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INHIBITION ASSAY OF ALA DEHYDRATASE BY SUCCINYL ACETONE IN TYROSINEMIA TYPE 1: A RAPID HT1 SCREENING TECHNIQUE FOR RESOURCE CONSTRAINED DIAGNOSTIC SETUPS

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Abstract

Succinyl acetone is a specific marker of tyrosinemia type I. The present study was undertaken to develop a simple and cost-effective quantitation method for succinyl acetone in dried blood spot (DBS) implementable in resource-constrained diagnostic settings. Spectrophotometric microassay based on the inhibitory effect of succinyl acetone on δ- aminolevulinate dehydratase (ALA-D) in erythrocytes, which catalyzes the formation of porphobilinogen from δ-aminolevulinic acid and is measured at 550nm. The reliability and suitability of the method for routine laboratory practice was assessed before the method was used for screening. Greater inhibition of ALA-D activity was observed when succinyl acetone was spiked to blood before spotting. The stability of succinyl acetone in DBS progressively deteriorated when stored at -80^{0}C. Among the 71 samples processed; an inhibition ranging from 2.75% to 55.47% was observed. The maximum inhibition of 63 - 73.47% was observed for a couple of the tyrosinemia true positive cases. The proposed method is useful for the mass screening of newborn babies for Tyrosinemia type I. This could ensure early detection and intervention while addressing the challenge of expenses involved in high-end technology, till the newborn becomes a universal reality.

INTRODUCTION

Tyrosinemia type I/ hereditary tyrosinemia type 1 (HT1) is an autosomal recessive disorder characterized by the absence or lesser functional fumaryl acetoacetate hydrolase (FAH) enzyme thus blocking the tyrosine metabolism [1, 2]. Lesser functional FAH lacks the efficiency to break down tyrosine. This leads to the accumulation of tyrosine and other toxic intermediates like succinyl acetone, maleylacetoacetate and fumarylacetoacetate in blood [3]. Accumulated succinyl acetone damages the liver and kidney causing weakness and pain [4]. The signs and symptoms of HT1 include damage and death to cells of the liver, kidneys, nervous system and other organs beginning in the first few months of life [5]. In the liver, tyrosine is broken down in a five-step process by the enzymes. The intermediates and final products of the metabolism are either excreted or used in the production of other essential metabolites. The FAH gene produces FAH which hydrolyses the fumarylacetoacetate to fumarate and acetoacetate. The epidemiology of HT1 is attributed to one of the three gene defects. The enzyme tyrosine transaminase produced from the tyrosine aminotransferase (TAT) gene is responsible for the breakdown of tyrosine into 4-hydroxyphenylpyruvate. The 4-hydroxyphenylpyruvate dioxygenase (HPD) gene is responsible for the production of the 4-hydroxyphenylpyruvate dioxygenase, which is involved in the second step of tyrosine breakdown i.e. 4-hydroxyphenylpyruvate into homogentisate [1-3]. Mutations in any of these three genes can lead to decreased activity and a suboptimal/ dysfunctional tyrosine metabolism.

Affected infants if not intervened in time, fail to gain weight and present with decreased growth rate, diarrhea, vomiting, severe abdominal pain, respiratory failure, symptoms of jaundice, a cabbage odored sweat and increased epistaxis. Some affected children may have neurologic problems. These characteristics remain for 7 days. Children with tyrosinemia type I succumb to death before the age of 10 years, if not treated [2]. Succinyl acetone is a specific marker of tyrosinemia type I [6].

HT1 with the global incidence of 1: 100000 live births[7], was found to be prevalent in the study area i.e. minimum 1 baby of every 1000 at-risk cases of IEM as per the data from the annual laboratory report of screened at-risk cases from local tertiary care hospital (of the study area) which may be even higher when accounted for the general population of the district or missed or under-diagnosed. The alarming prevalence data of the neighboring districts, Mysore and Bangalore, marked off the regional data i.e. 18.3: 100,000 [8], and vindicate the rationale of the present study.

