

Identification of Urinary Reducing Substances by Thin Layer chromatography: An Observational Study at a Pediatric Tertiary Care Hospital

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Abstract: Thin layer chromatography is a neglected test and is about to become obsolete from the clinical biochemistry laboratories. In this context, the aim of the present study is to determine the reducing sugar that is present in the urine samples sent to the lab as a routine investigation in a pediatric hospital setup.

Methods: A retrospective study of 383 sick children of neonatal to pediatric age group were selected during the previous one year period and their reports of Benedicts test for urine reducing substances, Multistix dipstick for Glucose and corresponding thin layer chromatography study were analysed. MS excel sheet used for statistical analysis and charts.

Results: Out of carefully selected 383 cases, 59% (226) were positive for reducing sugars in their urines. 14% were Glucosurics and 86% as Lactosurics. Lactose in the urine of infants was corresponding to

their intake of breast milk. None were positive for Galactosuria. Thus TLC showed 100% specificity.

Conclusions: Non Glucose Reducing Sugars in urine needs further evaluation with Thin Layer Chromatography to pinpoint the particular sugars, like Galactose. It helps in clinical decisions and there is a need for the rise in the awareness of the availability of such an economic and easier testing procedure that can be taken up as a tool for mass screening Inborn errors of metabolism in developing countries like India, where such a screening is not mandatory yet.

Keywords: Diabetes Mellitus, Inborn errors of metabolism, Lactosuria, TLC: thin layer chromatography, URS : urinary reducing substances.

I. INTRODUCTION

Thin layer chromatography (TLC) is a neglected test and has nearly become obsolete in the clinical biochemistry laboratories and is almost confined to laboratory literature (1-3). TLC in combination with Benedict's test and dipstick test for Glucose will render much comprehensive information in pediatric patients(4). Screening for inborn errors of metabolism is now, a common practice in the western countries but not yet in the developing countries like India, unless there is a high index of suspicion(5). In developing country like India, as there is a high birth rate and there is a greater risk of an apparently healthy newborn being affected with congenital malformation or inborn errors of metabolism (6). The reasons behind this scenario might be the low index of suspicion for these relatively rare inborn errors of metabolism and the costly investigations like HPLC/TMS (High Performance Liquid Chromatography/Tandem Mass Spectrometry) and lack of awareness of availability of

older but cheaper procedures like TLC. Reducing sugars of clinical interest are detectable in the urine with a weakly positive Benedict's test or a dipstick test, the diagnosis of which can be established using TLC (7). With this context, the aim of the present study is to determine the reducing sugar that is present in the urine samples, to emphasize the ease of technique and its specificity so that it can be adapted reliably in all clinical laboratories as a routine test for children to reduce the burden of preventable disabilities.

II. MATERIALS AND METHODS

After approval from the ethics committee, a retrospective analysis of data from the laboratory records, case sheets between the periods of April 2013 to March 2014 was done at Niloufer Hospital for women and children, Hyderabad. Inclusion Criteria: A total of 383 child urines were screened during this one year (TABLE 1). All the patients were admitted for evaluation of febrile illness or gastroenteritis, with no known previous history of illness. Exclusion criteria:

patients with hepatic and renal ailments were excluded from this study taking Total Serum Bilirubin and serum creatinine levels in to consideration. Age more than 12 years, premature newborns and known diabetics were not included to the data analysed. Urine was collected as a spot sample afresh in a sterile, dry, disposable sample collection bottle, and received at biochemistry lab, within 30 minutes of collection. Benedict’s test was done for detecting the presence of URS along with Multistix® SG reagent strips for dip stick Urinalysis. The Multistix (used to detect parameters such as Glucose, ketones, bilirubin, specific gravity, pH, blood, protein and urobilinogen) is manually dipped and visually interpreted at 30 seconds to compare with a range of reference color blocks printed on the box.

