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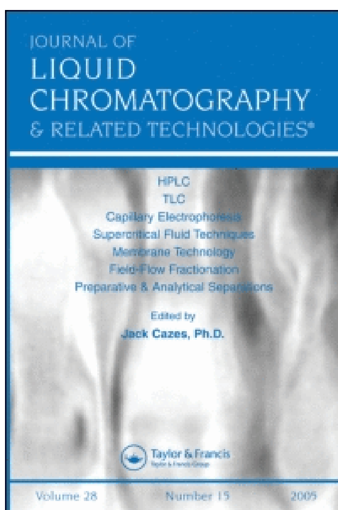

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A Simple Ion Exchange Thin Layer Chromatography Method for the Separation of Amino Acids in Clinical Samples

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A SIMPLE ION EXCHANGE THIN LAYER CHROMATOGRAPHY METHOD FOR THE SEPARATION OF AMINO ACIDS IN CLINICAL SAMPLES

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ABSTRACT

An ion exchange thin layer chromatography method for the separation of 16 amino acids is described. The effect of the pH of buffer on the resolution of the amino acids is discussed. Development in pH 3.3 buffer at 45°C resolved all the amino acids. The method is particularly well adapted to screening metabolic disorders in neonatal children.

INTRODUCTION

A number of metabolic diseases are characterized by the presence of amino acids in the blood and urine, e.g. phenylketonuria (PKU), in which the concentration of phenylalanine is elevated. The semi-quantitative Guthrie-Susi test (1) normally used to detect this condition is based on bacterial growth, and the results require confirmation by another method. The amino acid analyzer

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(2) could be used but this is an expensive approach which can still handle only one sample at a time. The same is true of high performance liquid chromatography. The amino acids are analyzed after derivatization with dansyl chloride (3,4) phenyl thiohydantoin (5) or o-phthalaldehyde/2-mercaptoethanol (6-8) because they are not readily measured by HPLC due to their low response to both UV and fluorescence detection. A recent method (9) was described for the determination (in 6 hours) of phenylalanine using immobilized bacteria and a lactate electrode. Although this method is sensitive it can analyze only one sample at a time and is specific for phenylalanine.

Ireland and Read (10) described a thin layer chromatography (TLC) method for use in neonatal screening to detect excess aminoacidaemia. In this method the extracted blood is spotted on cellulose plates which are then developed in two solvent systems. The plate is throroughly dried and then developed overnight in an ammonia-free atmosphere (a chromatography tank containing a dish of sulfuric acid) in the dark. Lepri et al (11) described a TLC method using impregnated reverse phase plates. However, the resolution of phenylalanine was poor on all reverse phase plates (C_2, C_8 and C_{18}) used.

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When ion-exchange thin layer chromatography was introduced (12), basic and aromatic amino acids could be separated on one plate. The technique was soon applied to the detection of metabolic disorders (13-15). We have developed a simple and rapid adapation of this technique which can be used to analyze amino acids in whole blood, serum, or even a blood sample dried on a piece of filter paper.

EXPERIMENTAL

Materials: Strongly acidic cation-exchange resin-coated TLC plates, Fixion 50 x 8, sodium form, were obtained from Chromatronix (Mountain View, CA). All chemicals used were reagent grade. Buffers were prepared in distilled deionized water. Standard amino acid solutions (table 1) were prepared in ethanol/HCl (95:5). Stock solutions were stored in the freezer. Small quanti-

TABLE 1
Concentration of standard amino acids
in ethanol:hydrochloric acid (95:5) solution.

Amino acid	mg/ ml	Amino Acid	mg/ ml
Aspartic acid	1.5	Isoleucine	6
Threonine	28	Leucine	12
Serine	21	Tyrosine	15
Proline	25	Phenylalanine	15
Glutamic acid	12	Lysine	36
Glycine	31	Ornithine	15
Alanine	34	Histidine	14
Valine	20	Arginine	12
Cystine	9	Tryptophan	10
Methionine	5.5		

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ties of samples diluted 4-fold will keep for several days in a refrigerator at 4°C. Regular TLC tanks and disposable micropipettes were used.

Eluting Solution: The buffer pH 4.2 in which the amino acids were separated was prepared by dissolving 14.4 g citric acid monohydrate, 8 g NaOH, 11.7 g NaCl and 7.7 ml concentrated HCl (37%) in 1L distilled deionized water.

