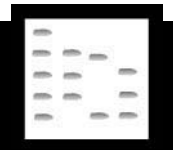
IN-BORN METABOLIC DISORDERS: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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The term inborn errors of metabolism or, more precisely, inherited metabolic diseases (IMD), is usually applied to a large group of relatively rare genetic disorders in the process of intermediary metabolism, transport defects or impaired receptors. The diagnostic procedure is complicated; the difficulty is the lack of sharp criteria for differential diagnosis, since the attendant symptoms are usually nonspecific. Comprehensive and specialized biochemical investigations therefore are the basis for the diagnosis of IMD. Using this approach, selective laboratory screening programmes can be prepared. The analytical programme includes a three-stage systematic procedure. Qualitative or semiquantitative procedures for urine metabolites are used when starting the investigation, the second step includes quantitative methods, while enzyme analysis or DNA testing belongs to the third stage, and completes the process of examination.

Multicomponent analysis of body fluids using gas and liquid chromatography combined with mass spectroscopy is the most important procedure used in the screening of IMD. However, at first one cannot do without simple and inexpensive methods like colour tests and thin-layer chromatography (TLC), which may give rapid qualitative information on metabolic conditions.

Selective laboratory screening concerns the analysis of individual groups of metabolites, usually those of amino acids and small peptides, catabolites of tryptophan, sugars, oligosaccharides, glycosaminoglycans, organic acids, purines and pyrimidines.

Separations are performed on pre-coated cellulose or silica gel high performance TLC (HPTLC) plastic or glass plates with or without fluorescent indicator F_{254}. The plates lay on a temperature-controlled surface (110–115°C), rapidly evaporating the elution solvent in the process of sample spotting. A volume, equivalent to a chosen amount of creatinine is used, when urine is applied.

Plates are developed with a mobile phase in either a horizontal DS chamber (Chromdes, Poland) or in a vertical pre-saturated glass tank. Chromatograms are dried and visualized under ultraviolet (UV) light of $\lambda = 254$ and 366 nm and/or by spraying with a detection reagent. Reference standards are used for metabolite identification. Quantification is carried out by linear scanning with a TLC scanner, operated with a PC software package. Interpretation of results is made according to age, diet, therapy, clinical symptoms and elementary biochemical results.

Amino Acids

Two-dimensional ascending TLC on cellulose with ninhydrin detection is used as the first approach in the screening for amino acid metabolic disorders. As a rule, 10 μL of plasma, serum, cerebrospinal or amniotic fluids (previously deproteinized with solid sulfosalicylic acid, 50 mg mL^{-1}) and a volume of urine, equivalent to 20 nmol of creatinine (previously desalted on Dowex-50WX8 in H^+ form, eluted with 2 mol L^{-1} ammonia) are applied to the 50×50 mm HPTLC cellulose layer. The plate is then developed in the solvent systems 2-propanol–formic acid–water, 80 : 4 : 20, in the first dimension and *tert*-butanol–acetone–25% ammonia–water, 50 : 30 : 10 : 10, in the second dimension. Chromatograms are sprayed with ninhydrin reagent, observed within 1 h and the next day, then heated for 3 min at 80°C (**Figure 1**). Aspartylglycosamine (in patients with lysosomal storage disease) can be detected as a blue-green spot when lightly overstained with a mixture of concentrated acetic and hydrochloride acids, 4 : 1 and heated at 60°C for 5 min. For detection of proline and hydroxyproline isatin (0.2 g in 100 mL acetone + 5 mL acetic acid) is recommended. When abnormalities are suspected, multiple spraying, in addition to ninhydrin, can be used: Ehrlich reagent (for tryptophan, hydroxyproline, citrulline and homocitrulline), Pauly reagent (for histidine and tyrosine metabolites), Sakaguchi reagent (for arginine) or platinic iodide (for sulfur amino acids).

Mono- and Disaccharides

The best results of screening for sugar defects have been achieved using two-dimensional vertical TLC on silica gel glass plates with orcinol detection.

