

Two tier analysis of organic acid disorders: a comprehensive approach for newborn screening

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Abstract

Background: The present study aims at two tier analysis for diagnosis and confirmation of organic acidemia using simultaneous analysis of dried blood spot (DBS) carnitine profile by tandem mass spectrometry (TMS) and urine metabolic analysis on gas chromatography mass spectrometry (GC/MS).

Material and Methods: This cross sectional study included 348 DBS samples and their dried filter paper urine sample came for routine and emergency screening of inborn errors of metabolism (IEM). All DBS samples were analysed for amino acid and acylcarnitine profile derivatized on TMS. All these cases were simultaneously tested for urine organic acid pattern on GC/MS which involved urease pretreatment, deproteinisation followed by derivatisation. Confirmation of IEM was based on the two tier approach containing the blood and urine metabolic pattern as recommended by panel of American College of Medical Genetics (ACMG).

Results: 17 cases were screened positive for IEM analysed separately for amino acid /acylcarnitine profile and urine organic acid profile. However the two tier testing screened and confirmed 8 cases of organic acidemia within 36 hours of receipt of sample which included 2 cases each of maple syrup urine disease (MSUD), glutaric acidemia Type I (GA I), and β -ketothiolase deficiency (BKT) and one each case of multiple carboxylase deficiency (MCD) and propionic acidemia (PA).

Conclusion: The simultaneous two tier metabolome testing for screening of IEM is more comprehensive, cost effective, delivering confirmatory results with significantly reduced turnaround time, thus reducing recall rate by eliminating false-positive results and help prevent unnecessary anxiety of parents.

Keywords: Organic acidemia, dried blood spot, tandem mass spectrometry, gas chromatography mass spectrometry.

1.Introduction

Inborn errors of metabolism (IEM) are typically associated with hereditary deficiency, which leads to defect in one or more enzymes that are important for normal metabolic activity.[1] Organic acid disorders are an important group of IEMs characterized by defect in enzymes involved in protein metabolism. Such defect results in metabolic blockade, deficiency of vital chemicals and buildup of toxic chemicals. These accumulated toxins eventually appear in blood as carnitine conjugates and in urine as organic acids and glycine conjugates which all serve as diagnostic markers in diseased

newborn. Being one of the common groups of IEMs, they have always been included in most of the newborn screening (NBS) programs.

NBS is a synchronized system employing various advanced technologies and comprises of screening, follow-up of abnormal test results, confirmatory testing, diagnosis, treatment, and assessment of periodic outcome and efficiency[2]. NBS is aimed for timely diagnosis of threatening clinical conditions that would otherwise hamper mental and physiological development of newborn. In general practice, samples are screened by various

analytical methods including enzymatic assays, enzyme linked immunosorbent assay (ELISA), tandem mass spectrometry (TMS) and gas chromatography mass spectrometry (GCMS). The screened positive samples are subjected to follow up testing (confirmatory testing), using additional methodologies. Upon confirmation of screened positive samples through follow up testing, treatment decisions are made.

Overall, routine practice requires 15 to 20 days to reach a confirmatory decision, if everything goes well (repeat sampling in due time, timely logistics and lab analysis). This time period is very crucial in NBS program, as ultimate goal is to provide a quality life to the affected newborn with proper medical guidance and treatment. Routine primary screening methods are designed to identify as many infants affected by IEM favoring diagnostic sensitivity over specificity for disorder detection. Such approach not only increases the numbers of false-positive test results, but also places families to unnecessarily increased stress and anxiety. [3] The number of false-positive results has increased manifold with growing number of disorders in the NBS test panels. One solution to this problem is to use improved methods or to couple primary screening methods with second-tier tests that improve selectivity. Another way for improving diagnostic specificity without compromising diagnostic sensitivity is to develop a second, linked test that is more specific than the original method. The linked second test can have a lower sample throughput but measures additional metabolites that either strongly support the presumption of a true positive case or refute the previous diagnosis.