Accurate quantitation of this biomarker employs a gold standard mass spectrometry platform [7]. The huge investment involved in the MS platform poses a major challenge in developing countries where economic concerns play a role in deciding the implementation of life-saving newborn screening programs and the prevalence and treatment accessibilities to many such disorders remain as issues of secondary concerns. The limited diagnostic laboratories offering the higher-end platforms (mass spectrometry technique) for the diagnosis of IEM disorders in developing countries like India and the huge live births also account for prolonged turnaround time [9]. The delayed turnaround time for screening would lose the very purpose of screening, i.e. the early identification of the disease for the timely intervention in agreement with Wilson Jungner Criteria. So the present study was focused to employ a methodology feasible for local diagnostic set-ups equipped with basic modalities (resource-constrained diagnostic set-ups) so that these basic labs could be used for screening programs. This could address the issues of increased cost and huge live births and the associated sample loads. Thus ensure that the sophisticated, well equipped higher end diagnostic labs could be optimally engaged for confirmatory diagnosis. The present study was undertaken to standardize a simple and cost-effective assay which is based on inhibition of ALA-Dehydratase by succinyl acetone in small scale laboratory settings to maximize the outreach of newborn screening.

Quantitation of succinyl acetone in dried blood spot samples using spectrophotometric micro assay method would help in the screening and diagnosis of HT1 in the resource limited settings of developing countries.

MATERIALS AND METHODS

Chemicals Used

MES monohydrate, aminolevulinate (ALA), succinyl acetone were from Sigma-Aldrich and tris (Hydroxymethyl) amino methane, mercuric chloride, trichloro acetic acid (TCA), dimethyl amino benz aldehyde, perchloric acid, dithiothreitol (DTT), glacial acetic acid, sodium hydroxide were from MERCK. All reagents were of analytical grade.

Preparation of Solutions

MES Buffer of 50mmol/L & pH 6.4, Tris of 2.5mol/L was stored at 4^0C. Dithiothreitol (21mmol/L), ALA (30mmol/L), modified Ehrlich reagent (2mol/L) was prepared freshly. Trichloroaceticacid (TCA) & mercuric chloride ($HgCl_2$) was prepared by dissolving 125g of TCA and 10g of mercuric chloride in distilled water, made it up to 1 liter and stored in a brown bottle at room temperature[4,5].

Sample Collection Procedure

Residual reversibly delinked samples of DBS specimens from neonates of tertiary care hospital at coastal Karnataka, India were obtained after routine screening of IEM disorders for seven conditions. The blood was collected by a heel prick on days 3-5 of life, spotted on Whatmann no.903 filter paper, allowed to dry at room temperature and stored at -20^0C until the day of analysis. The study obtained ethical clearance from Institutional Ethical Committee.

Assay Procedure

3mm discs of dried blood spot (DBS) on filter paper were punched into microtiter wells (one disc in one well). 50µl of DTT was added to each well. Microtiter plates were agitated at 100 rpm at room temperature for 15 minutes. 10µl of ALA solution was added to the test sample and 10µl of MES buffer to blank and incubated at 100 rpm for 4 hours at room temperature. The eluate from each well was transferred to different labeled microfuge tubes leaving the filter paper disc in the wells. 40µl of TCA. $HgCl_2$ solution was added to each tube and centrifuged at 390g for 30sec. 80µl supernatant from each tube was transferred to different wells of a microtiter plate and 100µl of Ehrlich reagent was added and kept for 10 minutes. Absorbance was read at 550nm in a microplate reader.

Color Reaction

A purple color is produced by the addition of 100µl of freshly prepared modified Ehrlich reagent. The color is due to the appearance of porphobilinogen, the maximum intensity is observed at the end of 10 minutes. Solution in the

blanks and samples of HT1 patients remained straw-colored due to the absence of substrate in blank solution and the presence of succinyl acetone in patients' blood respectively. The absorbance of porphobilinogen was measured at 550 nm in a microtiter plate using a spectrophotometric plate reader [10, 11]. Thus the method employs indirect quantitation of succinyl acetone i.e. the absorbance of porphobilinogen is indirectly proportional to the concentration of succinyl acetone meaning, with the presence of succinyl acetone in the blood inhibits ALA-D causing the decreased synthesis/absence of porphobilinogen and hence the decreased absorbance observed.