Filtered urine is diluted with 2-propanol, 1 : 1, and an aliquot, equivalent to 5 nmol of creatinine (or alternatively 5 μL equivalent of plasma, serum or cerebrospinal fluid, deproteinized with solid sulfo-

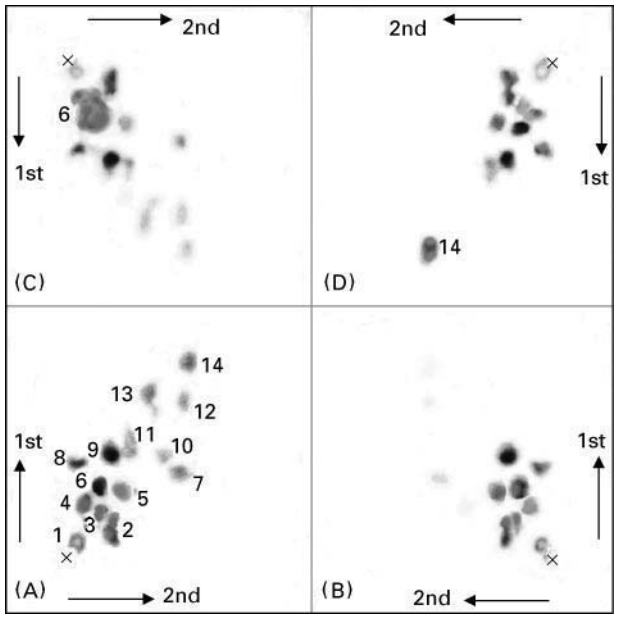

Figure 1 TLC of amino acids. The original 100 × 100 mm glass plate was divided into four by scraping off material. (A) Normal plasma; (B) normal urine (infant); (C) nonketotic hyperglycinaemia (urine, neonate); (D) leucinosis (urine, neonate). 1, Cys; 2, His + MeHis; 3, Lys; 4, Gln; 5, Ser; 6, Gly; 7, Tau; 8, Glu; 9, Ala; 10, Thr; 11, Tyr; 12, Phe; 13, Val; 14, Leu.

salicylic acid, 50 mg mL^{-1}) applied on the 50 × 50 mm layer. The plate is consecutively developed, with 0.5% boric acid in water–*n*-butanol–2-propanol, 20 : 30 : 50 (w/v) in the first direction and ethyl-acetate–acetic acid–water, 60 : 20 : 20 in the second direction. A freshly prepared mixture of orcinol (0.4% in ethanol, w/v) and concentrated sulfuric acid, 19 : 1, is used for detection. The plate is examined by transmitted light after 10 min warming at 100°C. Identification is made by comparison with standards, separated on the same plate (**Figure 2**).

Oligosaccharides

One-dimensional horizontal TLC on silica gel glass plates with fluorescent indicator F_{254} combined with orcinol or resorcinol detection is used for screening of certain lysosomal storage diseases and adenylosuccinate lyase deficiency. A supernatant of centrifuged native urine is applied in an amount, derived from urinary creatinine and the age (μL of sample = 40 × *F* per concentration of creatinine in mmol L^{-1}; $F = 0.75$, 1, 1.5 and 2 for the ages < 1, 1–2, 2–8 and > 8 years, respectively). The plate is developed twice under saturated conditions with a freshly prepared mixture of *n*-butanol–acetic acid–water, 4.5 : 2 : 2, with drying in between. The chromatogram is observed under UV 254 nm light to look for two dark bands of succinyl purines (absorbing at 254 nm) below raffinose. The oligosaccharides are then visualized by spraying with orcinol in the same way as described for mono- and disaccharides (**Figure 3**). Positive finding in UV light leads to rechromatography and detection with Pauly reagent and naphthoresorcinol, warmed for 10 min at 100°C. The same chromatographic procedure is convenient for screening of sialurias if using resorcinol reagent. For this a mixture of 1% resorcinol in 95% ethanol, w/v, and 2 mol L^{-1} HCl, 1 : 9, with the addition of 0.1 mol L^{-1} $CuSO_4.5H_2O$ in water (0.025 mL per 10 mL of the mixture) is used. After spraying, the chromatograms are covered with glass and warmed at 120°C for 30 min. Pathological glycopeptides in urine (on α-*N*-acetylgalactosaminidase deficiency, aspartylglycosaminuria and fucosidosis) should first be detected by ninhydrin (see section on amino acids, above), then overstained with orcinol.

