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Use of single solvent thin layer chromatography to diagnose different organic acidurias

Sir,

Organic acidurias (OADs) are an important class of inherited metabolic disorders arising due to a defect in intermediary metabolic pathways of carbohydrate, amino acids and fatty acid oxidation[1]. The usual clinical presentation includes central nervous system manifestations such as seizures, abnormal tone and acute metabolic encephalopathy, lethargy progressing to coma, poor feeding, recurrent vomiting and failure to thrive[2]. Late onset OADs in adolescents, present with loss of cognitive function, ataxia and recurrent ketoacidosis[3]. Laboratory findings suggestive of an OAD includes metabolic acidosis, ketosis, hyperammonemia, abnormal liver function tests, hypoglycaemia, neutropenia and tachypnea[4].

The cumulative frequency of OAD in a preselected high-risk group may be up to 200 times higher than that identified in the general population[5]. OADs are quite common in Indian population[6]. A study from Mumbai showed an incidence of 88 out of 1844 children suspected with inborn errors of metabolism (IEM) with organic acidemias (4.7%) associated with overall mortality of 37.5 per cent[7]. The prevalence is high in south India because of high birth rate and frequent occurrence of consanguineous marriages[1]. Nagaraja *et al*[8] identified 47 cases of OADs and 61 cases of amino acidurias in a total of 113 children with IEM. Study conducted on the incidence of OADs in India reported 365 patients with IEM diagnosed over a period of 20 yr, OADs accounted for 27 per cent of all cases (MMA 18%, PA 9.2%)[9]. Another study from south India screened 420 high-risk children and identified 45 children (10.7%) with OADs, including 16 cases of propionic aciduria, 15 cases of methylmalonic aciduria, 13 cases of Maple Syrup Urine Disease (MSUD)[6].

The analysis of organic acids in urine is of paramount importance for the diagnosis of OADs. More than 100 different organic acids are excreted in urine in these conditions. Confirmatory diagnosis of OADs requires expensive instruments such as liquid chromatography–mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS) or tandem mass spectrometry and enzyme assays or DNA analysis. High-field proton nuclear magnetic resonance (NMR) is a promising technique for the diagnosis of OAD[1]. Screening tests such as dinitrophenylhydrazine (DNPH) test and TLC are also employed but these do not confirm the diagnosis.

A hospital-based study from another tertiary care hospital in the vicinity of the study area found the prevalence of OAD to be 20.8 per cent of all high risk children investigated and 39.5 per cent of confirmed IEM[10]. The study results indicate the need for a feasible method to screen the OADs at least for the high risk cases till the newborn screening becomes a reality.

The present study checked for TLC as a technique for preliminary diagnosis and intervention, thereby reducing the fatal consequences before higher end diagnostic technology confirms the diagnosis.

This study was conducted at Centre For Excellence in Inborn Error of Metabolism (CE-IEM), department of Biochemistry, Kasturba Medical College and Hospital, Manipal. The study subjects (n=137) were suspected IEM cases aged under 18 yr referred for diagnostic work up of IEM. The ethical clearance certificate for the study was obtained from Institutional Ethical Committee.

The study employed the method of TLC where solvent used are amyl acetate:acetic acid:water mixture and 2 stains; bromocresol green[11] (bromocresol green in acetone); followed by dianisidine dye[12] (dianisidine in acetic acid : ethanol : water mixture).

As a pre-treatment procedure for samples, random urine samples were saturated with ammonium sulphate (Thermo Fisher Scientific Pvt. Ltd., Bengaluru) and

diethyl ether (Merck Specialities Pvt. Ltd, Mumbai) and acidified with concentrated hydrochloric acid then vortexed and centrifuged for 15 min at 2500 rpm, supernatant collected was kept for evaporation at 300°C in water bath. 100 µl of isopropanol was added to the sample tube after complete evaporation and spotted on TLC plates (pre-coated silica gel 60). Sample spotted TLC plates were then run in the solvent system and after the sample run, plates were stained first with bromocresol green indicator to reveal glutaric acid and lactic acid spots if present. The plates were then stained by dianisidine dye (Sigma Aldrich Chemie, Germany) to visualize the other organic acid spots on air drying such as succinylacetone, homogentisic acid, glutaric acid, acetoacetate, MMA and Malonic acid (MA).

