Vertical Sandwich-Type Continuous/Evaporative TLC with Fixed Mobile Phase Volume for Separating Sugars of Clinical Relevance in Paper-Borne Urine and Blood Samples in Newborn Screening¹

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We describe the history and current implementation of an inexpensive thin layer chromatography (TLC) method, vertical sandwich-type continuous/evaporative TLC with fixed mobile phase volume, that is convenient for detecting and identifying reducing sugars of clinical relevance in the paper-borne blood and urine samples collected in neonatal screening programmes. This method facilitates screening by providing a considerable degree of standardization of chromatographic results. Among some 555,000 newborns to which it has been applied, it has detected 10 cases of classical galactosaemia, 7 cases

of galactokinase deficiency, 2 cases of glucosuria, and 3 cases of transitory neonatal diabetes mellitus; the only false negatives we are aware of were two cases of galacto-4-epimerase deficiency detected by tandem mass spectrometry. Screening for sugars in urine has allowed the detection of galactosaemia when the accompanying blood sample was invalid because of transfusion or parenteral feeding. The conclusion is that this inexpensive procedure is very useful for the detection of relevant metabolopathies in circumstances where others fail. *J. Clin. Lab. Anal.* 24:106–112, 2010. © 2010 Wiley-Liss, Inc.

Key words: sandwich TLC; continuous TLC; sugars; saccharides; oligosaccharides; dried urine on paper; newborn screening; galactosaemia; neonatal diabetes; glycosurias.

INTRODUCTION

Paper-borne urine samples were first used for newborn screening by Helen K. Berry, in 1958, originally for the detection of phenylketonuria by testing for phenylpyruvic acid with FeCl_3 (1). They were soon also subjected to other tests, including a test for sugars with aniline phthalate (2,3); samples testing positive were eluted and subjected to paper chromatography for the identification of the sugars detected. Subsequently, Louis I. Woolf developed enzyme-based analyses of paper-borne urine samples that directly assayed galactose and glucose (and hence neonatal diabetes mellitus) in one procedure rather than two (4), but which thereby relinquished the detection of abnormalities affecting other reducing sugars. In 1978, when newborn screening was being established in Galicia (N.W. Spain), the enzymatic reagents used in Woolf's assay were taken off the market. Rather than switch to a procedure that

likewise only detected galactosuria and glucosuria, we adopted the two-stage strategy of Berry: since 1979 we have screened paper-borne urine samples from 555,000 newborns for reducing sugars, and thin layer chromatography (TLC) of positive samples has identified the reducing sugars responsible for positive test results (5).

Testing for reducing sugars in urine is routine in many pediatric clinics, but is included in few newborn-screening protocols. Its absence may in part be due to the fact that few programmes analyze urine samples obtained as early

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as blood samples, but also in part to Benedict's test (6) and others (7,8) being inconvenient and often unreliable when adapted for application to paper-borne samples (9). These considerations led us to develop a test specifically for paper-borne samples based on the yellow-to-blue/black color change accompanying the reduction of pentavalent vanadium in concentrated sulphuric acid (5). In the case of galactose, yellow turns to blue at concentrations in the range 100–500 mg/dl, and to black at higher concentrations; for other sugars, see ref. (5). Although some authors state that testing for reducing sugars in urine is not sensitive enough to detect galactosaemia (10,11), our experience with our test is otherwise (12): non-reducing disaccharides and oligosaccharides are hydrolyzed in the acid medium and give positive test results. Until the year 2003, paper-borne blood and urine samples were collected for analysis in our program between the fifth and eighth days of life; they are now collected on the third day of life, when the infant has been fed on milk for 48 hr.

Here we describe the second stage of our protocol, an inexpensive vertical sandwich-type continuous/evaporative TLC technique that uses a fixed volume of the mobile phase, and which has proved to be very convenient for identifying reducing sugars both in paper-borne urine samples with positive vanadium test results and in corresponding paper-borne blood samples. It is based on the principle of running samples with a known volume of mobile phase in a cell designed to ensure uniform conditions.