Glycosaminoglycans

One-dimensional multisolvent TLC on cellulose plastic sheet is one of the methods used for qualitative analysis of urinary glycosaminoglycans (GAGs, acid mucopolysaccharides), whenever the photometric screening test with azure A + B is repeatedly positive. GAGs are isolated from the sediment of centrifuged urine (10 mL aliquots of 24 h urine, adjusted to

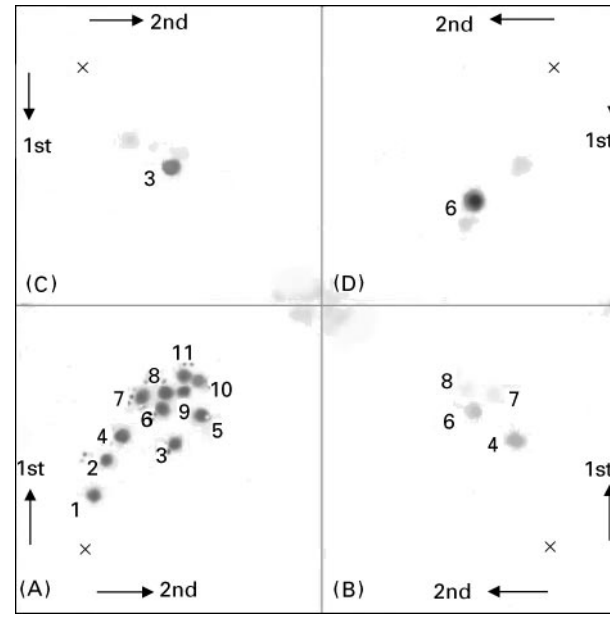

Figure 2 Separation of sugars (and oligosaccharides). The original 100 × 100 mm glass plate was divided into four by scraping off material. (A) Standard mixture; (B–D) urine samples: (B) normal neonate; (C) fructose intolerance (infant); (D) galactosaemia (neonate). 1, stachyose; 2, raffinose; 3, fructose; 4, lactose; 5, ribose; 6, galactose; 7, saccharose; 8, glucose; 9, xylose; 10, mannose; 11, arabinose.

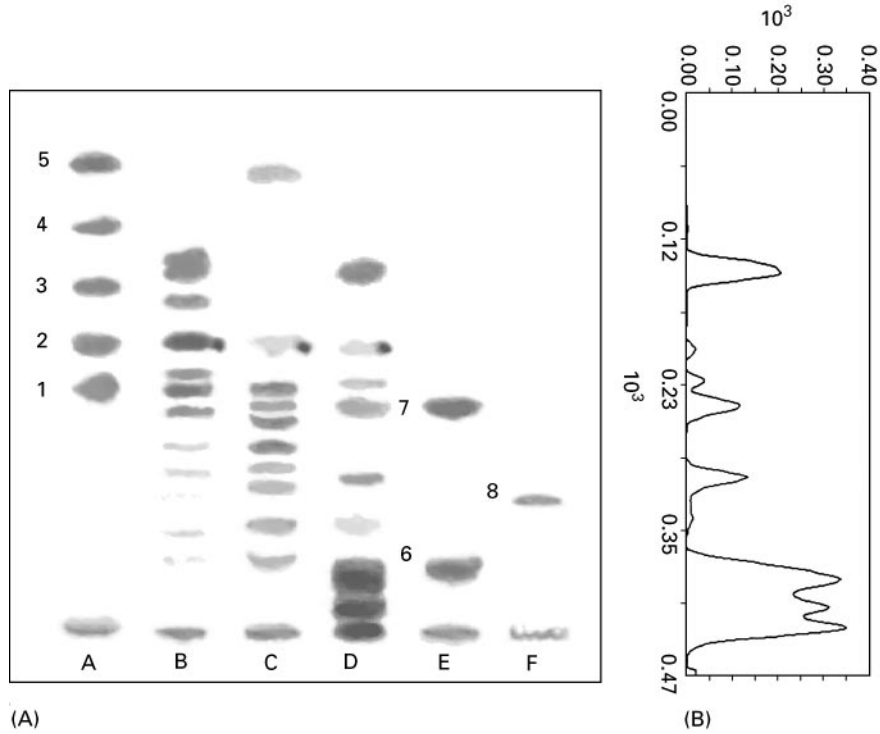

Figure 3 (A) TLC of oligosaccharides (and sugars), overlapping lactose as a standard. A, E, F, standards; B–D urine samples; A, normal infant; B, normal neonate; D, juvenile G_{M1}-gangliosidosis; F, overstained with resorcinol. 1, raffinose; 2, lactose; 3, glucose; 4, xylose; 5, ribose; 6, G_{M1}-octosaccharide; 7, glucotetrasaccharide; 8, sialic acid. (B) Densitogram of line D.

