

Correlation between Dried Blood Spot Thin Layer Chromatography and Plasma High Performance Liquid Chromatography of Leucine/Isoleucine Levels Among Filipino Patients with Maple Syrup Urine Disease (MSUD) seen at the Institute of Human Genetics, National Institutes of Health

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ABSTRACT

Introduction. Management of patients with maple syrup urine disease (MSUD) includes a low protein diet, supplemented with special formulas and constant monitoring of branched chain amino acids (BCAA). The gold standard for monitoring BCAA is plasma amino acid analysis using High Performance Liquid Chromatography (HPLC). In a developing country like the Philippines, however, the cost of this test is prohibitive to the majority of the patients. In our center, dried blood spot leucine/isoleucine (leu/ile) levels analysed by thin layer chromatography (TLC) is often used to diagnose and monitor these patients.

Objective. This study was done to determine the correlation of leu/ile levels using the two methods (TLC and HPLC).

Methods. A total of 46 MSUD patients were referred to the Biochemical Genetics Laboratory of the Institute of Human Genetics (IHG) from July 2001 to January 2004. Thirty five samples were obtained from 18 of these patients [some patients were seen at IHG more than once], and paired determinations of plasma amino acid using TLC and HPLC were made. The remaining samples were either hemolyzed or were not analyzed. The correlation coefficient [rho denoted as "ρ"] was estimated at a 95% confidence level using the Fisher's Z transformation.

Results and Conclusion. Of the 18 patients, 12 were males. The youngest was 1 day old and the oldest was 5 years old. The majority had the classical type of MSUD and dietary protein was restricted to between 0.6 gram/kg/day to 1 gram/kg/day of natural protein. Using the first pairs of observation for these 18 patients, the correlation coefficient was 0.76 (95% CI:0.462 to 0.907). This suggests a strong correlation between the two methods. It is recommended that further studies be done to determine the potential of the dried blood spot leu/ile level by TLC as an alternative method that can be used in the diagnosis and monitoring of MSUD patients especially in a developing country.

Key Words: Maple syrup urine disease, leucine, isoleucine, thin layer chromatography, high performance liquid chromatography

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Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive disorder that is caused by a defect in any of the subunits of the mitochondrial branched chain α ketoacid dehydrogenase (BCKAD) enzyme. The worldwide incidence of MSUD based on routine newborn screening data from 26.8 million newborns is approximately 1 in 185,000.¹ Although the precise birth incidence in the Philippines is unknown, 70 cases have been diagnosed from 1992-2008 (Padilla et al. personal communication).² This is the most common metabolic condition detected so far in the country.

Clinical manifestations are non-specific. Affected newborns present with lethargy, poor feeding and progressive encephalopathy. Diagnosis and management, if delayed, may cause irreversible brain damage and death. The gold standard for diagnosis is plasma quantitation of leucine, isoleucine, valine and allo-isoleucine using high performance liquid chromatography (HPLC). Management of affected patients includes lifetime dietary modification with supplementation of special MSUD formula and regular monitoring of plasma BCAA levels.

In a developing country like the Philippines, the cost of performing a plasma amino acid analysis is expensive for majority of the patients. Thus, there was a need to find alternative methods for the diagnosis and monitoring of these patients. This cross sectional study was done to determine the correlation of plasma BCAA levels using HPLC and dried blood spot leucine/isoleucine levels by thin layer chromatography (TLC) among Filipino MSUD patients.

Methods

Known cases of MSUD were ascertained through the Biochemical Genetics laboratory by thin layer chromatography, with elevated leucine/isoleucine levels in dried blood spots, or by the elevation of branched chain amino acid analysis in urine metabolic screening and plasma high performance liquid chromatography. Patient information i.e age, sex and protein intake at the time of sample collection was recorded. Samples for dried blood spot TLC and plasma

amino acid analysis by HPLC were collected at the same time (Figure 1). A 3.5 mL sample of venous blood was drawn aseptically from the patients. A small volume (0.5 mL) was blotted on the newborn screening filter card while 3.0 mL was collected in a heparinized tube. The latter specimen was centrifuged at 2700 rpm for 10 minutes to separate plasma. The plasma was stored in an ultra low freezer (-80°C) until the day that the plasma amino acids were run. Plasma amino acids were run monthly.