Spray Reagent: Solution A - 1 g ninhydrin in 100 ml 80% acetone.

Solution B - 1 g cadmium acetate in 150 ml 33% acetic acid

The reagent is prepared by mixing 100 ml of A with 20 ml B to which 1 ml of pyridine has been added. For maximum color development use a freshly prepared solution. Pyridine and cadmium acetate are added to enhance the color of the spots, and to exert a slight buffer effect which counteracts the acidity of the plate.

Extraction of amino acids from fresh blood or serum. 50 μl whole blood or serum are pipetted into a 1 ml vial containing 10 μl ethanol and 5 μl trifluoro-

acetic acid. Mix vigorously, preferably with a sonicator for 5 min, and then centrifuge for 5 min. Spot 10 µl of the supernatant on the plate, allow to dry and then develop for a distance of 10 cm in the buffer described above. The plate is again dried, then sprayed with the ninhydrin reagent and dried once again with a hair dryer on the hot setting. The amino acids will appear as pink spots which can easily be seen in white light.

Extraction of amino acids from dried blood: The filter paper on which the blood sample (50 µl) has dried is cut into small pieces which are placed in a test tube. 100 µl ethanol:HCl (95:5) and 10 µl TFA are added and the tube is allowed to stand for 10 min and then centrifuged. 10 µl of the supernatant is spotted on the plate and the procedure described above then followed.

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RESULTS AND DISCUSSION

Effect of pH on the Separation of Amino Acids. When plates are run in aqueous buffer it is possible to change the chromatographic conditions rapidly so that the necessary separation can be achieved. The analyst can change the pH, the ionic strength of the buffer, or both, as needed. In Fig 1 the separation of 16 amino acids on a plate developed in citrate buffer, pH 3.3 at 45°C is shown. Note that all the amino acids are well resolved and can clearly be identified.

When the plate was developed in citrate buffer pH 4.2 at room temperature, (Fig 2B) the amino acids arginine, histidine, lysine, ornithine, phenylalanine, and tyrosine were resolved from each other followed by leucine + isoleucine + methionine as one spot, and then valine. The remainder of the amino acids appeared as one spot at a high R_f. Note that lysine and histidine have changed their order of elution with the change in pH from 3.3 (Fig 2A) to 4.2 (Fig 2B).

At a pH of 5.1 (Fig 2C) lysine and ornithine are not resolved. At higher pH's, such as 6.0 (Fig 2D) it is possible to separate tryptophan from other amino acid. We conclude that, for the resolution of all 16 amino acids the best conditions are pH 3.3 citrate buffer at 45°C (Fig 1).