pH 5.5) by precipitation with 0.2 mL 5% aqueous cetylpyridinium chloride for 4 h in an iced-water bath. The dry precipitate is washed using 10 mL of 95% ethanol (saturated with sodium chloride) then diethyl ether, with centrifugation, decantation and drying in between. The precipitate is dissolved in 0.6 mol L^{-1} sodium chloride (100 μL) and 10 μL aliquots are spotted on the layer. GAGs are successively separated (incremental distances of 2 cm for each run without intermediate drying) according to the solubility of their calcium salts in six solvents of decreasing ethanol concentration (1 mol L^{-1} acetic acid–calcium acetate (g)–95% ethanol, v/w/v; I, 30 : 1 : 70; II, 40 : 2.5 : 60; III, 50 : 2.5 : 50; IV, 60 : 2.5 : 40; V, 70 : 2.5 : 30; VI, 100 : 5 : 0). After the sixth run, the plate is dried and immediately stained by immersing in toluidine blue in ethanol–acetic acid for 3 min. Excess stain is removed by rinsing in 10% acetic acid and the air-dried plate is evaluated by comparing with standards and using a densitometer (**Figure 4**).

Organic Acids

One-dimensional sequential TLC on cellulose glass plates in a horizontal arrangement with aniline–xylose detection is used in the screening for pathological organic acidurias.

Plasma, cerebrospinal fluid or vitreous humour is deproteinized with 95% ethanol, the supernatant is evaporated at 25°C under nitrogen, dissolved in water and the equivalent of 1 mL of the native material is further processed. A volume of urine, equivalent to 2.2 μmol of creatinine, is made up to 1 mL with deionized water. All samples are then spiked with phenylbutyric acid as an internal standard, acidified to pH 1 with concentrated HCl and saturated with NaCl. Organic acids are extracted with 6 mL of diethyl ether–ethyl acetate mixture, 1 : 1 (vortexed, three times for 30 s). The supernatant is mixed with 100 μL of 1 mol L^{-1} ammonia solution in ethanol (to protect the volatile organic acids), concentrated under nitrogen at 25°C to 1 mL, and 20 μL aliquots applied on the plate. Development is performed under saturated conditions in four consecutive steps (development distances increased in 1 cm steps in each run with intermediate drying), using the mobile-phase *n*-propanol–2 mol L^{-1} ammonia, 7 : 3. The plate is sprayed with aniline–xylose reagent (xylose and aniline in methanol) and evaluated with a densitometer (**Figure 5**).

Purines and Pyrimidines

Two-dimensional TLC on cellulose glass plates in the horizontal arrangement with UV detection at

