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ORIGINAL ARTICLE

Qualitative Analysis of standard Amino acid by Thin Layer Chromatography in examination of Inborn Errors of Metabolism

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ABSTRACT

Metabolism is divided into various pathways in which constituents are converted by enzymes that catalyze the transformation of the substrates (synthesis or degradation). Metabolic problems can occur when one or more of these enzymes are not functioning properly (i.e., high or low levels) or are absent. Most IEMs are inherited in an autosomal recessive (AR) manner.

The overall rate of inborn metabolic errors is estimated to be between 1:800 and 1:1000 live births, while the frequency is likely substantially higher due to clinical diagnosis challenges and diagnostic testing constraints. Incidence and prevalence of IEM differed between countries. Particular abnormalities of amino acid metabolism, disorders of carbohydrate metabolism, disorders of lipid metabolism, and disorders in the metabolism of purines and pyrimidines are all classified depending on the impaired metabolic pathway or enzymes involved in the generation of hazardous chemicals .aims or objectives in the study was to introduce a separation method of standard amino acid that could be effective for the detection amino acid disorder by producing distinguishable strategies based on silica gel TLC plates.

Methods: The present work included a case-control study. Samples were selected from the patients attending the rare diseases unit, AL-children teaching hospital, Karbala. Blood and urine samples were collected from the volunteers, participants had no history of IEM, and the standardization of 20 amino acid was done by TLC (10 times for each amino acid). After that, the separation of amino acid in the serum and urine was performed for the patient and control group.

Results: The study was includes 56 patients with age ranging from 7 days to 15 years. Infants of age < 1month were 7 (12%), newborn aged from 2 to 12 months numbered 39 (69%) and children aged >1year were 10 (17%), females was 22 (39%) and males represented 34 (60%), our study show that 41% of the suspected IEM patients were with a sign of jaundice, and 18, 5, 16, 29, 5, and 32% with diarrhea, dehydration, acidosis,

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vomiting, hepatomegaly, and flatulence respectively. Results were shown about 39%, 18%, 41%, 50%, 36% of suspected IEM patients were with Intellectual delay, Lethargy, Spasticity, Hypotonia, Epileptic seizures, respectively. Standardization of amino acids TLC was performed based on different strategies: the study results were established different strategies shown good demonstration of the standard AA throughout the retention factor (Rf) (mean and /or range), the arithmetic retention factor was result in subdivided of AA into 6 groups accordingly. Also, the standardization of AA were separated based on producing various distinguishable colors/ shapes. The above three methods were help to identify amino acid in the serum and urine samples.

Conclusion: This study was concluded that thin layer chromatography (TLC) was an easy qualitative method, convenient and inexpensive way that could use to determine types of components (AA) were in a mixture of a suspected IEM patients

INTRODUCTION

Metabolism is a collection of complex biochemical processes that occur in living organisms to keep key cellular functions running.[1] The majority of metabolic diseases are autosomal recessive characteristics. Characteristics of an inborn metabolic mistake include autosomal recessive inheritance and enzyme deficiency. Metabolic errors present during birth (IEM) are genetically determined by deficiencies of an enzyme involved in the synthesis, transport or degradation of molecules in a metabolic pathway. The occurrence of a block in one stage of a pathway results in the lack or excess of a particular substance and May, in addition, interfere in an alternative metabolic pathway.[2]