Organic Acid disorders are the intoxication type organic acidemias which often present in the neonatal period with myriad critical features like lethargy, poor feeding and vomiting, which may progress to coma if not identified and treated appropriately.[4] Although the organic acidemia is rare in western countries compared to amino acidemias[5]; the incidences in eastern part of world are more common, especially in India.[6] Almost 10% cases were screened positive for organic acidemia; during the period of January 2007-December 2008, amongst 420 suspects, showing significant symptoms.[7] These statistics are enough to say that, at a given population density in India, the figure of positive cases might reach, too many fold higher, and hence the focus on organic acidosis screening in newborn is the need of time today.

Many of the organic acid disorders can lead to irreversible damage in early days of life, especially

in first few weeks; each minute taken to reach confirmed diagnosis counts on the health of newborn. As a consequence, the present study focuses on confirmation of screened positive cases of organic acid disorders, using two tier testing on TMS and GC/MS simultaneously to reach a diagnostic decision within shortest possible time, which would benefit the affected baby.

2. Material and methods

2.1 Chemicals

We purchased isotopically labeled succinyl acetone, amino acids and acyl carnitine standards from Cambridge Isotope Laboratories (CIL). Chrom systems reagent kit for amino acid and acyl carnitine analysis purchased from Chrom systems, Germany. All other required chemicals and solvents of analytical reagent grade were purchased from Sigma Aldrich and used without further purification.

2.2 Clinical sample collection

The heel prick dried blood spot (DBS) sample was collected on preprinted circles of specially manufactured DBS card/paper (Whatman 903). This filter paper meets NCCLS and CDC specifications[8]. Similarly, urine samples were collected by adsorbing on filter paper (Whatman 903). Both samples (Blood and urine) allowed drying properly at room temperature for approximately two hours, before being shipped to the analyzing laboratory. Almost 348 heel DBS specimen collected randomly from different parts of the country were first taken for amino acid and acyl carnitine profile on TMS. Urine samples of all these cases were processed on GC/MS for metabolic acid pattern. The laboratory turnaround time for screening metabolic disorder began on the day when urine and DBS specimens were received. Written informed consent was obtained from parents of babies.

2.3 DBS analysis on TMS

DBS sample analysis for amino acid and acyl carnitine profile was performed on LCMS-MS 8030, Triple-Quadrupole Mass Spectrometer equipped with ESI probe (Shimadzu, Japan). DBS samples were processed by protocol stated in Chrom systems reagent kit. A positive and negative control sample was prepared and analyzed with every batch of patients tested. The data analyzed by using Neonatal Software version 3.2. The markers were expressed as $\mu\text{mol/L}$ based on its ratio with stable-isotope deuterium-labeled isomer (e.g., Leucine/deuterium-Leucine). Reference values for amino acid and acyl carnitine profiles have been calculated from the DBS concentrations measured in healthy newborns and early infancy.

2.4 Urine analysis by GCMS

Urine filter paper samples were extracted using suitable protocol and pretreated with urease at 37 °C to get rid of urea followed by deproteinisation with ethanol containing Heptadecanoic acid as internal standard. Further, sample was vacuum dried and residue was subjected to derivatisation by adding N, O,-bistrimethylsilyl) trifluoroacetamide (BSTFA) and Trimethylchlorosilane (TMCS) as described by Kuhara *et al.*[9] 1 µl Aliquots of derivatized sample were injected into Shimadzu QP- 2010 Plus GC/MS using auto sampler in split mode. The metabolites were chromatographically analyzed as trimethylsilyl compounds. The data analyzed with computer-assisted program and NIST library. Urinary creatinine was measured enzymatically using Microlab semi auto analyzer ARX-235. The markers were expressed as mmol/mol creatinine.

3. Results

Total 17 cases were picked up as presumptive positive when their blood carnitine profile and urine GC/MS organic acid analysis considered separately which showed one or the other finding indexing towards possibility of IEM. Amongst these 17 samples, 8 samples were confirmed for organic acidemia based on the consideration of both metabolic pattern (Table 1). Simultaneous analysis of blood and urine samples, detected and confirmed the presence of various metabolites for organic acidemia.