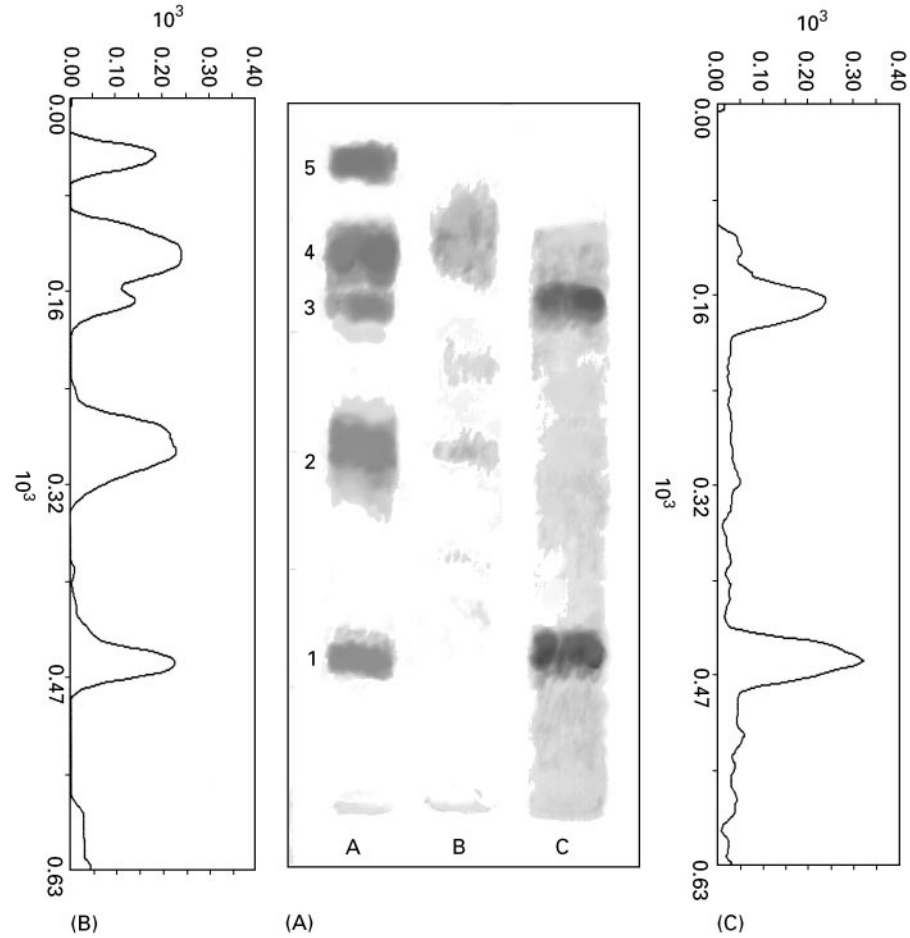

Figure 4 (A) TLC of glycosaminoglycans, multiple development. A, standards; B, control urine; C, mucopolysaccharidosis I–H. 1, dermatan-; 2, chondroitin-4-; 3, heparan-; 4, chondroitin-6-; and 5, keratan sulfates. (B) Densitogram of lane A; (C) densitogram of lane C.

254 nm is used to screen purine and pyrimidine defects. After 3 days on a low purine diet, 24 h urine is collected, warmed for 30 min at 50°C to dissolve precipitates and a filtered sample, equivalent to 50 μmol creatinine, spotted on the layer. The mobile phase is *n*-butanol–methanol–water–25% ammonia, 40 : 20 : 20 : 1, developed twice with 15 min drying between each run for the first direction and 2 mol L^{-1} ammonium sulfate in water for the second direction. Chromatograms are evaluated under UV light and by comparison with an age-control urine and the nucleoside and base standards, separated in parallel (**Figure 6**). For further identification, chromatograms are sprayed with mercuric acetate in 95% ethanol, then immediately with diphenylcarbazone in 95% ethanol and heated at 120°C for 10 min.

Tryptophan and its Metabolites

Screening method for both indolic and kynurenine metabolites of tryptophan (Trp) in urine is based on Sep-Pak C_{18} pretreatment, two-dimensional TLC on cellulose and detection at 254 and 366 nm, followed by staining with Ehrlich reagent. A volume of urine, equivalent to 2 μmol of creatinine, is acidified to pH 3.5 and the clear supernatant applied on the Sep-Pak cartridge. Impurities from the sample are washed out, successively with sodium dodecyl sulfate (SDS) and SDS–methanol. The Trp metabolites are eluted with a mixture of 1 mol L^{-1} ammonia and methanol (8 : 2). After evaporating the solvent under nitrogen the residue is dissolved in 250 μL methanol. An aliquot of 20 μL is applied on the dry cellulose layer, previously washed with deionized water. The plate is subjected to ascending development under saturated conditions at 4°C with two 0.2 mol L^{-1} sodium acetate buffers: pH 6 for the first and pH 3.3 for the second direction. The air-dried plate is examined under UV light (**Figure 7**) and then sprayed with Ehrlich reagent. Comparing the urine sample with a mixture of standards, identification of 13 metabolites of tryptophan is possible.

