

RAPID, HIGH-RESOLUTION, TWO-DIMENSIONAL AMINO ACID CHROMATOGRAPHY ON MICRO SCALE CHROMATOGRAMS

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SUMMARY

A convenient method is described for two-dimensional amino acid chromatography on micro scale chromatograms of 5×5 cm. Despite the short migration paths, excellent separation is obtained. The time of development is short and the technical procedure very simple. Extension to other classes of compounds is possible.

For the screening of disorders of the amino acid metabolism, two-dimensional methods are preferable because of their high resolving power. Their disadvantage is that they are time-consuming and comparatively difficult to perform. Recently, we have found that the newly developed cellulose-layered DC Alufolie (E. Merck A.G., Darmstadt) has unique properties. On this material the amino acids appear as very compact spots. With traditional solvent systems unexpectedly good separations can be obtained even when a run of only 5 cm in each direction is used. The time required for the development of such a 5×5 cm chromatogram is very short, even compared with the time given in the method of White¹ using fast running solvents. For the pyridine-butanol and the phenol solvents 20 and 50 min respectively are necessary. No previous equilibration of the chromatograms is required, nor is a constant temperature essential. By this method high-resolution, two-dimensional analysis of the urinary amino acids can be performed in less than half a day, making the diagnosis of amino acid disorders considerably more rapid.

MATERIALS

Stationary phase. D.C. Alufolien 20×20 cm, (cellulose 0.1 mm, E. Merck A.G., Darmstadt, No. 5552) are cut into 5×5 cm pieces. Scrape a narrow border of cellulose off each margin.

Mobile phases for general purposes. First solvent: butanol-pyridine-water (1:1:1, v/v). Second solvent: 88% phenol-25% ammonia-water (10:0.8:1, v/v). To 200 ml of this solvent 1 mg of *o*-oxychinoline is added.

Mobile phases for separation of fast-migrating substances (aromatic and branched-

chain amino acids; methionine). First solvent: 96% ethanol-water (86:14, v/v). Second solvent: *tert.* butanol-methyl ethyl ketone-25% ammonia-water (5:3:1:1).

Isolation of amino acids and preparations of a salt-free, concentrated solution

10 ml of filtered urine is passed through a column (diameter 0.7 cm) of 5 ml of Dowex-50 W X 8 (50-100 mesh, SO_3H resin), followed by an excess of water to remove anions and neutral substances. The effluent is discarded.

The amino acids are eluted from the column with 20 ml of 2 *N* ammonia (control for completeness by spot-testing with ninhydrin reagent). The eluate is evaporated *in vacuo* (40°); the dry material redissolved in 1 ml of water. This concentrated salt-free solution is ready for chromatography.

For rough estimation of α -amino-nitrogen the method of Pope and Stevens² or one of its modifications can be used.

Application of amino acids on the Alufolie

0.2-2.0 μl of concentrate, containing 1-1.5 μg of α -amino-nitrogen, is spotted at one corner, 9 mm from each side, by means of a mechanically driven microsyringe. A stream of air is blown on the point of application in order to evaporate the solvent. Sulfur-containing amino acids are oxidized: 0.3 μl of 30% H_2O_2 containing 0.2 mg ammonium molybdate per ml is brought on the dry sample spot.

Chromatography

Small trays of 6.0 cm (height) \times 5.5 \times 9.0 cm are used. Chromatograms are placed in grooves of a loose bottom plate. Freshly prepared solvent is carefully added to the tray until the level reaches 4 mm from the center of the spot.

Ascending chromatography: System A: first solvent 30 min; second solvent 50 min. System B: first solvent 2 \times 30 min; second 2 \times 30 min.

Drying: a few minutes at 100°.

Ninhydrin spray: 0.2% ninhydrin in ethanol.

Development of spots by heating for 3 min at 100°.