At any age, inborn defects can appear with a wide range of clinical signs and symptoms. The signs and symptoms of IEM can be split into two categories: early-onset and late-onset (coma, hypotonia or hypertonia, seizures, organomegaly) and late-onset forms(macrocephaly, microcephaly, attention deficit, mental and motor retardation, hypo/hypertonia, spasticity, seizures, movement disorders, stroke, ataxia, stupor, coma, psychiatric signs, hypo/hypertonia, spasticity, seizures, movement disorders, stroke, ataxia, stupor, coma, psychiatric signs, pancytopenia, thrombocytopenia, organomegaly).[3] The overall prevalence of inborn metabolic errors is estimated to be between 1:800 and 1:1000 live births.[4] Although there is limited information regarding this rate in Karbala, Iraq has a high rate of consanguinity, with an average of 47–60 percent, which may be related to socioeconomic, cultural, religious, and political variables. Only two provinces in Iraq (Baghdad and Karbala) have implemented a newborn screening program yet (which was started in 2013). no clear data was presented from Iraq. Up to now, more than 500 IEMs have been recorded. Locally, the number of participants who were screened in Misan province over one year (from 1 April 2017 to 1 April 2018) was 112. It has been recorded that 20 cases which about 18% were identified and confirmed to have IEM.[5] Other studies in Baghdad from December 2009 to December 2012 included 1758 participants selected from Al-Emamain Al Kadhemyian and the children's welfare teaching hospitals. The reported positive cases of IEM were around 224 (12.7%) cases.[6] While in the Mosul and Kurdistan regions (from January 2018 to January 2020), a study was conducted on 3000 participants, and only 4.4% of cases were reported and verified to have IEM.[7] Therefore, this paper was aimed to examine background information on the state of the scientific literature in this field of study Also, to highlight areas where additional research is needed, either to address knowledge gaps relating to develop and diagnose the disorders of IEM or high lighting the importance of early diagnosis to avoid the consequences.

MATERIAL AND METHODS

The present work included a case-control study. Samples were selected from the patients attending the rare diseases unit, Children Teaching Hospital, Karbala. The sociodemographic aspects of the patients were collected through the self-reported technique, including age, gender, BMI, Genetics History of family, congenital abnormalities, and having any current chronic diseases. They were also exposed to a medical examination for signs and symptoms of IEM by the sub-specialized doctor based on the World Health Organization (WHO) criteria. Patients groups were compared to a group who do not have a disease (apparently healthy) as a control subject.

Standard AA was prepared by dissolving 0.1 g of each stock amino acid in 1mL DW, mobile phase for (TLC) was prepared by mixing 14 mL of butanol (99.8 %), 14ml of acetone (99.9 %), 4 mL of Acetic acid and 8 mL of DW. The stationary phase for TLC was a silica gel 60F 254 precoated thin-layer chromatography (TLC) plates (20 cm×20 cm, 0.20 mm) from Macherey-Nagel (Germany) were used for amino acids analysis. The solutions (standard amino acids, patient and healthy control samples) were applied to the points marked with a pencil on a line at equal distances where the points are within one centimeter of the edge of the plate. All stains were dried with a dryer prior to placing the plate in the glass chamber. Each amino acid has appeared like specks strewn apart vertically. The distance over the total covered by the solvent is equal to the retention factor (RF) for each location.

RESULTS AND DISCUSSION

The study was attempted to examine the Thin Layer Chromatography: Basic Concepts and Importance as laboratory

techniques used for preliminary method prior to mass spectrophotometry. This could be carried out using a less sophisticated way to analyze amino acid disorder in patients with IEM. It has performed precisely the 20 amino acids and separated them according to different ways, which were listed below:

No	Separation principle	Table No
1	Depending on the arithmetic mean of the retention factor	1
2	Depending on the range (minimum-maximum) retention factor of ten readings	2
3	Based on amino acid color on TLC plates with ninhydrin spray reagent	3
4	Based on the shape of the amino acid spot on TLC	4
5	according to the distance traveled by a sample of each amino acid for ten readings	5

Standardization AA by TLC based on the Arithmetic Retention Factor

All 20 amino acids were standard by TLC on a regular silica plate based on the arithmetic retention factor. The retention factor (R*f*) values could reflect the Individual AA behavior in TLC is defined by a quantity known as R, which is given as a decimal fraction. The R is computed by dividing the distance traveled by the chemical from its initial position by the distance traveled by the solvent from its initial position (the solvent front). Generally, it is a constant for each component only when the experimental conditions are identical. Ten repeated runs performed the calculation of Rf for each amino acids. To identify the amino acids, Rf values were calculated and compared to the repeated values. If the development of the plate is done with the same solvent, kind of TLC plates, method of spotting, and under the same conditions, the Rf value for each known drug should remain the same.

Samples of Tyrosine in addition Phenylalanine amino acid standard runs were shown in supplementary martial (Figures 1 and 2), which illustrated the ten Rf reading.

