a solution of phenylalanine (1μl = 0.5μg) while in the next 5 strips were placed samples of serum (4μl) to be tested. The plates were then subjected to ascending chromatography using the solvent system described above. After the solvent front reached the 10 cm mark the plates were removed and the spots located. Normal samples of serum (4 μl) produced the same intensity of the spot obtained with 1 μl or less of the standard ; i.e. the normal value of phenylalanine in the serum is 12.5 mg%.

3. Results and Discussion

The one dimensional thin layer chromatographic separation of phenylalanine is shown in Figure 1. The amount of phenylalanine present in 4.0μl (0.004 ml) serum was estimated by this procedure. The quantitative estimation was made possible by comparing the intensity of the spots of the unknown with the intensities of known quantities of the phenylalanine. Attempts made to quantitate the method, by scraping off the spots, eluting, centrifuging and measuring in a colorimeter, have been unsuccessful. This was mainly due to the small amounts of standards used (0.2 to 1.0 μg) in the method.

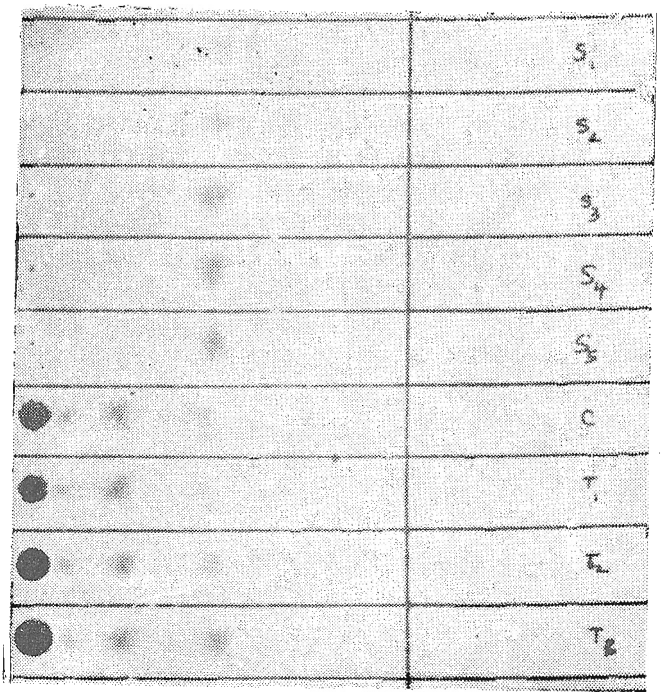

FIGURE 1. Thin layer chromatographic separation of phenylalanine in human serum.

Adsorbent Kiesel-Gel G, Solvent -n-butanol/acetic acid/water (4 : 1 : 1). Amino acids detected by spraying with ninhydrin and drying at 120°C.

S Standard phenylalanine expressed in mg%.
S_1, S_2, S_3, S_4, and S_5 = 12.5, 25.0, 37.5, 50.0 and 62.5 mg%.
C Control serum
T_1 Test sample of case (1), Table 1. Phenylalanine deficient diet for 7 days.
T_2 Patient fed on full cream milk for 12 days.
T_3 Patient again on phenylalanine deficient diet for 2 weeks.
(c.f. Table 1, case (1), experiments II, III and IV).

Among the many methods which are available for the estimation of phenylalanine of blood, the paper chromatographic method[9] needs comparison with the thin layer method described in this paper. The experimental details of the 2 methods are tabulated below.

	Chromatography	
	Thin layer	Paper
1. Solvent system	Butonal/acetic acid/H_2O (4:1:1)	Butonal/acetic acid/H_2O (4:1:5)
2. Number of runs	One dimentional	Two dimentional
3. Volume of serum requried	4 μl (0.004ml)	250 μl (0.25 ml)
4. Sensitivity of the method	0.5 to 2.0 μg	10 to 50 μg
5. Time taken to develop the chromatogram	90 min	48 h
6. Time taken for the whole estimation	3 h	54 h

The above tabulation reveals that the thin-layer method although open to errors associated with "eye estimation" of colour and not made quantitative by calorimetry, yet is very rapid and requires only one sixtieth of the serum necessary in the paper method. It should be emphasized that this test is normally carried out on new born babies and hence the volume of blood necessary is a key factor. The 4 µl serum necessary for the test can be obtained from the finger prick while the 250 μl serum necessary for the paper could only be obtained by venepuncture. This method is therefore very simple and can be adopted routinely in most of the provincial pathological laboratories.