The DBS sample of 6 day old female baby processed by TMS showed elevated propionylcarnitine (C3) levels up to 9.3µM/L of blood which was almost 30 times higher than the reference value. Propionylcarnitine is the primary marker used to screen for propionic acidemia (PA), methylmalonic acidemia (MMA), and multiple carboxylase deficiency (MCD). Its urine profile showed increased excretion of 3-hydroxypropionate and methyl-citrate which is consistent with PA (Fig.1). Absence of methylmalonic acid in urine ruled out the possibility of MMA. Due to aggressive identification by two tier analysis and initiation of

therapy surely prevented severe morbidity and mortality caused due to PA.

Similarly, increased level of glutaryl carnitine (C5DC) was detected in the two DBS sample of 1.5 year and 2.3 month old male baby when analyzed through TMS. C5DC is the primary marker for Glutaric acidemia type I (GA I) and a secondary marker for Glutaric acidemia type II (GA II). Apparent increases in C5DC have also been demonstrated in medium-chain acyl-CoA dehydrogenase deficiency, most probably because of acylcarnitines of identical mass.[7] On the other hand, the plasma levels of C5DC may not be consistently elevated, especially in the neonatal period due to carnitine deficiency, so that cases of GA-I may be missed by this method. More specific urine analysis thus played crucial role to resolve the dilemma showing increased excretion of glutarate, 3-hydroxyglutarate and gluconate (Fig 2), providing enough confidence in the diagnosis. The detection of C5DC alone is insufficient for the diagnosis of this disorder since it is known to be elevated in other conditions like GA II, respiratory chain disorder, branched chain organic acidurias as well as riboflavin deficiency and valproate therapy.

A 8 days old female baby had acylcarnitine profile in the blood sample and urine metabolome pattern both pointing out towards beta-ketothiolase deficiency (BKT) (Table 1).

Another case of 4 month old male baby had increased level of propionylcarnitine (C3) and 2-methyl 3-hydroxybutyrylcarnitine (C5OH) in the blood sample. Based on these findings, baby was suspected for MCD. Since MCD is characterized by deficient activities of three biotin-dependent enzymes, propionyl CoA carboxylase, pyruvate carboxylase, and β-methylcrotonyl CoA carboxylase. Urine analysis of same patient showed expected increased excretion of metabolic acids and glycine conjugates consistent with MCD (Fig 4).

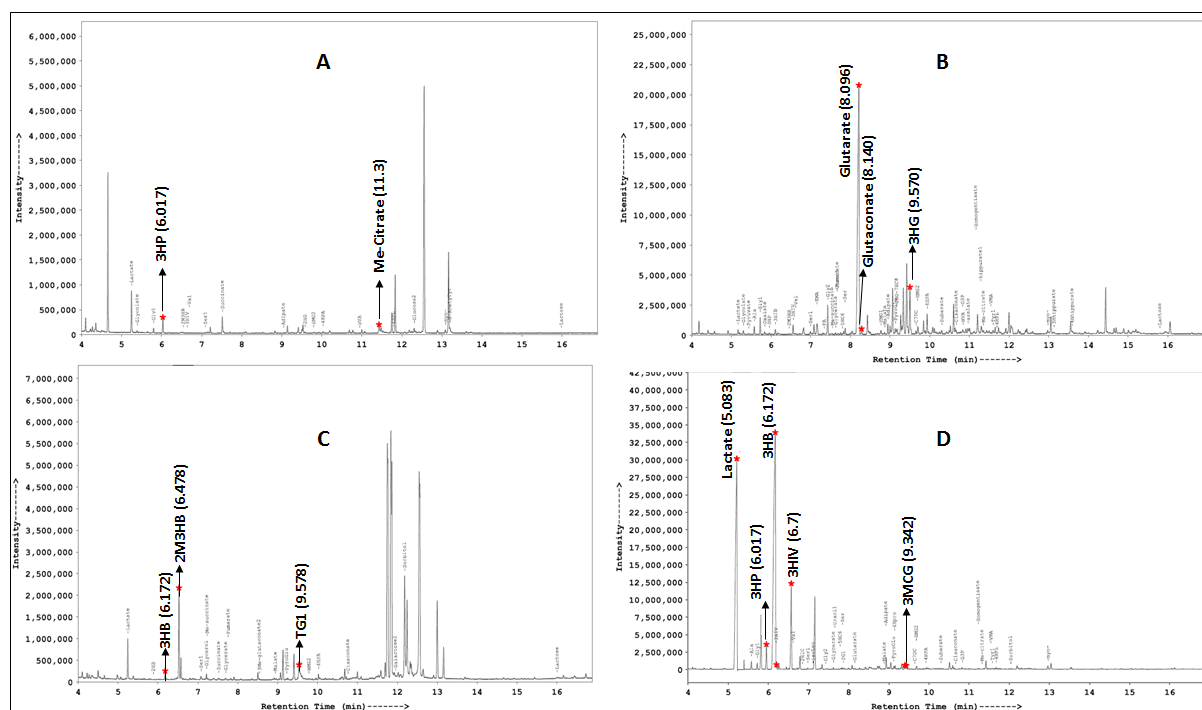
Results of both blood carnitine profile and urine metabolic profile were reported within 36 hours of receipt of samples in the laboratory for all the cases in the study group.