MATERIALS AND METHODS

Reference Sugars

Solutions containing 50–500 mg of each of the following sugars were prepared in water (MilliQ, from

Millipore, Billerica, MA): stachyose, raffinose, lactulose, saccharose, fructose, xylulose, lactose, galactose, glucose, arabinose, ribose, xylose, and 3-*O*-methylglucose. Table 1 lists the suppliers and catalogue numbers of these and other saccharides and oligosaccharides that were also used as reference sugars. Paper-borne reference samples were prepared by immersing pieces of Whatman 903 paper in the corresponding sugar solution and allowing them to dry in air in a horizontal position; the dried reference samples were placed in envelopes that were then sealed hermetically in plastic bags and stored at –20°C, under which conditions they are stable for several months.

Biological Samples

Urine and blood samples impregnated in Whatman 903 paper were obtained in the course of our neonatal screening program.

Other Reagents, Solvents, and TLC Plates

TLC plates: TLC was performed on silicagel 60 plates with a concentration zone and no fluorescent indicator (Merck 1.11844.0001). **Loading eluent:** Sugars were eluted from their paper substrate onto the TLC plate (see below for details) with freshly prepared 23:30:12 isopropanol/ethyl methyl ketone/30% ammonia (un-fresh eluent can subsequently give rise to horizontal interference bands); isopropanol was supplied by BDH (10224), ethyl methyl ketone by Scharlau (ME457), and 30% ammonia solution by Panreac (131130). **Mobile phase:** the mobile phase was 15:3:4 acetone/ethyl acetate/water, which is stable for several weeks when kept in a well-stoppered flask at 2–8°C; acetone was supplied by Merck (1.00014,1000), and ethyl acetate by Sigma (E4858). **Visualization reagent:** chromato-

TABLE 1. Reference Sugars

Saccharide or oligosaccharide	Supplier and reference	Saccharide or oligosaccharide	Supplier and reference
Galactosamine	Biochemika 48250	Palatinose	Sigma P2007
Glucosamine	Sigma G4875	Cellobiose	Sigma C7252
Maltoheptaose	Sigma M7753	Galactose	Merck 8689
Stachyose	Sigma S4001	Mannitol	Sigma M9647
Maltohexaose	Sigma M9153	Saccharose	Merck 7651
Maltotetraose	Sigma M9153	Glucose	Merck 8337
Glucuronic acid	Sigma G5269	Mannose	Sigma M6020
Raffinose	Sigma R0250	Fructose	Merck 5323
Maltotriose	Fluka 63430	Arabinose	Merck 1494
Isomaltose	Sigma I7253	Fucose	Sigma F8150
Sialic (<i>N</i> -acetyl-neuraminic) acid	Sigma A0812	Ribose	Merck 7605
Lactose	Merck 7657	Xylose	Merck 8689
Lactulose	Merck 5283	Rhamnose	Sigma R3875
Trehalose	Sigma T9531	3- <i>O</i> -methylglucose	Sigma M4879
Xylulose			

graphy spots were visualized with 0.1 M ammonium monovanadate (Merck 1226) in 1 M sulphuric acid (Scharlau Ac 2069) (13).

The Chromatography Cell

Figures 1 and 2 show the cells devised for the new technique; they are designed for TLC plates 10 cm wide and approximately 1.6 mm thick, including the silicagel layer. The front and back walls of each cell are glass plates 1 cm thick and are separated by a 2-mm-thick glass sheet that has been cut to form a right-angled

Fig. 1. The chromatography cell near the end of a run (note that the reservoir for the mobile phase is nearly empty).

Fig. 2. The chromatography cell, ready for mobile phase saturation. The mobile phase was injected with a digital pipette (right).