All the samples subjected for TLC separation were qualitatively identified using following parameters: (*a*) visualization of the 'coloured spots' developed, (*b*) retardation factor (Rf). Rf values for standard organic acids in the study are summarized in Table. In many of the disease conditions, multiple organic acids or metabolites are spotted together during the chromatographic run[1]. Out of 137 suspected samples processed in the present study, 27 samples were positive for different organic acids, two cases were positive for succinylacetone, five for homogentisic acid, one for glutaric acid, nine for acetoacetate, three for MMA, five for MA and two cases for combined presence of MMA and MA. When the chromatogram was sprayed with the locator reagent solution (bromocresol green reagent followed by o-dianisidine reagent), lactic acid exhibited light yellow spot near to the origin, and acetoacetic acid produced yellow-orange spot initially which gradually changed to purple colour, MMA produced purple spots lesser in intensity than acetoacetate. Succinylacetone, homogentisic acid and malonic acid produced yellow, clear spots with yellow background with brownish or purple border and orange red spots, respectively. However, when MA standard was used from a new and fresh unopened bottle after a period of 10 months, a difference in the Rf value was observed with the later trial yielding Rf value of 0.5. Furthermore, the same was with the case of lactic acid separation wherein the literature from Auray-Blais *et al*[13] confirms the separation near the solvent front. This discrepancy in the Rf value may be because of standard deterioration or because of changes in the lot or the separation system used. This discrepancy is one of the limitations of this method as there is a necessity of re-standardizing the method always if a change in the Rf is observed from the standard value expected.

In cases of tyrosinemia (n=2) and alkaptonuria (n=5), an unidentified purple spot was observed constantly which did not match with the Rf of acetoacetate. The urine sample of this unidentified spot was then subjected to GC-MS which revealed the presence of a phenyl ring. Hence, the spot was assumed to be phenyl acetoacetate since the colour matched with acetoacetate, but the GC-MS confirmed the presence of phenyl group. However, this hypothesis needs to be explored further. However, TLC did show a better predictive potential for detection of tyrosinemia as compared to the traditional Millon's and nitrosonaphthol test. Total five positive alkaptonuria cases were identified using the method, out of which two tested negative with ammoniacal silver nitrate test. The urine sample with suspected alkaptonuria showed a blackish brown precipitate with ammoniacal silver nitrate test, further dried urine spot of the same treated with 1M NaOH did not turn to brown colour which otherwise would have in the presence of homogentisic acid, causing ambiguity in confirmation. The proposed TLC method suggested it as a case of melanuria and not alkaptonuria as no spot coinciding with the Rf of homogentisic acid was detected. Hence, the proposed method (initially designed to identify methyl malonic acid) could effectively detect several OADs with only a few microliters of urine sample, thus bringing down the analysis cost and time and facilitate the clinician to start treatment in positive cases much before they got the access of reports from higher end techniques.

TLC being a reproducible and cost-effective qualitative separation technique has been a recommended method for the analysis of organic acids and ketones in urine[13]. There are evidences for using TLC technique for successful mass-screening by a single technician in Quebec Neonatal Urine Screening Programme (QNUSP) for 35 yr[13]. QNUSP reported studies on MS/MS indicating that the sensitivity of TLC overall exceeded 90 per cent when screening for 25 IEM disorders targeted in their programme[13]. Screening programme in Ludhiana, Punjab[14] and a hospital-based study in Karnataka[15] also justify the choice of TLC as preliminary screening method in developing countries like India.

Table. Colour developed and retardation factor of each standard, the number of positive detected on thin layer chromatographic run

Name of the organic acids/metabolites	Colour of spots[11,12]	R_f values	Number of cases (n)
Acetoacetate	Initially yellow-orange which turns to purple gradually	0.57	9
Succinyl acetone	Yellow	0.63	0
Homogentisic acid lactone	Dark brown	0.85	0
Homogentisic acid	Clear with yellow background and brownish or purple border	0.70	5
Methylmalonic acid	Purple	0.60	1
Malonic acid	Orange-red	0.5	4
Lactic acid	Light yellow spot at the origin	0.2	0
Glutaric acid	Pale yellow coloured spot on brown background	0.72	1
Pyruvic acid	Blood red turns to dark brown on drying	0.58	0
Multiple organic acids/metabolites spotted togthers			
Methylmalonic acid + acetoacetate	-		2
Malonic acid + acetoacetate	-		1
Methylmalonic acid + malonic acid	-		2
Succinylacetone + homogentisic acid lactone	-		2