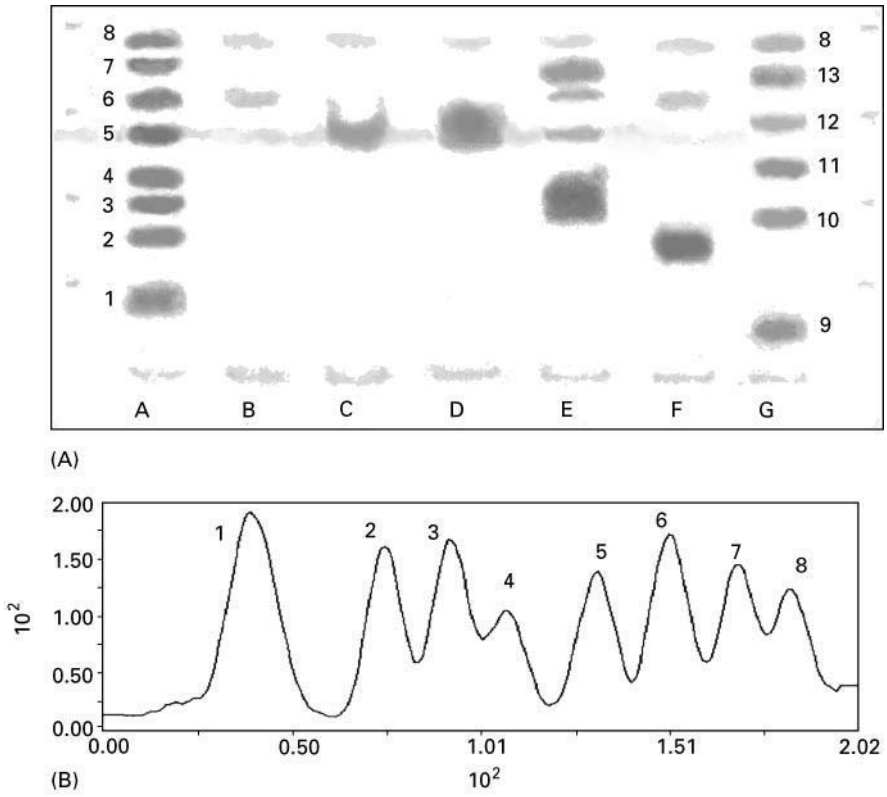

Figure 5 (A) TLC of organic acids, multiple development. A, standard mixture I; B, normal urine; C, normal plasma; D, lactic aciduria; E, 3-hydroxy-3-methylglutaric aciduria; F, methylmalonic aciduria; G, standard mixture II. 1, citric; 2, methylmalonic; 3, 3-hydroxy-3-methylglutaric; 4, ascorbic; 5, lactic; 6, hippuric; 7, isovaleric; 8, phenylbutyric (internal standard); 9, phosphoric; 10, adipic; 11, suberic; 12, sebacic; 13, 3-hydroxyisovaleric acids. (B) Densitogram of lane A.

Discussion

The protein-free filtrate from plasma and other material must be prepared rapidly to avoid binding of sulfur-containing amino acids to proteins. Some changes occur rapidly at room temperature or even when stored at $-20^{\circ}C$ for a week (glutamine and asparagine, in particular, disappear).

There are several amino acid metabolic disorders which can easily be detected solely by TLC screening