RESULTS AND DISCUSSION

In Figs. 1a and b, chromatograms are shown in order to demonstrate the possibilities of the method and also its limitations. Most amino acids appear as compact, circular spots, at least when the chromatogram is not overloaded. This is probably due to the short development time which minimizes diffusion, and to the regular structure of the micro-crystalline cellulose, carrier of the stationary phase. The latter property will cause the liquid-liquid distribution process to proceed uniformly over the whole area of the chromatogram. Also the time-dependency of the partition will be very constant. However, separation is also determined by the composition of the solvent system. When partition coefficients are equal or nearly equal, no separation will be obtained.

In Fig. 1a, chromatogram 1, a normal pattern of urinary amino acids as developed in system A is shown. Most positions are comparable with those on the 23 \times 23 cm chromatogram, but separation is better. Threonine is free and serine is just apart from glycine. Alanine and glutamine are separated. Chromatogram 2 repre-

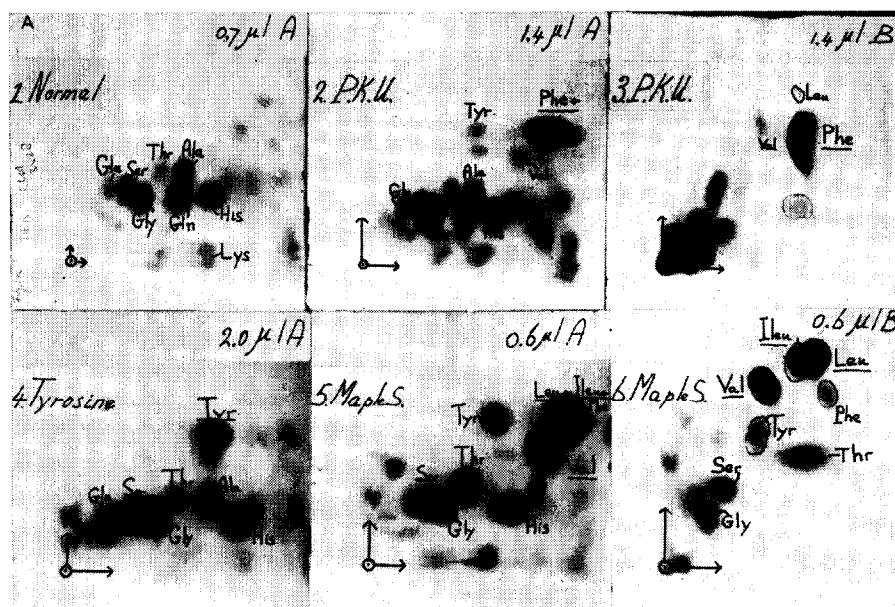


Fig. 1a. Chromatograms, 5×5 cm. First run: vertical direction. Second run: horizontal direction. 1, Normal urine. 2 and 3, Phenylketonuria: a strong spot of phenylalanine. 4, Tyrosinaemia: a strong spot of tyrosine. 5 and 6, Maple syrup disease (probable): strong spots of leucine, isoleucine and valine.