Overall the proposed method is simple, inexpensive and reproducible yielding the qualitative identification of few OADs in both mass and high-risk screening. Thirty five years of successful QNUSP on 2,500,000 newborns further justifies the implementation of this mass screening programme to start with, especially in the resource limited diagnostic setups. However, it is suggested that positive screens should be recalled for confirmation of the diagnosis using GC-MS.

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Prajna P. Shetty[1], Prince Jacob[2], Revathi P. Shenoy[1] & K. Nalini[1,*]
Departments of [1]Biochemistry & [2]Medical Genetics, Kasturba Medical College & Hospital, Manipal 576 104, Karnataka, India
For correspondence: nalini.kbhat@manipal.edu

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References

1. Vaidyanathan K, Narayanan MP, Vasudevan DM. Organic acidurias: An updated review. *Indian J Clin Biochem* 2011; *26* : 319-25.
2. Patil VS, Jailkhani R, Trivedi JD, Kulkarni P, Sagare AA, Mudaraddi R, *et al*. Screening for aminoacidurias and organic acidurias in patients with metabolic or neurological manifestations. *Biomed Res* 2012; *23* : 253-8.
3. Ramsay J, Morton J, Norris M, Kanungo S. Organic acid disorders. *Ann Transl Med* 2018; *6* : 472.
4. Lo SF. Organic acid disorders. In: Garg U, Smith Laurie D, editors. *Biomarkers in inborn errors of metabolism*. 1st ed. United States: Elsevier; 2017. p. 65-85.
5. Wanmacheer C, Wajner M, Giugliani R, Giugliani E, Costa M, Giugliani M. Detection of metabolic disorders among high-risk patients. *Rev Bras Genet* 1982; *5* : 187-94.
6. Narayanan MP, Kannan V, Vinayan KP, Vasudevan DM. Diagnosis of major organic acidurias in children: Two years experience at a tertiary care centre. *Indian J Clin Biochem* 2011; *26* : 347-53.
7. Jalan AB, Telawane N, Shinde R, Dasgupta D, Muehl A. Outcome of organic academia in Indian children. Available *from: http://www.metabolicerrors.com/media/websites/metabolicerrors.com/files/11(1).pdf.,* accessed on May 19, 2019.
8. Nagaraja D, Mamatha SN, De T, Christopher R. Screening for inborn errors of metabolism using automated electrospray tandem mass spectrometry: Study in high-risk Indian population. *Clin Biochem* 2010; *43* : 581-8.

9. Muranjan MN, Kondurkar P. Clinical features of organic acidemias: Experience at a tertiary care center in Mumbai. *Indian Pediatr* 2001; *38* : 518-24.
10. Sindgikar SP. Audit of organic acidurias from a single centre: Clinical and metabolic profile at presentation with long term outcome. *J Clin Diagn Res* 2017; *11 :* SC11-4.
11. Smith L. Chromatographic and electrophoretic techniques. *Vol 1, chromatography.* Available from: *https://www.journals.uchicago.edu/doi/10.1086/403548*, accessed on May 19, 2019.
12. Gutteridge JM, Wright EB. A simple and rapid thin-layer chromatographic technique for the detection of methylmalonic acid in urine. *Clin Chim Acta* 1970; *27* : 289-91.
13. Auray-Blais C, Cyr D, Drouin R. Quebec neonatal mass urinary screening programme: From micromolecules to macromolecules. *J Inherit Metab Dis* 2007; *30* : 515-21.
14. Kulshrestha MR, Uppal BK, Kalra R, Dahiya K, Kulshrestha R. Neonatal screening for aminoaciduria: Can TLC be used as an affordable method in developing countries? *Sch J App Med Sci* 2014; *2* : 3011-4.
15. Jailkhani R, Patil VS, Laxman HB, Shivashankara AR, Kulkarni SP, Ravindra MS. Selective screening for inborn errors of metabolism in children: Single centre experience from Karnataka. *J Clin Diagn Res* 2008; *2* : 952-8.