Glass plates of 20 X 20 cm size (Fig. 1), and using Butanol: Acetone: Acetic Acid: water (in a ratio 35:35:10:20) as solvent (mobile phase) and Diphenylamine (DPA) as staining agent (9, 10). TLC Silica gel 60 F₂₅₄ Aluminum sheets 20 X 20 cm (manufactured by Merck Millipore, Germany) were ready made pre coated plates were halved in size to 10 X 10 cm and were used, in case any repetitions were required (Fig.2).

III. OBSERVATIONS & RESULTS

Table 1. Age and sex of the subjects

Groups	Total	M	F
Neonates	91	69	22
Infants	188	113	75
Children	104	55	49
	383	237	146

Table 2.TLC detection of sugars

URS Positive 226	Neonates		infants		children	
	M	F	M	F	M	F
Glucose 32	11	2	7	5	6	1
Lactose 194	35	11	58	30	34	26
Total	46	13	65	35	40	27

IV. DISCUSSION

Benedict’s test suffers lack of specificity to determine the nature of the reducing substance in the

Multistix Glucose detection is based on a double sequential enzymatic reaction i.e., Glucose oxidase and Peroxidase enzymes which produces chromogens as green to brown colored end products of oxidation of glucose. This methodology is highly specific for glucose because of enzyme specificity but sensitivity ranges from 75-125 mg/dL, levels below which false negative results can be obtained due to the factors pertaining to the urine (8). The urine sample with either glucose reactive or URS positive, was further analysed with TLC to identify the presence of other sugars such as Galactose, Lactose, Fructose or pentose.

TLC was done with Silica gel slurry manually coated using a stahl type of hopper spreader on plain 383 patients were classified into 3 groups based on their age: as neonates 91, infants 188 and children above age one year 104 (TABLE 1). Out of 383 subjects, 226 patients (59%) tested positive for URS by Benedict’s test (TABLE 2).URS positive samples were subjected to TLC separation, to rule out false positive and negative Glucose test and to determine the reducing sugar. It was observed that 32 (14%) patients were Glucose positive by Multistix dipstick test and 194 (86%) patients were Lactose positives, by TLC. This is 8.4% and 50.6% of the total samples tested for the mentioned year.

urine. Other constituents like Ascorbate, salicylates and Uric acid etc., found in urine can also reduce the colorless cupric ions to colored cuprous ions. Ascorbic acid and ketones in higher concentrations can interfere with glucose oxidase enzyme of Multistix and raised specific gravity or temperature changes also influence the sensitivity of the test, there by contributing to false negative test (8). Reducing sugars such as Galactose and Fructose when present in urine, might point out a serious enzyme defect in liver and warrants confirmation by enzymatic assays. L-Xylulose is excreted in a non harmful condition, essential pentosuria which later on may lead to diabetes or some unrelated mellituria which requires elucidation by urine chromatography and blood studies (9). Endogenous Lactosuria is sometimes the only constant feature in pathological Galactorrhoea. Sucrose along with Lactose is found in urine of normal children who consume sweetened dairy products or artificial milk formulae (9). Demonstration of disacchariduria for lactose and or Sucrose is of practical value in fermentative diarrhea which improves when the offending sugars are removed from diet and is a life saving measure. In premature neonates, there is a characteristic augmentation of physiological mellituria due to the temporary inefficiency of hepatic function and raised intestinal permeability coupled with the high lactose

concentration (5 to 9 %) of human breast milk or sucrose- lactose combination in artificial formulae(9). Galactosemia and Hereditary Fructose Intolerance though rare inborn errors of carbohydrate metabolism, can be easily detected by TLC (10). Thus in all non Glucose URS, it must be a routine protocol to order for TLC evaluation of the sugar. This would either rule out or confirm the presence of a particular sugar, which can aid in clinical decision both in sick children as well as adults, especially pregnant and lactating women. Thus Sugar Chromatography is indispensable when more comprehensive information is required.