Table 1: New born screening results of screen positive cases by two tier testing approach

No. of cases	Observed TMS markers	Presumptive diagnosis based on Carnitine profile	Urinary markers by GCMS	Confirmed Screening based on second tier approach
2	Leucine, valine	MSUD	Lactate, 2-hydroxybutyrate, 3-hydroxybutyrate, 2-hydroxyisocaproate, 2-hydroxyisovalerate	MSUD
1	Propionylcarnitine (C ₃), 2-methyl 3-hydroxybutyrylcarnitine (C ₅ OH)	HMG, 3MCC, MCD, BKT, 3MGA	Lactate, 3-hydroxybutyrate, 3-hydroxypropionic acid, 3-methylcrotonylglycine, tiglylglycine, 3-hydroxyisovalerate, 2-hydroxyisovalerate.	MCD
2	Alanine, glutaryl carnitine (C ₅ DC)	GA I, GA2	Glutarate, 3-hydroxyglutarate, glutaconate	GA I
1	Propionylcarnitine (C ₃)	PA, MMA, MCD, CUD	3-hydroxypropionate, methylcitrate	PA
2	Tiglylcarnitine (C ₅ :1), 2-methyl 3-hydroxy butyrylcarnitine (C ₅ OH)	BKT	3-hydroxybutyrate, 2-methyl 3-hydroxybutyrate, tiglylglycine.	BKT

MSUD: Maple syrup urine disease; MCD: Multiple carboxylase deficiency; GA I: Glutaric acidemia Type I; GA II: Glutaric acidemia Type II; PA: Propionic acidemia; BKT: β -ketothiolase deficiency; HMG: 3-Hydroxy-3-methylglutaryl CoA lyase deficiency; 3MCC: 3-Methylcrotonyl- CoA carboxylase deficiency; 2M3HBA: 2-Methyl 3-hydroxy butyric aciduria; 3MGA: 3-Methylglutaconic aciduria; MMA: Methylmalonic acidemia; CUD: Carnitine uptake defect

Fig 1: Urine total ion chromatogram of positive cases of organic academia



A) Propionic acidemia

B) Glutaric Acidemia type I

C) β -ketothiolase deficiency

D) Multiple carboxylase deficiency.

Figures in the bracket indicate retention time of chromatographic run.

3HP: 3-hydroxypropionate; 3HG: 3-hydroxyglutarate; 3HB: 3-hydroxybutyrate; 2M3HB: 2-methyl 3-hydroxybutyrate; TG: tiglylglycine; 3HIV: 3-hydroxyisovalerate; 3MCG: 3-methylcrotonylglycine.