On the other hand, the Amino acids were divided into six subgroups according to the rounding Mean of Rf for each amino acid in an attempt to make the identification of the amino acid more and easily separated since In terms of time and cost, preparative TLC can be significantly more efficient than mass spectrophotometry. It is groups of AA listed in the Figures below. Group A has included the amino acids (Glycine, Serine, Histidine, Alanine, Aspargine) the range of their Rf was (0.4, 0.42, 0.42, 0.43, 0.43) respectively as demonstrated in Figure 1; Group B has included the amino acids (Threonine, Glutamic, Valine, Cysteine) the range of their Rf was 0.49, 0.51, 0.54, and 0.55, respectively as demonstrated in Figure 2, Group C has included the amino acids (Proline, Glutamine, Aspartic) the range of their Rf was (0.46) as demonstrated in Figure 3, Group D has included the amino acids (Methionine, Isoleucine, Leucine) the range of their Rf was (0.66, 0.68, 0.69) respectively as demonstrated in Figure 4, Group E has included the amino

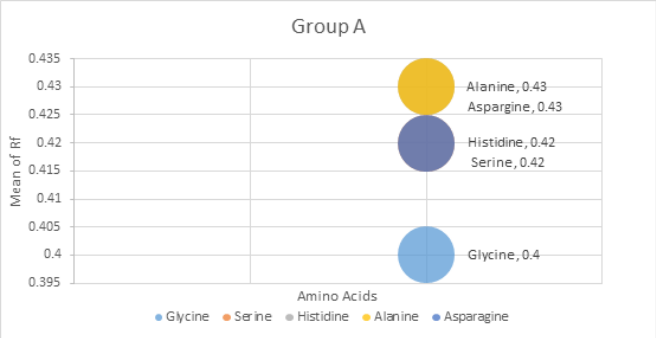

Figure 1: The mean of retention factor (R*f*) values of standard Amino acids (Group A), including (Glycine, Serine, Histidine, Alanine, Asparagine) separated by TLC

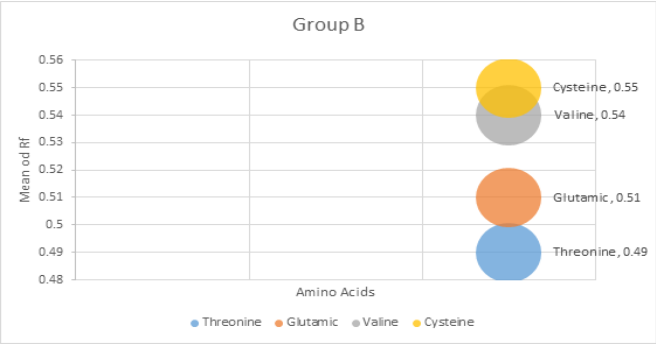

Figure 2: The mean of retention factor (R*f*) values of standard Amino acids (Group B), including (Threonine, Glutamic, Valine, Cysteine) Separated by TLC

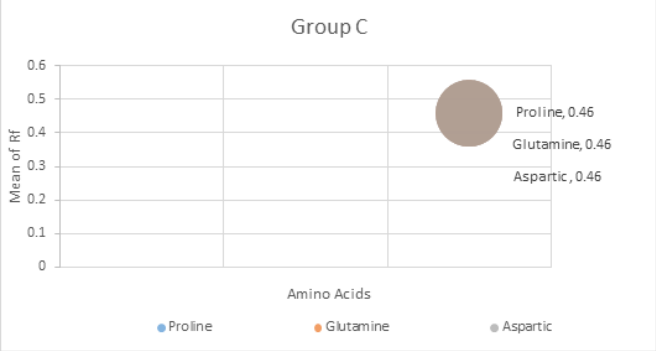

Figure 3: The mean of retention factor (R*f*) values of standard Amino acids (Group C) including (Proline, Glutamine, and Aspartic) separated by TLC