TLC is a type of partition chromatography technique where liquid in the silica gel acts as the stationary phase. It is superior to paper chromatography (where cellulose in paper acts as stationary phase) in terms of compactness of spots, improved resolution and quicker run is made possible (10). Earlier, plain glass plates (Fig. 1) were used with manual coating with adsorbents, silica gel or cellulose. This instrumentation was economic but particular set of skill is needed to prepare the TLC plates. Later, TLC glass plates were replaced by commercially available Aluminum sheets (Fig 2) pre-coated with Silica gel or Cellulose. This made TLC, convenient to practice in clinical laboratory as a routine test for determination of sugars and amino acids on a large scale. Though TLC technique was improved to automation like HPLC, the conventional

one is cheaper and convenient to adapt in a routine biochemistry laboratory even at rural setup.

Partition coefficient is a characteristic of a particular substance for a given pair of solvents. If a mixture of different substances were subjected to redistribution in a mixture of any two immiscible liquids, the substances have their unique progressive separation. Based on this principle of Partition chromatography, substances like sugars or aminoacids or drugs are elucidated with their *Relative fractions, R_f*.

$$R_f = \frac{\text{distance travelled by the substance}}{\text{distance travelled by the solvent front}}$$

Thus, *R_f* value is a unique figure for a substance for a given mixture of solvents. The sugar with less polarity (pentoses) would ascend far greater distance (*R_f* nearer to unity) than the one with more polarity. Disaccharides are more polar hence they run slower (*R_f* is lesser than unity). Fructose, Glucose and Galactose are having intermediate *R_f* in that order for the Butanol Acetic acid water solvent mixture. This is the basis of determination of the particular sugar in the sample of urine.

In our study, the importance of TLC is emphasized by taking up fresh cases for urine reducing substance determination and Lactose was found to be the most frequent URS in all the three groups 77%, 88% and 89% respectively, followed by Glucose.

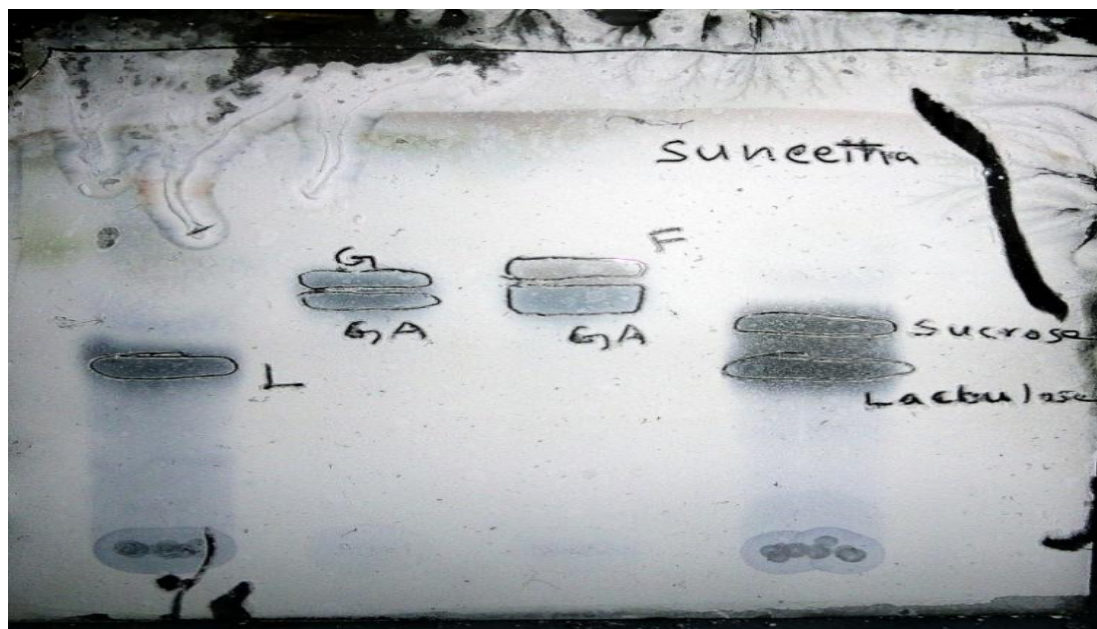


Figure 1. Glass TLC plate prepared manually showing patient's urine (s) sample (L) Lactose; G=Glucose;F=Fructose;GA=Galactose in mixed standards