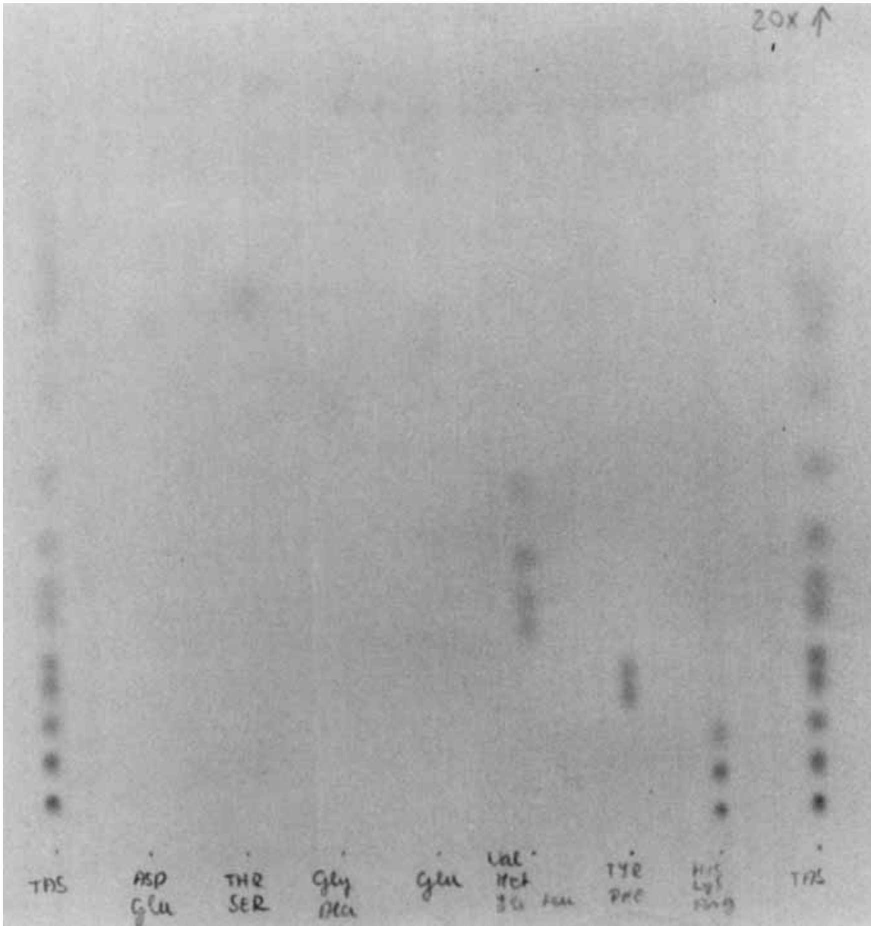

Figure 1. Separation of 16 amino acids in citrate buffer, pH 3.3 at 45°C.

In blood samples from PKU cases the levels of phenylalanine are elevated, and the best conditions to use are buffer pH 4.2 or 5.1 and to develop the plates at room temperature. A comparison of the phenylalanine levels in the blood of a normal child with that from a PKU patient is shown in Fig 3. Note that better results were obtained when serum was used (Fig 4) rather than whole blood. Unlike the Guthrie-Susi test (1) the present method allows the simultaneous

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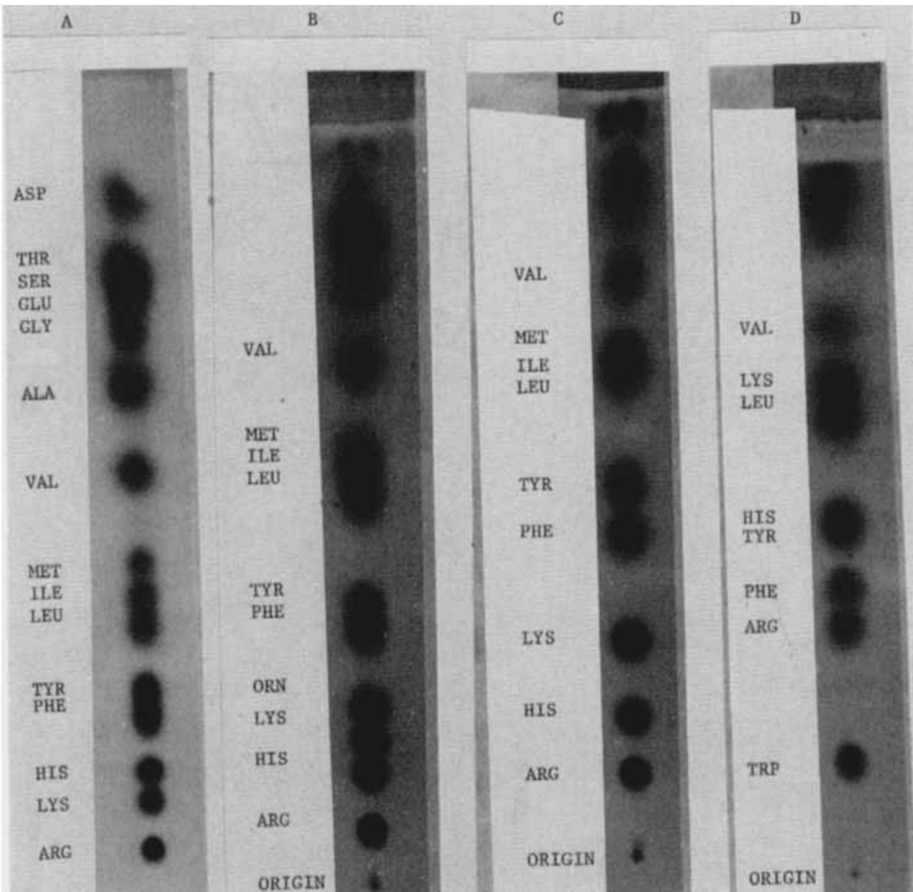

Figure 2. Comparison of the separation of amino acids in a citrate buffer having a pH of 3.3 (A), 4.2 (B), 5.1 (C) and 6.0 (D).