U-shape of inside width 105 mm, so that the internal horizontal cross-section of the cell is for most of its height a 105×2 mm rectangle. These three glass plates are held together by five nuts and bolts: two at the top (one through each vertical limb of the U), and three at the bottom, which also attach the metal feet of the device. A reservoir for the mobile phase is formed by $115 \times 10 \times 7$ mm horizontal recesses cut into the front and back walls with their lower edges 10 mm above the top of the horizontal portion of the U (which constitutes the bottom of the cell), together with semi-discs 1 cm in diameter cut in the vertical limbs of the 2-mm-thick sheet with their centers 1.5 cm above the bottom of the cell so as to coincide with the ends of the horizontal recesses; the reservoir is longer than the width of the cell so as to avoid distortion due to the meniscus of the mobile phase.

The height of the back wall above the bottom of the cell is the same as the length of the TLC plate; the front wall and the 2-mm-thick separator are shorter, and their top edges are coated with a silicon "bed." Silicon guides installed on the back wall where it overtops the front wall ensure that when the TLC plate is inserted in the cell its silicagel-coated face makes no contact with the front wall. Once the TLC plate has been inserted, the cell can be "closed" by a lid consisting of a 2-mm-thick glass slip that is placed on the silicon bed with its edge flush against the silicagel layer (to which end the silicon guides terminate 2 mm short of the top of the front wall, and two glass pieces cut from a TLC plate are stuck on the back wall laterally to the guides to help ensure that the lid is not placed askew). The lid is held in place with a strong rubber band that passes under the top two nuts (Fig. 1). The closed cell only communicates with the outside atmosphere via the silicagel layer.

Procedure

Loading

Discs 5 mm in diameter are cut with a paper punch from the paper-borne urine and blood samples, and from reference samples, and are placed with tweezers in the 2.5×10 cm concentration zone of a TLC plate, with their centers approximately 1 cm from the lower edge of the plate, opposite their identification codes (Fig. 3a). The discs are then covered with a 2×10 cm glass slip that is fastened with bulldog clips to keep the paper discs sandwiched in place (Fig. 3b). This assembly is stood for 1 min in a conventional chromatography tank containing loading eluent to a depth of 1 cm, after which it is transferred to a drying stand where a current of room-temperature air from a household hair-drier is directed at the exposed part of the concentration zone for 3 min (Fig. 4); hot air leads to development problems similar

Fig. 3. Sample discs being placed on the concentration zone of the TLC plate (a) and covered with a glass slip held in place by bulldog clips (b).

Fig. 4. Drying a loaded TLC plate with a stream of room temperature air directed by a hair-drier at the half-centimeter of concentration zone that is not covered by the glass slip holding the sample discs in place.

to those caused by the use of loading eluent that has not been freshly prepared.

Development

A precisely measured volume of mobile phase (1.5, 3, or 5 ml, depending on the purpose of the run; see below) is injected into the cell with a digital pipette (Thermo Scientific Finnpiquette or similar); a silicon slab 100 mm wide and 2 mm thick is slid down the silicon guides as though it were a TLC plate until its edge closes the cell (Fig. 2); and to allow the atmosphere inside the cell to become saturated with mobile phase, the cell is placed for 3 min in a forced air chamber (e.g. a Hot-Cold S from J.P. Selecta, Barcelona, Spain) that is thermostatted at 25.5°C. The TLC plate is then introduced and the cell is closed with the glass lid. Since the silicagel layer lies only about 0.4 mm from the front wall, the method is of sandwich type. To separate less mobile sugars (listed in Table 2), a 20 cm TLC plate is used in a

TABLE 2. Rt and Rg Values, and Limits of Detection (LoDs), of less Mobile Sugars Run over 4 hr on 20 cm TLC Plates using 5 ml of Mobile Phase (means of two or more runs)