4. Discussion

NBS has significantly improved the detection of inherited metabolic disorders, but proportionately has increased burden of reporting false positive results. We must recognize these false positive test results has adverse effect and devote more attention for its minimization. It is desirable for any NBS program to design and adopt the strategies like improved technology and two tier testing which increases sensitivity without reducing specificity. The strategy that is used by any screening program is determined by the technology available, the relative expense of measuring the respective analytes, and the beliefs of those who establish the program. Feasibility of implementing two tier strategies has first been demonstrated during large scale screening for congenital hypothyroidism wherein primary testing is done with thyroxine hormone and only those individuals with low T₄ are tested for Thyroid stimulating hormone (TSH). Such two tier approach not only improved efficacy without the need for collecting a second sample but also reduced the false positive rate.

TMS is rapidly being adopted by NBS programs to screen dried blood spots for >40 markers of disease in a single assay[10]. Its revolutionary technique and analysis time per specimen is of few minutes, compared to GC/MS analysis which requires pretreatment and proper sample preparation. [10][11] TMS has recently gained much attention due to new method development, significant high throughput and low cost and hence been accepted as primary test by most of the NBS program. However an attempt to increase the number of disorders in TMS screening panel increased the burden for lowering the cutoff values which proportionately increased the chances of false-positive rate to a reasonable level. Many of the abnormal analyte elevation found in TMS screening are not pathognomonic of a single disorder and can be produced by several different genetic disorders. Moreover conditions like gestational age, birth weight, liver or kidney dysfunction, parenteral nutrition, hyper alimentation, drug treatment, sepsis can confound the screening results.[12] Preanalytical errors like hemolysis, layering of DBS, anticoagulant use, moisture add to the total error of analysis. In such context, additional, more-specific diagnostic tests are required. In such scenario, GC/MS is the preferred confirmatory technique, at least in diagnosing organic acidemias, but it requires another level of laboratory sophistication and resources apart from its low throughput and cost involved.[13][14]

Moreover expert opinion and peer-reviewed data strongly support a follow-up protocol that includes a plasma acylcarnitine analysis along with urine organic acid analysis in order to lower down the diagnosis and to institute acute specific therapy.[15] Many investigators have proposed better precision and reproducibility of GC/MS in quantification of organic acids than TMS. Another reason for integrated two tier approach is based on the fact that amino acid analysis does not contribute to clarifying the initial differential diagnosis but do play a chronic therapeutic role if the eventual diagnosis dictates protein/amino acid restriction. Therefore we have proposed a novel NBS algorithm incorporating second tier testing of urine by GC/MS following presumptive positive testing on the initial blood spot for the increased number of abnormal results from the primary test for improving the performance of NBS.

The present manuscript provides insights to diagnosis and timely confirmation of common organic acid disorders assigned to the core panel of American College of Medical Genetics (ACMG) which meets all the criteria established by the expert group like availability of screening test, efficacious treatment and adequate knowledge of natural history. [16] The encountered positive cases of organic acid disorders belong to the class of common organic acidemia as reported earlier. [6][7] Primary markers of carnitine profile were expected to be consistently expressed with their associated disorders, whereas the secondary markers add credibility or help with the differentiation. For example any DBS sample which has elevated propionylcarnitine (C3), is screened positive for PA. [17] It has been suggested that urine organic acid analysis plays a major role in the initial follow-up testing of such suspected inborn errors marked by specific acylcarnitines. Thus demonstration of 3-hydroxypropionate and methylcitrate in urine invariably confirms the diagnosis of PA (Fig 1). Propionyl glycine is a specific marker of PA which could be lost during sample storage and/or transportation. Similarly notable presence of C5DC, a primary marker of GA I, must follow analysis of urine metabolic study of presumptive suspected case. The urinary profile of suspect showing increased excretion of glutarate, 3-hydroxyglutarate and glutaconate is almost certain (refer Fig 2), since 3-hydroxyglutarate excretion has not been found in any other condition. [18] The urinary profiles are usually abnormal with increased glutaric acid, 3-hydroxyglutarate and glutaconate in GA I. [19]