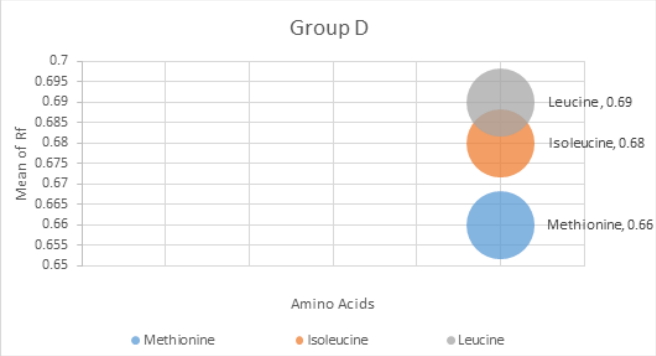

Figure 4: The mean of retention factor (R*f*) values of standard Amino acids (Group D) including (Methionine, Isoleucine, and Leucine) separated by TLC

acids (Phenylalanine, Tyrosine, Tryptophan) the range of their Rf was (0.7, 0.72, 0.73) respectively as demonstrated in Figure 5. In contrast, Group F has included the amino acids (Lysine, Arginine) the range of their Rf was (0.19, 0.33) as demonstrated in Figure 6.

Standardization of the AA by TLC Depending on the Range (minimum-maximum) retention factor

Since the different AA was shown a similarity in the mean of Rf due to their traveling in the same rates owing to their affinity for the stationary phase additionally/or their solvent solubility, therefore another standardisation was done depends on the range (minimum-maximum) of the retention factor for ten repeated readings. The range of Rf of each AA was listed in Table 1.

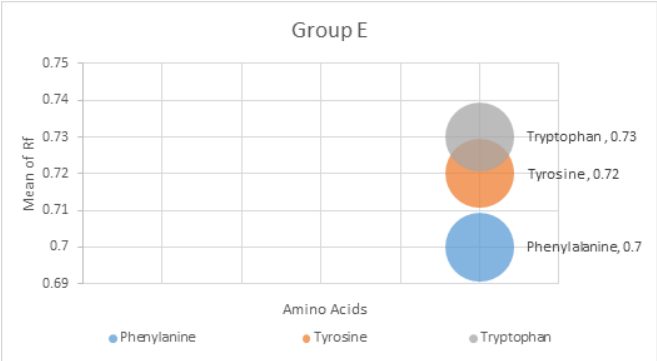

Figure 5: The mean of retention factor (R*f*) values of standard Amino acids (Group E) including (Phenylalanine, Tyrosine, and Tryptophan) Separated by TLC

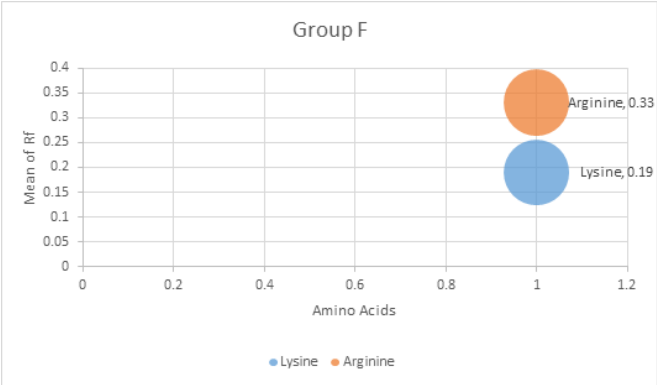

Figure 6: The mean of retention factor (R*f*) values of standard Amino acids (Group F), including (Lysine, Arginine) separated by TLC

Standardisation the AA by TLC based on Amino Acid Colour in the TLC Plates with Ninhydrin Spray Reagent

Some amino acids were impossible to distinguish by their Rf due to showing a sort of interference. Therefore, a new suggested way was performed to get a more appropriate separation of the Standard solutions of amino acids by examining the differences of the amino acid colors and shapes in the TLC plates with ninhydrin spray reagent. Ninhydrin is used to detect amino acids because they are colorless substances. Ninhydrin interacts with - amino acids, resulting in a rainbow of colors. Colors were observed visually, and the colour pictures of chromatograms by digital camera were recorded. Table 1 (in the supplementary material) was illustrated the color of the 20 standard amino acid. Colors were observed visually, and the color pictures of chromatograms by digital camera were recorded. Table 1 (in the supplementary material) was illustrated the color of the 20 standard amino acid. The newly designed Standardization of AA which was established to detect the twenty amino acids on thin-layer chromatography plates based on different strategies shown good demonstration throughout the Rf and producing various distinguishable colors.

Although TLC can be used easily for routine Analysis, this chromatographic system could be involved in a combination of ways, which might result in better performance.