Sugar	Rt	Rg	LoD (mg/dl)
Galactosamine	2.3	2.8	25
Glucosamine	4	4.6	100
Maltoheptaose	4.9	5.8	90.3
Stachyose	8	10.1	< 50
Maltohexaose	8.9	11.6	100
Maltopentaose	10.5	13.8	92.2
Maltotetraose	17.3	21.7	90
Glucuronic acid	26	31.3	80
Raffinose	26.8	35.3	< 50
Maltotriose	33.7	41.8	80
Sialic acid	35	42	100
Lactose	42.4	55.6	< 50
Lactulose	44.3	57.9	< 50
Galactose	64.7	84.6	< 50
Saccharose	71	93	< 50
Glucose	76.3	100	< 50
Fructose	81	105.2	< 50
Arabinose	90.2	117.5	< 50
Ribose-xylose	102.3	–	< 50
3-O-methylglucose	102.7	–	

cell with an internal height of 18 cm; the sugars are run with 5 ml of solvent, which is exhausted in 4 hr. More mobile sugars (Table 3) are run in 2 hr with 3 ml of solvent using the same-sized TLC plate and cell (sugars of intermediate mobility can be run under either conditions, and are listed in both these tables). To identify glucose and galactose, 1.5 ml of solvent is run in about 40 min on a 10 cm TLC plate in a cell with an internal height of 9 cm.

Visualization

Once the solvent is exhausted, the TLC plate is dried in a fume cupboard, immersed in the visualization solution, and heated for 5 min in an oven at 120°C.

TABLE 3. Rt and Rg Values, and LoDs, of more Mobile Sugars Run over 2 hr on 20 cm TLC Plates using 3 ml of Mobile Phase (means of two or more runs)

Sugar	Rt	Rg	LoD (mg/dl)
Stachyose	1.9	4.4	< 50
Raffinose	10.8	26.5	< 50
Isomaltose	14	34.4	25
Lactulose	19.7	48.2	< 50
Lactose	20.6	57.8	< 50
Trehalose	25.3	59.1	40
Palatinose	29	69.6	25
Cellobiose	30	70	75
Galactose	32.5	80.5	< 50
Mannitol	34	89.2	25
Saccharose	36.5	89.6	< 50
Glucose	40.1	100	< 50
Mannose	42.5	108.9	40
Fructose	42.7	109.7	< 50
Arabinose	48	119.1	< 50
Fucose	55.3	142.7	25
Ribose-xylose	59.4	146.8	< 50
Xylulose	67.5	171.7	< 50
3-O-Methyl-glucose	71.2	176.4	< 50
Rhamnose	74	189.7	< 50

Fig. 5. Blood and urine samples from a patient with classical galactosaemia, run in 45 min using 1.5 ml of mobile phase, together with aldose and ketose standards. Note that galactose is detected in urine but not in blood.

So treated, sugars appear in the chromatogram as blue spots (or as black spots if an exceptionally high sugar concentration causes reduction to vandadium(III) instead of vanadium(IV)) that remain visible for at least 24 hr (Figs. 5–7).

RESULTS

Tables 2 and 3 list the Rt and Rg values, and detection limits, of the sugars run; runs are illustrated in Figs. 5–7. Rg and Rt, or cRf (14), are like Rf, except that instead of dividing the distance run by the analyte by the

Fig. 6. Blood and urine samples from a patient with galactokinase deficiency, run in 45 min using 1.5 ml of mobile phase, together with aldose and ketose standards. Note that galactose is detected in urine but not in blood.

Fig. 7. Blood and urine samples from two patients with MS/MS-detected galacto-4-epimerase deficiency, run in 45 min using 1.5 ml of mobile phase, together with aldose and ketose standards. Note that galactose is not detected in blood, and is only detected in urine at age 29 days.

distance traveled by the solvent front, the divisor is the distance to the top of the cell (for Rt) or the distance run by glucose (for Rg; using glucose as the reference corrects for environmental variation). Note that galactokinase and galactose-1-*P*-uridyltransferase deficiencies show up clearly (Figs. 5 and 6).