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determination of more than one amino acid in more than one sample on the same plate. This is important because it was found by Haltzman et al (16) that false PKU positives can be reduced if a tyrosine determination is performed together with phenylalanine. The use of pH 5.1 buffer allows such a determination (Fig 2C). In a recent review article of screening for inborn errors of

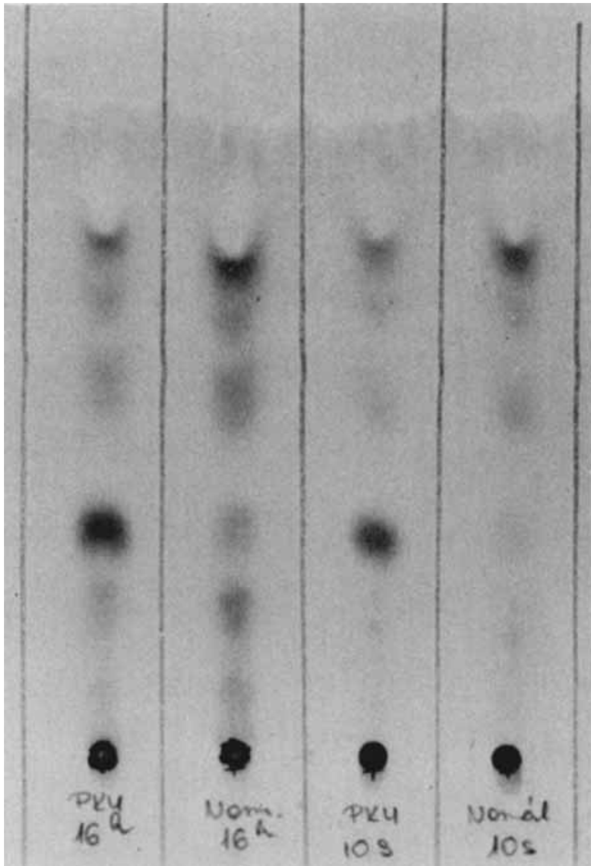

Figure 3. Comparison of phenylalanine levels in the blood of PKU patient and a normal child. See Figure 2(B) for order of elution.

metabolism, Watts (17) suggested that a screening method should be (a) reliable and simple; and (b) produce no false negatives, and few false positives. The test described here is simple, reliable and quantitative (when densitometry is used) and can be used for screening more than one amino acid disorder simultaneously, for example tyrosinaemia, histidinaemia, lysinaemia, and maple syrup urine disease.

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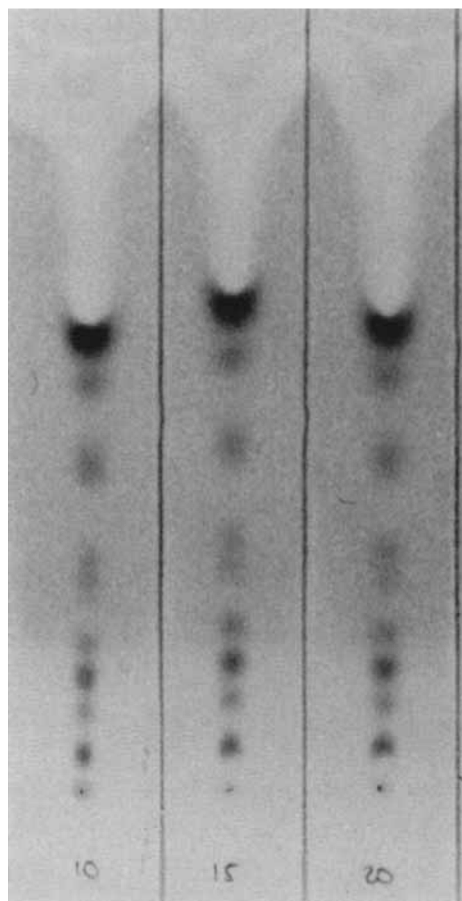

Figure 4. Separation of amino acids in serum using a citrate buffer, pH 4.2 at room temperature. See Figure 2(B) for order of elution.

CONCLUSION

Ion exchange thin layer chromatography using strongly acidic resin-coated plates affords a simple and straight forward method for the separation in a single one-dimensional run in aqueous buffer of the basic and aromatic amino acids. The technique is ideal for use in screening amino acid disorders in the blood and other biological samples. It can be operated with as little as one drop of blood dried on a piece of filter paper.

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