It can be concluded that based on the results obtained from the simple fingerprint TLC analysis will be helpful in the identification and standardization of AA and can be utilized as a reference for the identification of AA disorder such as in IEM.

Clinical Application of Standardisation AA by TLC

Generally, Physicians in the public health service who wish to request any screening test for suspected IEM patients have to send it to an outpatient clinic because no higher performance methods are available locally. Because the clinical presentation of AA disorder/IEM is non-specific and might mimic common illnesses, definitive laboratory diagnosis is critical in validating clinical suspicion. Early diagnosis using

Table 1: Range of the retention factor for the 20 Amino acids

No.	*Amino acids*	*Range of RF*	*No.*	*Amino acids*	*Range of RF*
1	Glycine	0.34–0.50	11	Serine	0.40–0.48
2	Alanine	0.37–0.49	12	Threonine	0.45–0.55
3	Proline	0.38–0.61	13	Cysteine	0.50–0.63
4	Valine	0.53–0.59	14	Asparagine	0.41–0.48
5	Leucine	0.65–0.79	15	Glutamine	0.42–0.52
6	Isoleucine	0.62–0.72	16	Lysine	0.13–26
7	Methionine	0.62–0.76	17	Arginine	0.30–0.36
8	Phenylalanine	0.67–0.79	18	Histidine	0.39–0.48
9	Tyrosine	0.66–0.78	19	Aspartic	0.43–0.50
10	Tryptophan	0.65–0.79	20	Glutamic	0.50–0.55

Tandem Mass Spectrometry (TMS) in the expanded newborn screening program in developed countries, followed by early intervention during the presymptomatic or early symptomatic period, has been shown to improve the outcome of patients with IEM, which is only available outside and usually sent to a neighboring country (Jorden). In our case series, delayed diagnosis is common. This might be involved in the increasing numbers of IEM among children that death even before getting results.

Since we need to probably way forward for our country, the Standardisation of AA by TLC was applied in sera patients of suspected IEM. If the screening procedure is properly applied, the tentative diagnosis of Amino acids disorder might be possible on the same day of the test.

Samples of patients suspected of inborn errors of metabolism were analyzed for any amino acid disorder using TLC exactly same as the method of the standard of AA, and in the same time, in order to confirm the results of TLC, blood samples of some patients (12 cases) were sent for screening using Tandem Mass Spectrometry (TMS) to Amman/ Jorden. The results were back after 1-month. Samples of TLC run for separation of AA in serum and urine of suspected patients with IEM shown in Figure 3, 4 (in the supplementary material), and also TLC were run for samples of healthy control as shown in Figure 5, 6. Neither the blood samples nor the urine samples of the suspected patients have demonstrated any AA disorder compared to the standard AA's color and shapes. These patients' results for no AA disorder were confirmed by comparing the blood and urine sample of the healthy control groups and confirmed by patients' results of mass spectrum.

Knowledge Gap and Implications for Future Research

Early, undetected, and untreated IEM may result in disability, posing a significant economic hardship. Unfortunately, only a few hospitals in Iraq have built proper programs for IEM illnesses, notably Al-Kadhmiya Hospital. In Iraq, more research is needed to monitor and investigate the IEM. Consanguinity is the primary cause of the disorder in our region, so public education efforts through the media, schools, and universities are recommended to educate the public about the potential health concerns associated with marriage between close relatives. Genetic counselors also play an important role in educating and assisting parents and affected siblings in preventing the birth of another affected child in the future by exposing them to primary preventive methods such as prenatal diagnosis and Preimplantation Genetic Diagnosis (PGD). One efficient way to reduce IEM is for governments to enact policies that require every baby born to undergo a screening test. Physicians, scientists, lab technologists, and governments should promote training programs to compensate for the scarcity of specialist expertise in this field. As a final conclusion: IEM disorders must be diagnosed and treated as early as possible to prevent the unpleasure complication. Generally, to get the diagnosis, HPLC and MS MS must be done for the suspected patients; if they have an amino acid disorder, both methods are expansive, time-consuming, and available only in a few locations in Iraq. To exceed these adverse effects, this study suggested starting with TLC (easy, unexpansive, time effective), which could help screen the suspected patients most likely to have amino acid disorders. The more specific and sensitive methods (HPLC & MS) could be used only to prove the diagnosis.

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