Urinary excretion of 2- methyl 3-hydroxybutyrate is the most characteristic feature of BKT. [20] C₅:1 and C₅OH carnitines are established primary and secondary markers for BKT. [21]

However Sarafoglou *et al* demonstrated that affected BKT cases can have normal acylcarnitines when their urine organic acids show severe ketosis. [22] Blood carnitines and Urinary metabolic studies taken together usually confirm BKT deficiency. Similarly the cumulative picture of elevated C₃ and C₅OH carnitines showing excretion of 3-hydroxypropionate, 3-methylcrotonylglycine, tiglylglycine, 3-hydroxyisovalerate. Metabolic profile on two matrices, biotinidase activity and estimation of biotin level provided findings all attributed to possibility of MCD.

Newborn screening in India is still in infancy where very high prevalence of IEM to the extent of 1 in every thousand newborns was observed. [23] Several investigators stressed the importance of screening in India, necessitating nation-wide large-scale screening. [24][25][26] A case study reported previously showed, 55% of mental retardation due to organic acidemia. [6] These Indian studies clearly demonstrate higher incidence of IEM in the background of poor development of NBS model. Several cases of neonatal deaths related to metabolic disorders go undiagnosed while dealing with deaths due to neonatal sepsis. Additionally most of the previously unscreened babies present in the late childhood for which the NBS cut-offs may not find applicable and difficult to interpret. Since treatments and responses for organic acidemia have significantly improved over the decade, such integrated two tier approaches enhance to pinpoint the diagnosis. [16] Another notable benefit of urine analysis in two tier approach is the measurement of analytes which may not be available through TMS providing comprehensive organic acid model and improved specificity.

5. Conclusion

The application of two tier newborn screening metabolome analysis is precise and robust and therefore suitable for implementation in routine clinical screening programs having shorter turnaround time. In our opinion, this will reduce or even eliminate false-positive results and prevent a great deal of unnecessary anxiety for parents and associated problems. [3][8] On this ground, the present study brings significant approach, where two tier analysis system applied to NBS programs reduces recall rate and overall expenses achieving the ultimate purpose of screening of IEM.

References

- [1] Garrod AE. The incidence of alkaptonuria: a study in chemical individuality. *Lancet*. 1902; 13:1616–20.
- [2] Das SK. Inborn Errors of Metabolism: Challenges and Management. *Ind J ClinBiochem* 2013; 28(4):311–313.
- [3] Tarini BA1, Clark SJ, Pilli S, Dombkowski KJ, Korzeniewski SJ, Gebremariam A *et al*. False-positive newborn screening result and future health care use in a state Medicaid cohort. *Pediatrics*. 2011; 128(4):715-22.
- [4] Seymour CA, Thomason MJ, Chalmers RA, Addison GM, Bain MD, Cockburn F, Littlejohns P, Lord J, Wilcox AH. Newborn screening for inborn errors of metabolism: a systematic review. *Health Technol Assess*. 1997; 1(11):1–95.
- [5] Green A. ACP Broadsheet 120: January 1989. Guide to diagnosis of inborn errors of metabolism in district general hospitals. *J Clin Pathol*. 1989; 42(1): 84–91.
- [6] Vaidyanathan K, Narayanan MP, Vasudevan DM. Organic Acidurias: An Updated Review. *Ind J ClinBiochem* 2011; 26(4):319–325.
- [7] Narayanan MP, Vaidyanathan K, Vinayan KP, Vasudevan DM. Diagnosis of major organic acidurias: two years' experience at a tertiary care center. *Indian J ClinBiochem*. 2011; 26(4):347–353.
- [8] Mei JV1, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr*. 2001; 131(5):1631S-6S.
- [9] Kuhara T, Shinka T, Inoue Y, Ohse M, Zhen-wei X, Yoshida I, Inokuchi T, Yamaguchi S, Takayanagi M, Matsumoto I. Pilot study of gas chromatographic–mass spectrometric screening of newborn urine for inborn errors of metabolism after treatment with urease. *J Chromatogr B Biomed Sci Appl*. 1999; 731(1):141-7.
- [10] Napolitano N, Wiley V, Pitt JJ. Pseudo-glutaryl carnitin aemia in medium-chain acyl-CoA dehydrogenase deficiency detected by tandem mass spectrometry newborn screening. *J Inherit Metab Dis*. 2004; 27:465–471.
- [11] Zytovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, Strauss AW, Comeau AM, Eaton RB, Grady GF. Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England newborn screening program. *Clinical Chemistry* 2001; 47(11):1945–55.