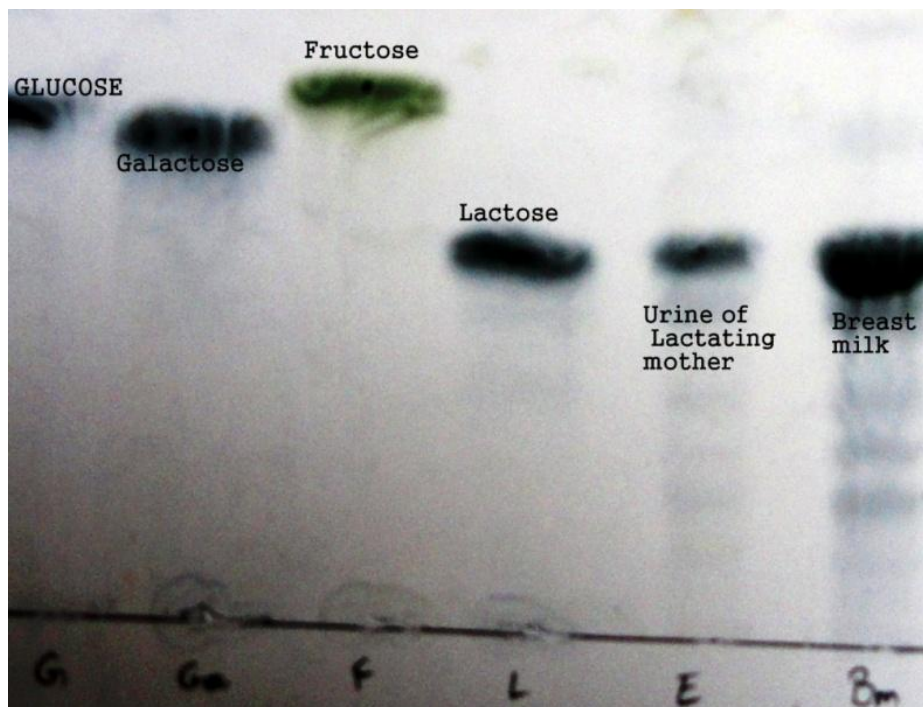


Figure 2. TLC silica gel Aluminum sheet (plate) showing various standards

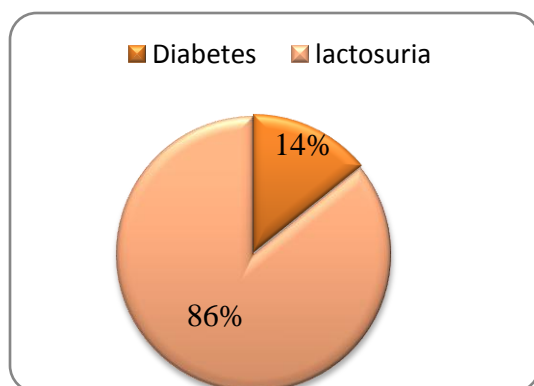


Figure 3. Outcome of TLC testing

This is due to increased availability of Lactose in the gut which tends to increase the un hydrolyzed Lactose absorption to enter the blood stream. As Lactose is not metabolizable in the liver, it gets excreted in the urine (9). But Lactosuria peaks in infancy in the breast fed group (Fig 4) where milk is the major source of their food. The present study demonstrated 59% of URS positive patients this finding was consistent with previous study by Shams S., et al (7). 86% of those who were positive for URS, were diagnosed as non diabetes mellitus and 14% were diagnosed as Diabetics which were later confirmed by Blood sugar studies (Fig.3). Incidence of Diabetes Mellitus in neonates and infants are same with decreased appearance after infancy. Incidence of Diabetes Mellitus in neonates and infants are same with decreased appearance after infancy. But Lactosuria peaks in infancy which is the breast fed group

