Thin-Layer Chromatographic Screening of Amino Acids in Plasma and Urine of Newborns

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We describe a simple, rapid, and inexpensive unidimensional high-resolution thin-layer chromatographic procedure for screening for aminoacidopathies in newborns. Protein-free filtrates are prepared for ultrafiltration of as little as 10 µl of plasma. A rapid, inexpensive photographic method provides large, high-contrast pictures of the chromatograms for permanent records. A day’s samples can be completed in 3 h, including all phases of the procedure.

Additional Keyphrases: tyrosinemia in premature infants • photographic records of chromatograms • inherited disorders

Thin-layer chromatography is currently a widely used method for screening plasma and urine specimens for amino acid abnormalities (1–3). Despite improvements over paper chromatography, the amino acid patterns for plasma are seriously obscured by the protein. Procedures proposed for eliminating this problem include, for example, the use of centrifuged supernates from sera that have been heated in boiling water (2). Other methods of deproteinization involve unacceptable dilutions and (or) coprecipitation of some of the amino acids.

In a preliminary report, it was pointed out that ultrafiltration of plasma will remove this interference (4). Here, we describe our experience of over a year in using this technique to prepare protein-free filtrates from extremely small samples of plasma. In addition, we describe an inexpensive and simple method in which an ordinary photographic paper is used to provide large, clear pictures for our permanent records.

Materials and Methods

Equipment

Ultrafilters were made from polycarbonate rod, 17 mm (¼ inch) in diameter (Figure 1). Bodies of upper and lower parts were 28 mm (1 1/8 inches) long. Male and female screw connectors were 10 mm (⅜ inch) in diameter, with 16 threads per inch. Initially, set screws were 5 mm (¼ inch) with 28 threads per inch, but later units were made with 20 threads per inch, because these were easier to insert and remove without damaging the bottom unit threads. Center bores were 1.5 mm (⅛ inch) in diameter, and were beveled to 3 mm (⅛ inch) at the end of the male screw connector, to provide a large filtration area. Plastic washers were cut with a No. 3 power cork borer, and center holes were punched with a 13-gauge syringe needle, rounded and sharpened at the tip. After each use, these ultrafilter units should be well cleaned with distilled water.

Other items include a cork borer, manual, No. 3; an Allen wrench for inserting bottom set-screws; a microcentrifuge, Clay-Adams or similar, with about 15 000 × g capability; a hot air blower; a chromatography tank, rectangular, glass, capable of holding 20 × 20 cm thin-layer sheets; and a drying oven at 70 °C. In addition, a photographic dark room is needed with a bellows camera, Rembrandt (Burke and James Co., Chicago, Ill.), on a fully adjustable stand, capable of holding 5 × 7 inch film holders, equipped with a flat field lens [89 mm (3.5 inch), F6.3, Ilex Process Paragon, mounted on a Copal shutter, Ilex Optical Co., New York City] and a shutter timer; two flood lamps (Luxo) with circular fluorescent lamps, on universally adjustable stands; an Ednalite "Lightable," Model 150B-B, with circular fluorescent lamp for uniform backlighting; a red safety light; a refrig-

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erator; film holders for 5 × 7 inch film; squeegee sheet, ferrotype, with rubber roller; and trays for developer, fixer, and water washes. All photographic equipment and supplies were obtained from Standard Photo Supply Co., Chicago, Ill. 60611.

Supplies

Nitrocellulose membrane, 1 inch by 10 foot rolls (No. VSWP025C1; Millipore Corp., Bedford, Mass. 01730).

Capillary micropipettes, 5-, 10-, and 50-μl.

Control sera: “Validate” is suitable, with phenylalanine and tyrosine added to concentrations of 5 and 10 mg/100 ml each.

Thin-layer cellulose chromatogram sheets: These are 20 × 20 cm, plastic backed, without fluorescent indicator (No. 13255; Eastman Kodak Co., Rochester, N. Y. 14650).

Developing solvent: Mix n-butanol:acetic acid: water (120:30:50 by vol) in a chromatography tank, and saturate the atmosphere with the help of solvent-soaked filter paper sheets on the walls of the tank. This system is stable for at least five days.

Ninhydrin spray (No. NIN-3; Sigma Chemical Co., St. Louis, Mo. 63178).

Developer: A container of “Ethol LPD” single mix is dissolved in three liters of warm water and diluted with cold water to four liters for stock, and kept refrigerated. Working developer is made by adding one volume of stock plus two volumes of water. Use at room temperature.

Fixer: Contents of Edwal Quick Fix containers, including hardener, are diluted in water to one gallon. Use undiluted at room temperature. Store refrigerated.

Photographic paper: Resin type, plastic backed, high contrast Kodak KIND-2200 paper is obtained in 11 × 14 inch sheets. Cut into 4 7/8 × 7 inch pieces for insertion into 5 × 7 inch film holders.