Calculation

Two standard curve graphs were plotted. 1) Absorbance (y-axis) versus Concentration (X-axis) and 2) Percentage inhibition of succinyl acetone versus Concentration of succinyl acetone. To plot a standard curve graph for Percentage inhibition by the increased concentration of succinyl acetone was calculated by the following formula.

$$\text{Percentage inhibition} = [(\text{Absorbance of Standard} - \text{Absorbance of blank}) \div \text{Absorbance of Standard}] * 100$$

Percentage Inhibition for the samples are calculated by relating the final absorbance of the test sample (obtained by the difference of Absorbance of test sample and test control) against the standard curve graphs.

The study of linearity and limit of detection was done at two different levels by taking whole blood from healthy volunteer and spiked with different concentration (20, 40,100 µmol/L) of succinyl acetone and spotted and other by adding different concentration (10,100,500 µmol/L) of succinyl acetone to DBS extract[10].

RESULTS

It was observed that there was increased inhibition of ALA-D activity when succinyl acetone was added to the blood before spotting as compared to the inhibition observed when succinyl acetone was added to DBS extract (Figure 1 & 2). The minimum detection amount (limit of quantitation) of succinyl acetone was 5µM/L and the limit of detection was found to be 0.09 to 0.27 without succinyl acetone in blood.

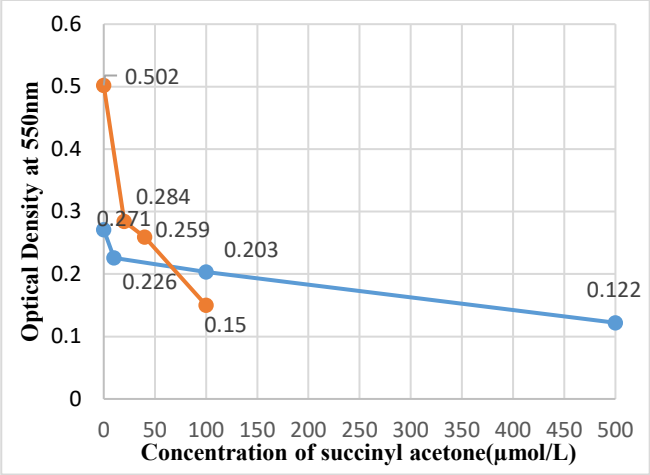

Figure 1: Graph shows the optical density at 550 nm for different concentration of succinyl acetone. Red line indicates the optical density of succinyl acetone added to blood before spotting. The blue line indicates the optical density of succinyl acetone added to DBS extract. Optical density obtained is the mean absorbance of six replicates with SD-0.002

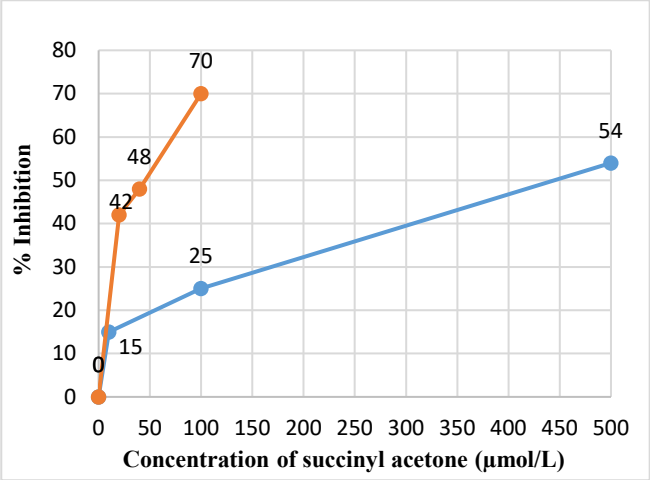

Figure 2: Graph shows the percentage of inhibition of ALA-D with different concentrations of succinyl acetone. Red line indicates % inhibition of ALA-D when succinyl acetone added to blood before spotting. Blue line indicates % inhibition when succinyl acetone added to DBS extract. The values represented are mean of six replicates with SD of 0.0021.

Linearity

The inhibition proportionately increased with increased concentration of succinyl acetone however maximum inhibition was reached at 100µM/L.

DBS specimen enriched with different concentrations of succinyl acetone was stored at -80^{0}C. It was noted specimen storage led to progressive deterioration in its stability, with analysis of fresh samples producing the most reliable results.