DISCUSSION

The chromatographic method described here has evolved continuously since the establishment of the Galician neonatal screening program in 1979 (15–20). At that time (15), samples were run on TLC plates in a conventional tank using a 25:15:8 mixture of *tert*-butanol, ethyl methyl ketone, and 25% aqueous NH₄ as loading eluent (salts being virtually immobile in this

kind of solvent (25), and 60:30:5:15 ethyl acetate/pyridine/acetic acid/water as mobile phase (21)). The distinctive features of the method are the vertical sandwich configuration and the use of a fixed volume of mobile phase. This volume, which depends on the analytic task to be performed, controls the distance run by the analytes on a TLC plate of given width under controlled conditions; to ensure reproducibility, chromatograms are run at a fixed temperature in a thermostatted chamber of fixed volume and atmospheric composition. The method described may be considered as an improvement on the short bed continuous development concept published by Regis Chemical Co. in 1979 (14,22), which was insufficiently supported by the chromatography chamber they marketed (Fig. 8) (23). However, the Regis method is not of sandwich type, does not depend critically on mobile phase volume as the present method does, does depend critically on development time, uses larger solvent volumes, and provides less reproducible results.

The method described has allowed the detection of galactosaemia, transitory neonatal diabetes mellitus, and glucosuria in newborns. Among the newborns screened (over 555,000), we have found 10 cases of galactose-1-*P*-uridylyltransferase deficiency ("classical galactosaemia"; Fig. 5), 7 cases of galactokinase deficiency (Fig. 6), 2 cases of glucosuria, and 3 cases of transitory neonatal diabetes mellitus. Somewhat paradoxically, the cases of galactosaemia were all detected via the urine sample; the chromatographic method is not sensitive enough for the detection of galactosaemia in blood samples (Fig. 5), although it does detect high serum glucose levels. Nor are either the vanadium test for reducing sugars or the TLC procedure sensitive enough for timely detection of galactose-4-epimerase deficiency, although galactose does show up in the urine chromatograms of these patients at an age of about

1 month (Fig. 7). However, the utility of testing urine as well as blood is highlighted by one of the classical galactosaemia cases, in which our tandem vanadium-TLC urine test showed high galactose excretion even though the serum galactose concentration was normal because, unknown to the laboratory staff, the newborn had received a blood transfusion before samples were taken, the transfusion and sampling having been performed (the latter during parenteral feeding) in another center. The ability of the method to distinguish between high urine glucose and galactose concentrations is highlighted by another case in which a high galactose concentration was detected in the presence of a strong glucose spot due to glucose-based parenteral feeding (12).

Except for two cases of galactose-4-epimerase deficiency detected since March 2002 using tandem mass spectrometry to measure hexose monophosphates in blood samples (24), no cases of galactosaemia with false-negative urine tests attributable to the test procedure have come to our attention, as they almost certainly would have done had there been any, given the case reporting instructions in force in the catchment area of our neonatal screening laboratory (Galicia, N.W. Spain).

CONCLUSIONS

When employed in tandem with the vanadium test for reducing sugars, the TLC procedure described here constitutes an efficient protocol for neonatal screening for galactosaemia and other defects of sugar metabolism other than galactose-4-epimerase deficiency. It is particularly valuable in that the urine test is not subject to interference from transfusions or parenteral feeding as blood-based tests are.

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Fig. 8. Regis Short Bed Continuous Development Chamber. Internal measurements: 22 × 8.5 × 2.2 cm. For TLC plates up to 20 cm wide and 10 cm high. For plates between 10 and 20 cm wide, the teflon "wings" are slid toward the center of the tank; for plates less than 10 cm wide, a teflon auxiliary piece is needed. From the SB/CD-Short Bed Continuous Development Operation Manual 1979, p 2, Regis Technologies, Inc., Morton Grove, IL.

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