Clinical Material

Blood from newborn infants was obtained in heparinized pediatric-size collecting tubes and centrifuged. In one survey covering 80 consecutive days, samples were gotten from all newborns who were routinely screened for plasma phenylalanine. Several specimens of plasma and urine with known aminoacidopathies were obtained from Dr. Paul W. K. Wong, University of Illinois Department of Pediatrics.

Preparation of Plasma and Urine

Plasma: Cut and presoak the pieces of nitrocellulose membrane in distilled water for at least 15 min. Place the plastic washers in the bottom halves of the micro-ultrafilter units, over the center bore in the female connectors (Figure 1). Cut discs of moist, blotted nitrocellulose membrane with the No. 3 cork borer, on a hard plastic surface, and place them over the washers, glossy side up, by using special flat-surface forceps. Fill the upper part of the center bore with 20 to 50 μl of plasma, by using 50-μl capillary micropipettes. Screw the top and bottom parts together, being careful not to invert the washer or the membrane. Tighten the set screw to the bottom of the unit, and centrifuge in a high-speed Clay-Adams microcentrifuge (Figure 2) for 10 min, or until the center well is about half filled with filtrate.

Urine: Centrifuge the urine in an ordinary tube if the specimen is cloudy. Ultrafilters may be prepared from urine if initial chromatograms show protein spots at the origin sufficient to interfere with the interpretation.

Fig. 2. Micro-ultrafilters as they fit into the Clay-Adams high-speed centrifuge
Chromatography

Unscrew the ultrafilters, remove the washers, transfer as much as 10 μl of filtrate to capillary micropipettes, from which the filtrate is spotted onto pre-marked application points on the cellulose thin-layer sheet. Best results are obtained if applications are placed to form a small band rather than a round spot on the application point. In the case of urine, similarly spot two samples (3 and 5 μl) of each clear specimen. Develop the chromatograms for over 2 h, or until the solvent front is within 3 cm of the top. Remove and dry the chromatograms, spray them with ninhydrin, and place them in a 70 °C oven for 10 min. Interpret the chromatograms, and keep them refrigerated until they are photographed (ninhydrin-treated chromatograms do not fade appreciably for several weeks when kept cold).

Photography

Position the chromatogram sheets on the Lightable 18 cm from the camera, with both backlighting from the Lightable and top-lighting from flood lamps, each positioned 30 cm from the center of, and 18 cm above, the chromatogram. Set the shutter aperture to f11, and exposure to B, so that exposure time is under control of the shutter timer. Take pictures directly onto photographic paper loaded in the film holders. With proper adjustments, exposure times should be between 6 and 10 s. Place exposed photographic paper sheets into working developer for about 30 s, rinse briefly, place in fixer for 1 min, wash in water, blot briefly on the squeegee and allow to dry.

Results

Figure 3 illustrates differences between chromatograms of filtered and unfiltered plasma and urine. Streaking caused by plasma protein seriously interferes with the slower components and also results in poorer resolution of the more mobile components as compared with the chromatogram of filtered plasma.

Although urine does not normally contain protein sufficient to interfere with the chromatogram, abnormally high concentrations of urinary protein may decrease the migration of all of the components. In such cases, urinary protein may be easily removed by micro-ultrafiltration (Figure 3).

Migration of Amino Acids

Amino acids were added to plasma, and chromatograms of the filtered samples are shown in Figure 4. Preliminary trials showed that separation and sharpness of bands were considerably improved if the standards were chromatographed with plasma, rather than if they were spotted as pure standards. With this system, both phenylalanine and tyrosine are readily separated, and may be quantitated to an acceptable degree.

Migration of various amino acids and their RF values are shown in Table 1. Variations in color permit distinctions that are not as apparent in these photographs (Figures 3–6). Although there is some overlap of some of the amino acids, separations are distinct enough so that further procedure for identification of abnormal components is greatly facilitated.

Phenylketonuria and Tyrosinemia

Figure 5 shows the ease with which one can detect supranormal plasma concentrations of phenylalanine and tyrosine in patients. Concentrations of 8 and 15 mg of phenylalanine per deciliter are readily seen, as well as 5 and 10 mg/dl concentrations of tyrosine, in comparison with duplicate chromatograms made from plasma of a normal subject.

