Two-Dimensional Thin-Layer Chromatography on Two-Layer Plates of Amino Acids

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Separation of the amino acids in urine by use of thin-layer chromatography (TLC) has hitherto required that the specimen be first desalted and then chromatographed in two dimensions with at least two pairs of developing solvent systems. We wished to simplify both steps. The customary method of desalting on a column is replaced by desalting on a plate that supports a strongly acid cation-exchanger and a cellulose layer. This method, originally developed for one-dimensional TLC, is used here for two-dimensional TLC. Urine is applied to the ion-exchange layer and strong acids and neutral substances are removed with water. The amino acids are then chromatographed into the cellulose layer, and are separated there two dimensionally with a newly devised pair of developing solutions. This pair of solvents separates nearly all of the amino acids in urine.

Additional Keyphrases novel developing system • desalting on the TLC plate • screening technique for inherited amino acid anomalies

Separation of the urinary amino acids is necessary for the recognition of certain anomalies of amino acid metabolism. Ninhydrin-positive substances are so numerous in urine that only procedures with a high resolving power are useful. Because so many analyses must be performed during a screening program, column chromatography in amino acid analyzers, the best method, can only be used in selected cases. Two-dimensional thin-layer chromatography (TLC) offers a compromise. Several TLC procedures (1, 2) perform impressively, but are laborious. Our investigation was directed toward finding a way to decrease work expenditure without limiting diagnostic usefulness.

Urine samples are generally desalted before chromatography by means of small ion-exchange columns (1, 2). We described a procedure for one-dimensional chromatography in which a two-layer plate was used that circumvented the need for desalting on a column (3). An ion-exchange strip on the lower edge of the plate fulfills the function of the ion-exchange column so that urine can be applied directly to the plate, without pretreatment. Eluate concentration and column regeneration are circumvented.

This procedure was modified for two-dimensional chromatography. We think that most of the pairs of developing solutions previously described for use in the TLC of amino acids in urine do not resolve them sufficiently. Hitherto, the best results have been obtained by preparing two chromatograms for each urine sample, with two different sets of developing solvent pairs. We have devised a developing solvent system that better separates the amino acids in question so that one two-dimensional chromatogram will suffice.

Materials and Methods

Supplies

Equipment used included chromatography tanks, special applicator (from Basic Equipment Kit for EM-Diagnostic TLC, Brinkmann Instruments, Westbury, N.Y. 11590, Catalog No. 6860 470-2), source of warm air (hair dryer), drying oven, 2-μl microcapillaries, and a two-layer plate.
(Figure 1). The preparation of the plate is described in (5). The following chemicals (all reagent grade) were used: methanol, ammonium hydroxide, pyridine, dioxane, n-butanol, acetone, acetic acid, and ninhydrin.

Procedure

Two microliters of distilled water are first applied as a streak at the lower left corner of the plate (Figure 1). Two microliters of urine are then applied as a streak on the wetted application line with the help of the applicator and a 2-μl capillary. The microcapillary should be 6 to 8-mm long; longer capillaries do not discharge completely when applying a streak. After the urine is applied, the plate is developed to its upper edge with water (about 3 h). (Since no damage results from leaving the plates in water for a longer time, the development can be started in the evening and left overnight.)

After the plates are dried with warm air, the amino acids are moved to the first solvent-front line by double development with methanol and 25% aqueous ammonia (50:50); the time for each development is 10 min. The ion-exchange strip and that part of the plate (aluminum foil as backing) above the second solvent front line are now cut off and discarded. The desalting is followed by the usual two-dimensional chromatography. The TLC plate is doubly developed in the solvent system pyridine:dioxane:ammonium hydroxide:water (35:35:15:15) (time for each development is 90 min), then doubly developed with the solvent system n-butanol:acetone:acetic acid:water (35:35:10:20) (time for each development is about 60 min). A 0.4 mol/liter solution of ninhydrin in butanol:acetone (50:50) is added to the solvent before the last development (3 ml of ninhydrin solution per 40 ml of solvent). After development in the solvent containing ninhydrin, the plates are dried in warm air, then heated in the drying oven at 80°C for 3 min. Should a strong background discoloration result, the temperature of the drying oven must be set 5 to 10°C lower. When a fume hood is available, the ninhydrin can be sprayed onto the plate [0.5% ninhydrin in ethanol:glacial acetic acid (98:2)]. When supports other than aluminum foil are used for the sorbent layer, other heating times are more appropriate. Glass plates, for example, require 5 min at 80°C.

Evaluation

Most amino acids react with ninhydrin to form a violet-blue dye. The following amino acids are exceptions and give different shades: 1-methylhistidine and 3-methylhistidine spots are grey-blue or reddish-violet, proline and hydroxyproline spots are yellow, and asparagine spots are orange.