(Fig.4). No significant differences were observed in rate and degree of positive results with respect to gender which supports the findings by Shams S., et al (7). Many laboratories used TLC successfully (11, 12, 13, 14). TLC as a routine test in our laboratory showed 100% specificity by proving all the dipstick Glucose reactive samples to be Glucose present and other URS positives to be Lactose positive. Galactose positivity was ruled out assuring the clinician with a focused reporting. Québec Newborn Urine Screening Program (QNUSP) reports on TMS indicate > 90% sensitivity of TLC (15) where as we achieved a specificity of 100% and with the use of all those clinically important sugars as standards, 100% sensitivity is attained.

V. CONCLUSION

TLC can be used for mass screening of urine sugars and amino acids but is not popular among health providers and general public as a reliable and affordable test to screen children for inborn errors of metabolism like Galactosemia. TLC is a comparatively cheaper and time tested procedure, thus routine urine screening by TLC in both public and private sectors, must be emphasized especially in the background of high birth rate and preventable morbidities in our country.

ACKNOWLEDGEMENTS

Authors would like to thank Prof.Dr N Vani, HOD, dept.of Biochemistry, Osmania Medical College and Prof.Dr K.Devaraj, Medical Superintendent

Niloufer Hospital for their moral support during this work

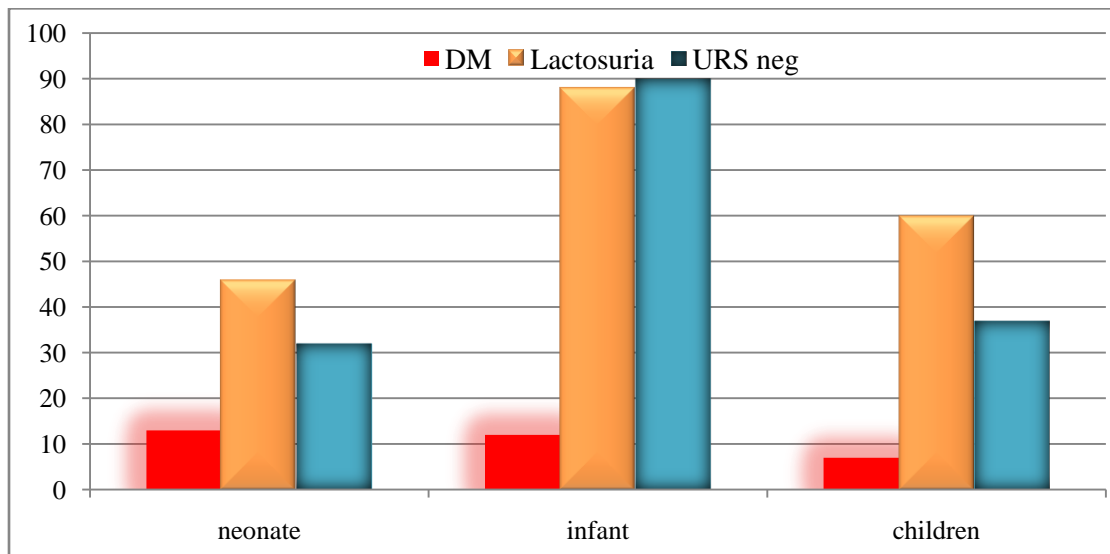


Figure 4 . Group wise data showing total number of Diabetics, Lactosurics and URS negative subjects

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