Imprecision

Within run imprecision and between the run imprecision was calculated using discs of 4 DBS samples. The between run coefficient of variation (CV) was calculated using discs of 4 different DBS samples assayed in 3 successive runs taking 2 replicates each time. The absorbance readings were used for the calculation of % CV. The within run % CV ranged from 7-15% which is acceptable but the between run % CV ranged from 17-27%. The between run CV was high which necessitated the use of a daily calibration curve.

Effect of Blood Additives

At the time of standardization, the samples with EDTA and heparin were used for the study. The absorbance of samples with EDTA was found to be decreased as compared to the heparinized sample. EDTA chelates zinc present in ALA-D and hence inhibits its activity, causing a decrease in absorbance. Hence heparinized blood was used for standardization.

ALA-D Assay in DBS Spots

A total of 74 samples were processed during the study. Graphs obtained by adding standards of succinyl acetone to blood and spotted at different concentrations (Figures 1 & 2) were used to determine the concentration of succinyl acetone and the percentage of inhibition of the test samples. Eleven samples showed more than 50% inhibition out of which three samples showed 73% inhibition which was the maximum value we could calculate from the graph.

DISCUSSION

This method employs the inhibition of erythrocytes ALA-D activity by succinyl acetone as the basis of quantitation of succinyl acetone in the blood samples. The extent of inhibition was studied by adding different concentrations (20, 40, 100 µmol/L) of succcinyl acetone to the blood before spotting (DBS). It was observed that the maximum inhibition achieved at 100µmol/L concentration of succinyl acetone. With the addition of increasing concentrations of succinyl acetone to DBS extract(instead to the whole blood), there was a proportional decrease in the absorbance concerning the increasing succinyl acetone concentration, but the optical density difference did not vary much after 100µmol/L. Hence 100µmol/L of succinyl acetone was taken as maximum concentration for the study. When succinyl acetone was added to the blood samples, percentage inhibition observed at 100µmol/L was 70% as compared to 25% inhibition obtained when the same concentration of succinyl acetone standard was added to the
extract. The higher % inhibition obtained in the blood samples could be due to the inhibition of ALA-D which would have started before the preparation of the extract. The maximum inhibition obtained from our study corroborated with the maximum inhibition reported (89.88%) elsewhere[11].

In the present study, all the samples showing 50% -60% inhibition were clinically confirmed to be cases of transient tyrosinemia (as confirmed by treating neonatologist) and followed up subsequently till the resolution of their clinical manifestation. Three samples out of eleven cases (showing more than 60% inhibition) were subsequently confirmed to be cases of tyrosinemia type1/classical tyrosinemia (as confirmed by treating neonatologist and TMS assay) which is comparable to the findings of a similar study reported by Gautham et al [11]. Hence any sample showing inhibition of more than 60% inhibition can be considered as positive for tyrosinemia and warrants immediate action in terms of confirmation and intervention. The remaining eight cases were periodically followed up and declared to be cases of transient tyrosinemia by the treating clinician.

The present method would be an effective tool in the detection of tyrosinemia type I in the primary health care centers where the cost of the test is a major criterion to be considered. The approximate expense involved for the present method is Rs. 100/- per test which is about ten times lesser than that of the gold standard, Tandem mass spectroscopic (TMS) method. (Cost estimated includes the cost of consumables and sample logistics). The simplicity of the method along with the cost-effectiveness and reliability, encourages the inclusion of screening for tyrosinemia type I in the routine newborn screening program using DBS samples. Moreover, there are evidences for employing this method for successful mass-screening of newborns in the Quebec Neonatal Screening Program for the years 1970 to 1997 as second-tier and for the years 1998–2014 as first test for HT1 though not widely embraced. The gold standard, TMS could be employed for the confirmatory diagnosis of those with more than 60% inhibition obtained by the proposed method [12]. This proposition is based on maximum inhibition obtained in eleven of 74 cases from our study which were directed for further confirmatory investigation(TMS and GC-MS) and found only three confirmed with Tyrosinemia Type 1.

Thus the proposed method can be effectively used for the quantitation of succinyl acetone in the dried blood samples. The analytical recovery study and the reproducibility observed justify the efficiency of this method. The method proves to be sensitive enough to quantitate minute concentration of succinyl acetone (detection limit of 5µmol/L) which would help to differentiate the true tyrosinemia cases from the normal babies, as well as address concerns pertaining to cost-intensive/ sparsely accessible high-end MS platforms and high sample loads, relevant to our Indian scenario.