Separation of plasma amino acids via thin layer chromatography (TLC) was performed on cellulose TLC plates. Dried whole blood discs (3 x 3 mm) of standards and samples were placed in designated microtubes and extracted with 50 μ L of 70% ethanol. Aliquots of 20 μ L from the extraction solution were spotted 1 cm from the bottom edge of a 10 x 20 cm cellulose plate. The plate was placed in a developing tank where a solution composed of 35% acetone, 35% butan-1-ol, 10% glacial acetic acid and 20% distilled water was previously equilibrated for 30 minutes. This solvent was allowed to run on the plate for a period of 45 minutes to one hour or until the solvent front had reached one half centimeter from the top edge of the plate. The plate was air-dried for 30 minutes and then transferred to another tank with an equilibrated solution of the following constitution: 35% of 0.2% ninhydrin, 35% butan-1-ol, 10% glacial acetic acid and 20% distilled water. The plate was removed when the solvent reached the height traveled by the previous solvent. Color development occurred after air-drying for at least 15 minutes. Leucine/isoleucine eluted at approximately 0.86 Rf. The concentration of standards used for this study were 285, 375, 775, 1130, 1680, 1950, and 2375 μ mol/L of leucine/isoleucine. The intensity of the purple spot of the sample was compared with each standard for estimation of leu/ile concentration (Figure 2). Each plate was read by two medical technologists for initial reading and verification.³

Determination of plasma amino acid was done using the Shimadzu HPLC LC-10AT system where a unique post column derivatization detection unit was connected to an ordinary separation unit. This system used the Shim-Pack Amino Acid Series 6 mm x 10 mm Lithium-type column packed with a strong sulfonic acid cation exchange resin composed of styrene-divinylbenzene copolymer (gel type) of 5.0 μ m particle size as base. Protein removal for each sample was done by adding 1.0 mL of plasma to 50 mg of cooled sulphosalicylic acid (SSA). This was then centrifuged at 14000 rpm at 4°C after an hour of refrigeration. The free amino acid solution (supernatant) was transferred to a vial and diluted with the mobile phase A at a 1:1 ratio. The resulting solution was filtered with a PTFE 0.2 μ m filter then injected into the column. The amino acids were separated by means of a binary gradient elution method using two liquids, a standard solution (mobile phase A) consisting of 0.15 N lithium citrate with 7% methyl cellosolve adjusted to pH 2.6 with perchloric acid, and an eluent (mobile phase B) composed of 0.30 N lithium citrate and 0.20 M boric acid,

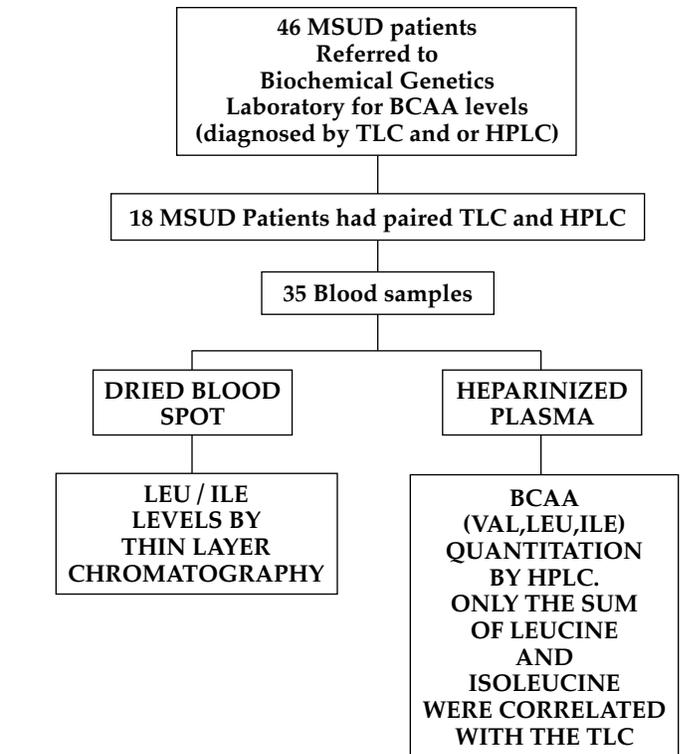


Figure 1. Paired 35 blood samples from 18 MSUD patients were analyzed for branched chain amino acid levels using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