- [12] Slaughter JL, Meinzen-Derr J, Rose SR, Leslie ND, Chandrasekar R, Linard SM *et al.* The Effects of Gestational Age and Birth Weight on False-Positive Newborn-Screening Rates. *Pediatrics* 2010; 126(5):910-16.
- [13] Matsumoto I, Kuhara T. A new chemical diagnostic method for inborn errors of metabolism by mass spectrometry—rapid, practical, and simultaneous urinary metabolites analysis. *Mass Spectrometry Review* 1996; 15(1):43–57.
- [14] Shoemaker JD, Elliott WH. Automated screening of urine samples for carbohydrates, organic and amino acids after treatment with urease. *J Chromatogr.* 1991; 562 (1-2):125-38.
- [15] Dietzen DJ, Rinaldo P, Whitley RJ, Rhead WJ, Hannon WH, Garg UC *et al.* National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: Follow-Up Testing for Metabolic Disease Identified by Expanded Newborn Screening Using Tandem Mass Spectrometry; Executive Summary. *Clinical Chemistry* 2009; 55(9):1615–1626.
- [16] Newborn screening: toward a uniform screening panel and system--executive summary. American College of Medical Genetics Newborn Screening Expert Group. *Pediatrics* 2006; 117:S296-307.
- [17] Fenton YA, Rosenberg LE. Disorders of propionate and Methylmalonate Metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease.* New York, NY: McGraw-Hill; 1995:1423-43.
- [18] Stokke O, Goodman SI, Thompson JA, Miles BS. Glutaric aciduria; presence of glutaconic and β -hydroxyglutaric acids in urine. *Biochem Med* 1975; 12:386.
- [19] Goodman SI, Frerman FE. Organic acidemia due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease.* New York, NY: McGraw-Hill; 1995:1451-1459.
- [20] Merinero B, Perez-Cerda C, Garcia MJ, Carrasco S, Lama R, Ugarte M *et al.* β -Ketothiolase deficiency; Two siblings with different clinical conditions. *J Inherit Metab Dis* 1987; 10(2):276-78.
- [21] Schutgens RBH, Middleton B, vdBlij JF, Oorthuys JWE, Veder HA, Vulsma T, Tegelaers WHH. Beta-ketothiolase deficiency in family confirmed by in vitro enzymatic assays in fibroblast. *Eur J Pediatr* 1982; 139:39.
- [22] Sarafoglou K, Matern D, Redlinger-Grosse K, Bentler K, Gaviglio A, Harding CO, Rinaldo P. Siblings With Mitochondrial Acetoacetyl-CoA Thiolase Deficiency Not Identified by Newborn Screening. *Pediatrics* 2011; 128: e246–e250.
- [23] Verma IC. Burden of genetic disorders in India. *Indian J Pediatr.* 2000; 67(12):893–8.
- [24] Kumta NB. Inborn errors of metabolism (IEM)—an Indian perspective. *Indian J of Pediatr.* 2005; 72:325–32.
- [25] Devi AR, Naushad SM. Newborn screening in India. *Indian J Pediatr* 2004; 71: 157-160.
- [26] Kapoor S, Gupta N, Kabra M. National newborn screening program still a hype or a hope now? *Indian Pediatr.* 2013; 50(7):639-43.