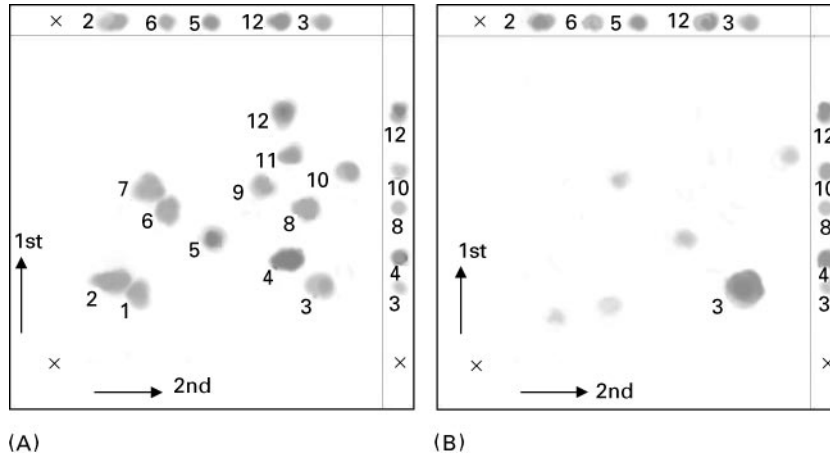

Figure 6 TLC of purines and pyrimidines. (A) Standards; (B) uric aciduria (adult). 1, xanthine; 2, guanine; 3, uric acid; 4, xantosine; 5, hypoxanthine; 6, adenosine; 7, adenine; 8, orotic acid; 9, uracil; 10, cytosine; 11, inosine; 12, thymine.

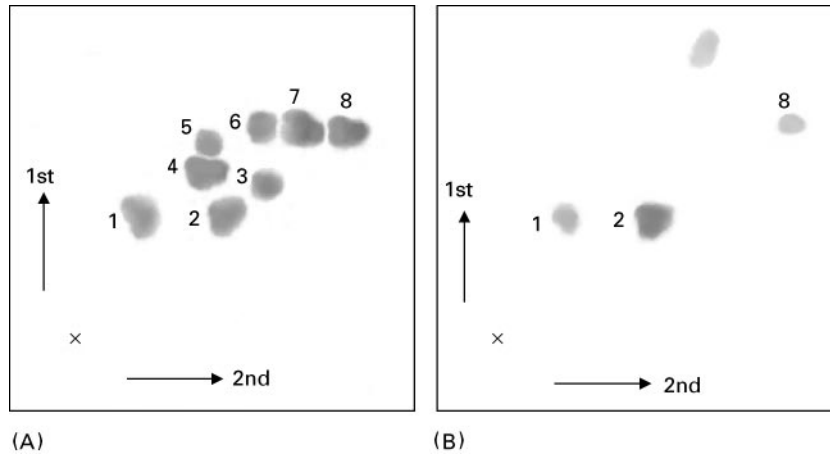

Figure 7 Separation of some indolic and kynurenine metabolites of tryptophan, UV 254 nm detection. (A) Standards, (B) xanthurenic aciduria (urine, child). 1, indolylacryloylglycine; 2, xanthurenic acid; 3, kynurenine; 4, kynurenic acid; 5, 5-hydroxyindolic acid; 6, 3-hydroxyanthranilic acid; 7, anthranilic acid; 8, indoxyl sulfate.

of urine, plasma or cerebrospinal fluid, such as leucinosis, phenylketonuria (where infants have not had newborn mass screening), hypermethioninaemic or -uric conditions, iminoglycinuria or cystinuria. An increase of glycine indicates primary nonketotic hyperglycinaemia or can be a secondary indication of an organic acid disease. Plasma glutamine is an important indicator of disorders of ammonia removal. Amniotic fluid is useful for prenatal detection of citrullinaemia, argininosuccinic aciduria or homocystinuria. High glycine in urine during therapy with valproate or ninhydrin-positive metabolites of antibiotics may confuse the interpretation of results.

On other occasions TLC only leads to a suspicion of IMD and a more precise method (amino acid analysis or HPLC) must be used for final diagnosis.

TLC is a simple screening technique for sugars, provided that their precursors are present in food. Under normal conditions no melituria is detectable. In neonates, traces of lactose and galactose appear in urine. Malabsorption, sugar intolerance, type I tyrosinosis and liver diseases, besides the genetic defects in metabolism of sugars, are the conditions related to pathological melituria. Two ribose-containing succinylpurines appear as blue-grey spots close to saccharose and galactose on chromatograms from urine and cerebrospinal fluid of patients with adenylosuccinase deficiency (IMD of purines).

Further investigation of sugar defects can be performed by specific enzyme assays, GC and HPLC.

Only slight banding with the most prominent glucose below raffinose-tetrasaccharide is detectable in normal urine when analysing oligosaccharides. Diagnosis may be difficult in neonates, where physiological lilac bands should be differentiated from the brownish ones excreted in mannosidosis, or from the pink-brown stripes seen under fucosidosis.