The validity of the method was checked by 2 procedures : (1) by carrying out the clinical norms ; no sample gave more than 16 mg% (w/v) and (2) two children in the same family with phenylketonuriya were investigated for phenylalanine levels before and after feeding with phenylalanine rich and deficient milk. The results (Table 2) show that when the children were fed with phenylalanine deficient milk, the phenylalanine levels of the blood fell to 6 and 8 mg%. However, when they were fed with the normal full cream milk, the values rose to 50 and 40 mg%. Therefore this method is quite accurate enough to detect small changes in the phenylalanine levels of blood.

3.1. Clinical norms for Ceylonese children

All blood samples tested were obtained from children of both sexes of the age group 3 months to 6 years, who were warded at the Lady Ridgeway Hospital, Colombo, for various ailments ; the group did not include those who showed any signs of phenylketonuria. Fifty samples of blood were estimated for phenylalanine content. The value of serum phenylalanine ranges from 0 to 16 mg/100 ml with a mean of 7.07 ± 4.86 mg/100 ml.

3.2. Changes in serum phenylalanine content with diet of cases of phenylketonuria

Two cases of phenylketonuria in the same family were investigated. The changes in serum phenylalanine of these 2 subjects with diets deficient in phenylalanine (Lofenalac) is reported in Table I.

TABLE 1. Cases of phenylketonuria and changes in serum phenylalanine content with diet.

Case (1)

	Age	Date of collection of blood	Diet	Serum Phenylalanine (mg/100 ml)
(i)	1 yr 7 months	5.6.69.	Full Cream milk	25
(ii)	1 yr 7 months	12.6.69.	*Phenylalanine deficient milk	6
(iii)	—	24.6.69.	Full Cream milk	50
(iv)	—	8.7.69.	*Phenylalaine deficient milk	12.5
(v)	—	13.7.69.	—do—	10
(vi)	—	2.8.69.	—do—	10

Case (2)—Sister of Case (1)

	Age	Date of collection of blood	Diet	Serum phenylalanine (mg/100 ml)
(i)	5 days old	26.6.69	Breast fed	25
(ii)	8 days old	1.7.69	Breast fed	40
(iii)	15 days old	13.7.69	*Phenylalanine deficient diet	16
(iv)	40 days	30.7.69	—do—	8

Clinical norm for Ceylonese children 7.07 ± 4.86
Range : 0—16 mg phenylalanine/100 ml.
*(Lofenalac)

3.3. R_F values of other amino acids

In order to ascertain that the other amino acids had no effects on the separation, pure samples of 24 amino acids were tested. Of these 24 amino acids, 10 are known to be concerned in metabolic diseases.[3] The R_F values are provided in Table 2 Of the 24 amino acids tested only phenylalanine had an R_F of 0.67 while all others had values ranging from 0 to 0.73.

TABLE 2. RF — Values of amino acids in human plasma after spraying with Ninhydrin — Thin layer

Amino Acid	RF — Values
Asparagine	0.22
Alanine	0.29
Aspartic acid	0.24
Amino-butyric acid	0.33
Arginine	0.09
Cystine	0.11
*Citrulline	0.22
Glutamic acid	0.36
*Glycine	0.23
Glutamine	0.20
*Histidine	0.09
*Leucine	0.62
*Iso-Leucine	0.57
Lysine	0.09
*Methionine	Did not move
Ornithine	0.07
*Phenylalanine	0.67
*Proline	0.21
Serine	0.24
Threonine	0.31
Taurine	0.30
Tryptophane	0.72
*Tyrosine	0.60
*Valine	0.44

* Amino acids known to be concerned in metabolic diseases.

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