Fig. 1. Application scheme. Dimensions are in millimeters

After evaluation of the chromatogram, the plate can be reheated for 15 min at 120°C. Spots corresponding to the following amino acids, otherwise only visible at very high concentrations, then appear: β-aminoisobutyric acid, phosphoethanolamine, taurine (violet-red), carnosine (grey-blue), and β-alanine (blue). The ethanolamine and putrescine spots are intensified and the color of the proline and hydroxyproline spots changes from yellow to violet-red. The same effect is obtained by allowing the plate to stand for 29 to 48 h at room temperature.

Amino acids on the chromatogram are first identified by use of a chromatogram of a normal adult urine (Figure 2). The triangle of spots, glutamine:glycine:serine, is especially characteristic. The most important amino acids can be identified from Figure 2. The position of other amino acids that are less often present can be found by comparison with Figure 3. The following points should be especially noted:

1. Separation is poorer if the amino acid concentration is too great. When the concentration of the basic amino acids is too great, double spots are obtained in acidic solvents; very high concentrations of the acidic amino acids give double spots in basic solvents.

2. After development in ammonisocal solvents, methionine is partly oxidized in the drying process. This is the cause of the supplementary methionine spots shown in Figure 3.

3. Taurine and phosphoethanolamine are eluted from the ion exchanger with water. Should one
wish to detect these amino acids, the plate can be developed with water to the first solvent-front line, and the remaining chromatography then is done as usual.

**Results**

Figure 4 shows the separation of a mixture of pure amino acids. Because the concentrations of the individual components in urine are different, chromatograms of urine look different.

On comparing chromatograms of urine that have been desalted by means of a two-layer plate (Figure 5) with chromatograms of urine that have been desalted on a column, the following differences are found: The amino acids, cystine and cystathionine, appear on the chromatograms of urine on two-layer plates as slightly distorted spots. Their position is changed as compared to the chromatogram of pure amino acid solutions; however, this effect is less pronounced when desalting is done on a column. Also, some black lateral bands are noticeable on the two-layer plates, which do not, however, affect the detection of the amino acids. Apart from these effects, the quality of the chromatogram obtained after desalting on a two-layer plate is equivalent to that of a chromatogram obtained after desalting on a column.

The following separations are critical with the solvent pair described: leucine and isoleucine, glutamine, citrulline, and glycine. When these last three amino acids are present in substantial amounts, they are hardly separated. When one of the three amino acids is missing, a separation is possible. With very high concentrations of 3-methylhistidine, the separation from serine is sometimes poor. The approximate detection limits for various amino acids are shown in Table 1.

Changes in the amino acid concentration (Figure 6) or the presence of unusual metabolites is in every case easily recognizable (Figures 7 and 8).
Fig. 4. Chromatogram of a mixture of pure amino acids

Fig. 5. Amino acid chromatogram of a urine from a normal adult

Fig. 6. Chromatogram after addition of histidine to a normal urine (20 mg/100 ml). The histidine spot is intensified

Fig. 7. Chromatogram after addition of argininosuccinic acid to a normal urine (20 mg/100 ml)

Discussion

Compared with desalting on a column, desalting by means of a two-layer plate does not require the regeneration of a column or the concentration of an eluate. A procedure has been described by Copley and Truter (4) in which an ammoniacal solvent at elevated temperature (40 °C) simultaneously elutes the amino acids from the ion-exchange layer of a two-layer plate and separates them. According to our experience, this procedure, which, apart from the use of unusually high temperature, is very simple and elegant, does not provide the necessary resolution.

About 38 amino acids, amines, and peptides that we have tested can be separated by use of the recommended solvent pair described under Procedure. The separations that are difficult to achieve with the solvent pair we have chosen are, according to present knowledge, not important in the screening of urinary amino acids. In the presence of maple-syrup disease, excretion of the branched-chain amino acids, leucine, isoleucine, and valine, is increased. Such an increase is immediately appreciated—the amino acids, leucine and isoleucine, which our suggested procedure does not separate, are both increased. Citrulline is normally present in only very small amounts in urine. An increase
in this amino acid is conspicuous, because the area between glutamine and glycine is then filled. In addition, a suspected case of citrulline can be easily identified by spraying with Ehrlich’s reagent (5).

The use of the pair of solvent systems described generally eliminates the need for preparing a second chromatogram with a second pair of solvent systems. Although the development time for the chromatograms is long, this hardly can be counted into the work expenditure. The sensitivity of detection of the method for the various amino acids is so great that a false negative result caused by the detection limit being too high never comes into question.

The purpose of the work under consideration was the development of a suitable and effective separation method for screening, one that is reliable in routine use. In the practical use of the method, the standardization of the volume of applied urine related to the creatine content is of importance. We suggest that a volume of urine containing 1 µg of creatinine be applied (1).

We wish to thank Mr. Erkens for his meticulous performance of the experiments.

References