It is possible to combine a successive detection in UV light and spraying by ninhydrin, orcinol and resorcinol without losing sensitivity substantially. Some authors emphasize the importance of sample desalting; we have not found this necessary, desalting may lose some metabolites.

The final diagnosis can be achieved by enzyme activity assessment or structural analysis of the excreted oligosaccharides by gas chromatography–mass spectrometry (GC-MS) or nuclear magnetic resonance spectroscopy.

Most of the mucopolysaccharidoses (MPS) known so far present patterns of urinary GAGs which are clearly different from normal, even if the total urinary GAGs are not always present in excess (some cases of type III MPS), or the typical component does not react (keratan sulfate in MPS type IV). On the other hand, some pathological chromatograms may not reliably differentiate between MPS types I and VI, for example, without enzyme analysis. The main advantage of TLC of GAGs is to facilitate selection of the probable defective enzyme to be analysed for the definite diagnosis.

Urinary organic acid analysis is a vital diagnostic tool in the investigation of patients with suspected IMD, although not many laboratories perform TLC of organic acids. We consider this approach to be an important and convenient way of getting prompt orientation, allowing exclusion of negative samples in the first place. In healthy controls, organic acids are present at trace levels, the most prominent being hippuric acid. Pathological specimens are conspicuous by either unusual or very dark bands. Unclear or suspicious results are further checked by GC-MS, employing the remaining sample which is immediately lyophilized after an aliquot has been applied on the TLC layer.

Organic acid analysis may help in the diagnosis of saccharides, amino acids, fatty acids and respiratory chain defects.

Using two-dimensional TLC, some purine and pyrimidine defects can be revealed. Unfortunately, specific and sensitive visualization agents of these strongly UV-absorbing substances are not available. Large unusual spots in urine are easily detectable, but some false positives, due to other metabolites, drugs or food additives, should be carefully interpreted and verified by HPLC. HPLC-MS combined with an ion exchange pretreatment of urine is a promising method for the final diagnosis of these defects.

Metabolites of Trp are difficult to analyse because of their instability and low concentration in urine. To minimize losses, bright light should be avoided throughout the procedure. The method used is simple and reliable enough to detect increased excretion of Trp metabolites. In the case of any abnormality observed, the same pretreated urine sample is further processed by HPLC.

Conclusion

TLC might seem to be slightly overshadowed since the introduction of more sophisticated methods in the screening of IMD. However, these methods are not available everywhere and their cost may discourage their use for screening, so that they are only applied in rather advanced cases. Using TLC, more samples can be separated simultaneously on one plate under identical conditions, which decreases the price per analysis. Horizontal arrangement of plates can be economically employed for routine use because mobile-phase consumption is very low. Availability of pre-scored layers makes analyses more flexible, more rational and economical. Advantages of HPTLC precoated layers, compared to the standard ones which were previously used, consist in sharper separation, shorter migration distance, minimal diffusion and increased detection sensitivity.

Various TLC techniques in screening for IMD have been described. In general, we prefer such procedures, when the sample, analysed by TLC, may be subject to more detailed analysis by HPLC or GC-MS, if necessary. Using creatinine concentration as a basis for the volume option of the urine processed, misinterpretation of results can be minimized.

In children with unexplained neurological disease it is highly recommended that amino acid and sugar analysis of cerebrospinal fluid and urine before and after acid hydrolysis is performed systematically. In this way defects of purines or (*N*-acetylated) amino acids and peptides can be detected by virtue of a large increase in aspartate, glycine or ribose levels.

TLC represents only one part of the whole IMD screening procedure and it is necessary to emphasize that negative TLC results do not prevent more detailed investigation if the patient shows continuous signs of an acute disease or progression of neurological symptoms.

It should be emphasized that negative findings are also important, because metabolic disease can be excluded.

Acknowledgement

This work has been supported in part by the Ministry of Health, Czech Republic, Project No. 4097-3.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Instrumentation; Modes of Development: Conventional. **III/Acids:** Thin-Layer (Planar) Chromatography. **Amino Acids:** Thin-Layer (Planar) Chromatography. **Carbohydrates:** Thin-Layer (Planar) Chromatography. **Clinical Chemistry: Thin-Layer (Planar) Chromatography. Nucleic Acids:** Thin-Layer (Planar) Chromatography.

Further Reading

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