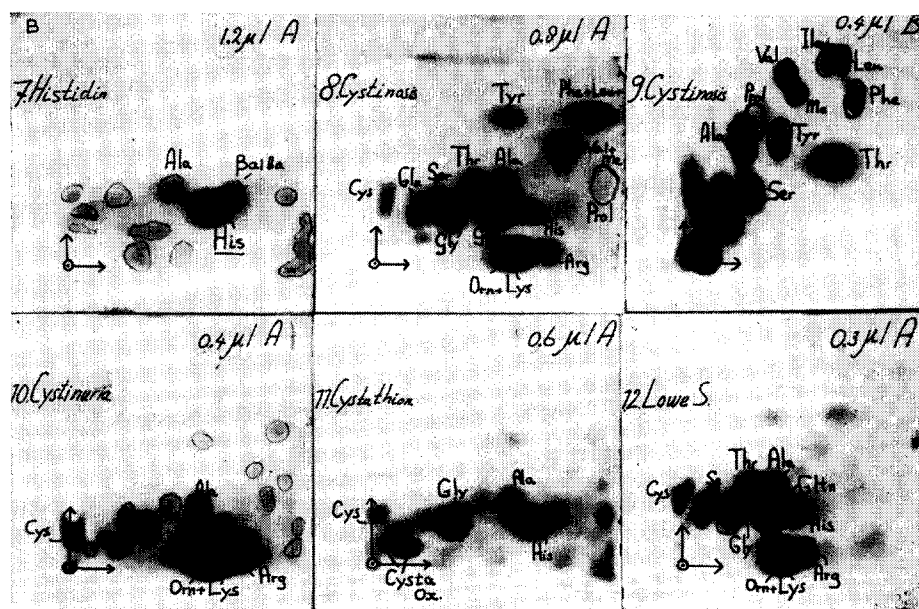


Fig. 1b. Chromatograms, 5×5 cm. First run: vertical direction. Second run: horizontal direction. 7, Histidinemia: a strong spot of histidine. 8 and 9, Cystinosis: generalized amino aciduria. 10, Cystinuria: strong spots of cysteic acid (oxidized cystine) and the basic amino acids lysine+ornithine and arginine. 11, Cystathioninuria: 2 spots of oxidized cystathionine. 12, Lowe syndrome: several amino acids increased.

sents a patient with phenylketonuria. In system A a strong spot of phenylalanine can be seen, however, phenylalanine is not separated from leucine and isoleucine. In system B these compounds are obtained apart from each other (chromatogram 3). The same can be seen on the chromatograms 5 and 6, showing a maple syrup excretion pattern (heavy spots of leucine, isoleucine and valine). On chromatogram 4 a strong excretion of tyrosine in an adult female patient with permanent tyrosinaemia and tyrosyluria (described elsewhere³) is shown. Chromatogram 7, Fig. 1b, is a pattern of histidinaemia; histidinuria is obvious. On chromatogram 8, the generalized amino aciduria of cystinosis is shown. A beautiful separation of fast-running amino acids can be seen with system B (chromatogram 9). Methionine and valine appear as separate spots (not oxidized with H_2O_2). Chromatogram 10 represents cystinuria (homozygous patient). Cystine appears as cysteic acid* (after oxydation with H_2O_2). As can be seen lysine and arginine are separated but not lysine and ornithine. The positions of the basic amino acids are highly dependent on the NH_3 content of the second solvent. Freshly prepared solvents must be used. In the first run basic amino acids tend to streak somewhat; for strong bases as putrescine, cadaverine and colamine this effect is very pronounced (spots in lower right corner). Chromatogram 11 is representative for cystathioninuria. Two spots of oxidized cystathionine, probably of the sulfoxide and the sulfone, are present close to the origin. On chromatogram 12 the generalized amino aciduria of a patient with the Lowe syndrome is presented.

The use of the method is not limited to the analysis of urinary amino acids, but can be adapted for serum and cerebrospinal fluid as well, introducing the removal of protein as an extra step. We obtained excellent separations with as little as 0.05 ml of cerebrospinal fluid, so that detailed, systematic screening of amino acids in the cerebrospinal fluid is now possible.

We also found that this technique was suitable for the two-dimensional chromatographic analysis of other classes of compounds, such as sugars, imidazoles and indoles, providing suitable solvent systems are used and a convenient method for the isolation of the class of compounds investigated is employed.

We hope that the method described here will contribute to the general acceptance of the highly informative two-dimensional chromatography for the screening of inborn errors of amino acid metabolism.

REFERENCES

- 1 H. H. WHITE, *Clin. Chim. Acta*, 21 (1968) 297.
- 2 C. G. POPE AND M. F. STEVENS, *Biochem. J.*, 33 (1939) 1070.
- 3 S. K. WADMAN, F. J. VAN SPRANG, J. W. MAAS AND D. KETTING, *J. Mental Deficiency Res.*, 12, part 4 (1968) 269.

* Homocysteic acid and cysteic acid normally coincide, but are separated when the chromatogram is developed $3 \times$ in the phenol solvent (system A). This is important for the recognition of homocystinuria.