LIMITATIONS

The sample size was 74 and increasing the sample size could make the study more specific and informative.

CONCLUSION

This method is simple, reliable, sensitive and cost-effective and can be employed in any laboratories with minimum facilities. The above features support the application of the method for the mass screening of newborns for HT-1 which would help in early detection and intervention of the condition. Those cases showing percentage inhibition more than 60% should be recalled for confirmatory diagnosis by mass-spectrometry platform and clinical correlations. This stratification of high risks is based on the proposed preliminary line of investigation and making them undergo confirmatory diagnosis would reserve the economic concerns associated with the concept of newborn screening implementation. Ultimately proposed spectrophotometric microassay though seems to be outdated still holds relevance to overcome the expenses of high-end technology till newborn screening becomes a universal reality in developing countries and also helps in early detection and intervention.

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CONFLICT OF INTEREST

The authors declare no known conflict of interest.

REFERENCES

1. Grompe, Markus (2016-12-20). "Disorders of Tyrosine Metabolism". www.uptodate.com. Retrieved 2018-02-23.
2. The Maternal and Child Health Bureau, Health Resources and Service Administration, Genetic Services Branch, MCH Project #:1H46 MC 00189-03 http://mchb.hrsa.gov.
3. Lindblad B, Lindstedt S, Steen G. Enzymic defects in hereditary Tyrosinemia. Proc Natl Acad Sci U S A. 1977;74(10):4641–5
4. de Laet, C., Dionisi-Vici, C., Leonard, J.V., McKiernan P, Mitchell G, Monti L, *et al.* Recommendations for the management of tyrosinaemia type 1. *Orphanet J Rare Dis*. 2013;8,8. https://doi.org/10.1186/1750-1172-8-8
5. Nelson, David; Cox, Michael (2013). Lehninger Principles of Biochemistry (6th ed.). New York: WH Freeman and Co. p. 719. ISBN 978-1-4292-3414-6.
6. Stinton C, Geppert J, Freeman K,Clarke A, Johnson S, Fraser H, et al. Newborn Screening for Tyrosinemia type 1 using succinyl acetone- a systematic review of test accuracy. *Orphanet J Rare Dis*. 2017; 12(1):48.doi:10.1186/s13023-017-0599-z;PMID:28274233;PMCID:PMC5343414.
7. Priestley RC.J, Alharbi H, Callahan P.K, Guzman H, Walters P.I, Smith L, et al. The Importance of Succinylacetone: Tyrosinemia Type I Presenting with Hyperinsulinism and Multiorgan Failure Following Normal Newborn Screening. *Int. J. Neonatal Screen*. 2020, 6, 39; doi:10.3390/ijns6020039
8. Rao AN, Radha Rama Devi, Savithrsi H.S., Venkatr AO, Bittles A.H. Neonatal screening for amino acidaemias in Karnataka, South India. Clinical Genetics 1988: 34: 60-63
9. Shetty, PP., Nagendra, K., Nalini, K., Rao P, Bhaskaranand. N, Belle V. S. Amino Acid Profile and Lactate Pyruvate Ratio: Potential Adjunct Markers for Differentiating Inborn Errors of Metabolism. Ind J Clin Biochem (2019). https://doi.org/10.1007/s12291-019-00840-1
10. Schulze A, Frommhold D, Georg F. Spectrophotometric Microassay for δ-aminolevulinate Dehydratase in Dried- Blood Spots as Confirmation for Hereditary Tyrosinemia Type I, *Clinical Chemistry*. 2001; 47(8), 1424-1429.
11. Kumar Gautam, Sharma Akanksha, P S Y Shivam. Confirmation of Hereditary Tyrosinemia Type I in Neonatal stage Using Spectrophotometric Micro-assay Based on the Determination of Succinylacetone level in Dried Blood Spots. *J. Experimental Biology and Agricultural Sciences.* 2014; 2(2S), 293-299.
12. Giguère, Y.; Berthier, M.-T. Newborn Screening for Hereditary Tyrosinemia Type I in Québec: Update. In Hereditary Tyrosinemia: Pathogenesis, Screening and Management; Tanguay, R.M., Ed.; Advances in Experimental Medicine and Biology; Springer International Publishing: Cham, 2017; 139–146, doi:10.1007/978-3-319-55780-9_13.