In an 80-day survey of the incidence of tyrosinemia in consecutively evaluated premature and full-term newborns, about 20% of the premature infants showed abnormally high plasma tyrosine concentrations, as compared with about 0.4% of the full-term infants (Table 2).
Table 1. Migration of Amino Acids on Cellulose Thin-Layer Sheets*, and Color Produced with Ninhydrin

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Rf</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystine</td>
<td>0.12</td>
<td>Violet-gray</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.21</td>
<td>Red-violet</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.22</td>
<td>Gray</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.23</td>
<td>Gray</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.24</td>
<td>Blue-violet</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.27</td>
<td>Violet</td>
</tr>
<tr>
<td>Serine</td>
<td>0.30</td>
<td>Violet</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.31</td>
<td>Violet</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.32</td>
<td>Violet</td>
</tr>
<tr>
<td>Homocystine</td>
<td>0.32</td>
<td>Violet</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.35</td>
<td>Yellow</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.38</td>
<td>Violet</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.38</td>
<td>Violet</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.40</td>
<td>Violet</td>
</tr>
<tr>
<td>Proline</td>
<td>0.45</td>
<td>Brown</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.50</td>
<td>Blue-violet</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.56</td>
<td>Brown-violet</td>
</tr>
<tr>
<td>Methionine</td>
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<tr>
<td>Valine</td>
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<td>Violet</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<tr>
<td>Isoleucine</td>
<td>0.67</td>
<td>Violet</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.68</td>
<td>Violet</td>
</tr>
</tbody>
</table>

* Solvent system: n-butanol: acetic acid: water (120: 30: 50 ml).

Table 2. Incidence of Tyrosinemia in Full-term vs. Premature Newborns*

<table>
<thead>
<tr>
<th></th>
<th>Full-term</th>
<th>Incidence</th>
<th>Premature</th>
<th>Incidence</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>823</td>
<td>3</td>
<td>78</td>
<td>15</td>
</tr>
</tbody>
</table>

* Estimated concentrations greater than 5 mg of tyrosine per deciliter were considered positive.

Aminoacidopathies

Several abnormal amino acid patterns found in urine and plasma samples from patients are shown in Figure 6. Increased phenylalanine and tyrosine are easily seen in plasma samples A and B, as well as increased glycine in plasma D and in urine H. Increased urinary homocystine can be seen in the sample from a patient with homocystinuria (urine G), but a plasma sample, C, from a homocystinuric patient demonstrates only increased methionine. Argininosuccinic aciduria, F, and hyperlysinsuria, I, are seen with several of the characteristic associated elevations of other metabolites and amino acids.

Discussion

The advantages of using plasma prepared by ultrafiltration are as follows: (a) Proteins are efficiently removed, and no interference can be seen on the chromatograms (Figure 3). (b) As little as 10 μl of plasma can yield sufficient filtrate for a good chromatogram, although 20- to 50-μl samples are ordinar-

ily processed. (c) Filtrates are prepared with only 10 min of centrifugation, because the beveled bores on the top parts of the ultrafilters provide a large filtration area relative to the sample volume (Figure 1). The entire procedure, including chromatogram development, can easily be completed within 3 h.

The availability of a good-quality plastic-backed resin-type photographic paper (Kodak KIND-2200) made it possible to take large, high-resolution pictures without negatives (Figures 3–6). The use of both backlighting and top illumination permits better light control and higher contrast without glare. Each 5 × 7 inch sheet costs about 5 cents. This paper dries quickly without curling, and requires only a minimum of blotting on a squeegee. The same photographic process is now being used to obtain very clear and large reproductions of immunoelectrophoretic patterns.

Although resolution was excellent, some overlapping of the amino acids was still observed (Table 1, Figure 4). Apparent abnormalities in urinary and plasma amino acid patterns are currently being followed up by gas–liquid chromatography of n-propyl, N-acetyl derivatives of sample amino acids, and also by use of a high-resolution amino acid analyzer. The clarity of our chromatograms greatly facilitates our followup procedures.

About 20% of newborn premature infants were found in a consecutive 80-day survey of newborns in our institution to have increased plasma tyrosine concentrations (Table 2). None of the phenylalanine assays gave any indication of high tyrosine concentrations, which were readily seen on the chromatograms (Figure 5), although increased tyrosine is known to interfere slightly with our fluorometric

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assay for phenylalanine. One group of investigators found 10 of 18 low-weight newborns to have plasma tyrosine concentrations greater than 5 mg/dl (5); another reported plasma tyrosine concentration in low-weight newborns to average 15.8 mg/dl (6). Thus such increased tyrosine concentrations in premature infants is a common finding, and may not be as harmless as commonly thought (7).

Some of the less rare aminoacidopathies, such as histidinemia (4 to 7 per 100,000), hyperprolinemia (5 per 100,000), Hartnup disease (4 to 7 per 100,000), iminoglycinuria (5 to 6 per 100,000), and cystinuria (7 per 100,000) have been reported to occur almost as frequently as phenylketonuria (7 to 15 per 100,000) (7–9). The thin-layer chromatographic procedure reported here provides a simple unidimensional method for detecting not only a wide variety of rare genetic defects (Figure 6), but also tyrosinemia in premature infants, which occurs frequently and is treated readily. The versatility, wide applicability, and sensitivity of this method should make it a procedure of choice in screening for aminoacidopathies.

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