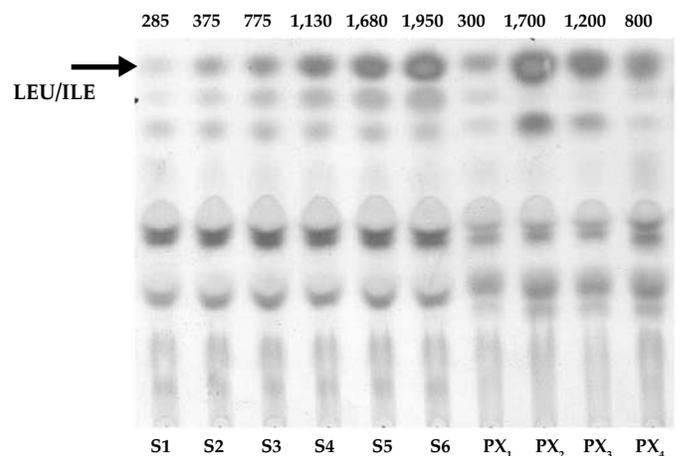


Figure 2. A sample thin layer chromatogram for leucine/isoleucine levels showing standards at increasing concentrations in μ Mol/L (from S1 to S6) and monitoring samples from MSUD patients (Px1 to Px4).

adjusted to pH 10.0 with 4M lithium hydroxide. The post column eluent was mixed with o-phthalaldehyde (OPA) reagent, and was fed to the detection unit which transmitted the data to the Class VP software, to enable quantitation. When arginine (the final peak) eluted, the column was automatically cleaned and equilibrated with a regenerator solution of 0.20M lithium hydroxide to prepare for the analysis of the next sample.⁴

Combined leucine/isoleucine levels on TLC were then correlated with the total sum of the leucine and isoleucine levels on HPLC. A scatter plot was generated for the 35 pairs of observations obtained from the 18 patients (Figure 3). Another scatter plot was generated only for the baseline sample from each of the 18 patients (Figure 4). To determine the strength of the association of the TLC method with the Plasma Method, the correlation coefficient was estimated at a 95% confidence level using the Fisher's Z transformation.

Results and Discussion

A total of 35 paired samples from 18 patients were analyzed. Among the 18 patients, 12 were males. The age ranged from 1 day old to 5 years old. The patients were on protein restriction at 0.6 gram per kg per day to 1 gram per

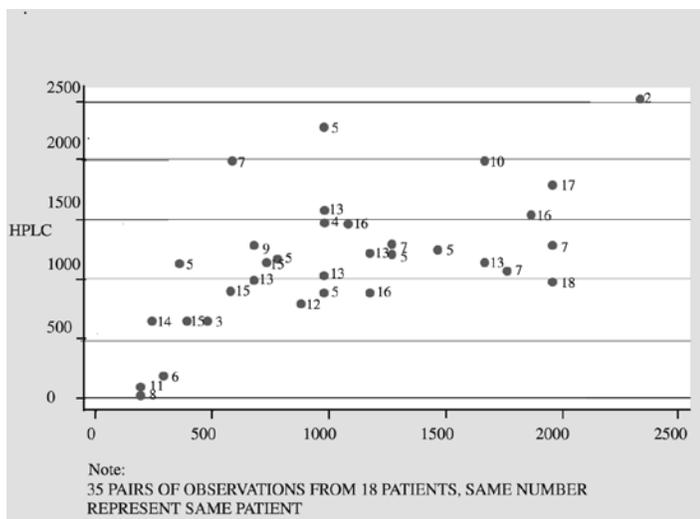


Figure 3. Scatter plot of the 35 pairs of observations obtained from 18 patients.

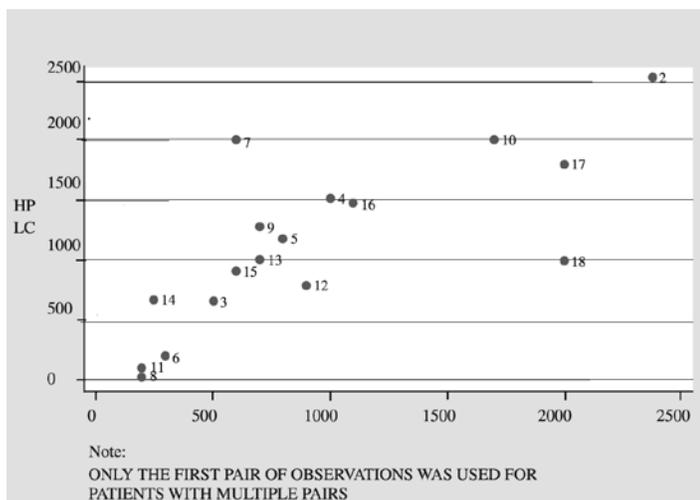


Figure 4. Scatter plot using 18 pairs of observations. The correlation coefficient, r is 0.7643 [95% confidence interval: 0.462-0.907]. These estimates were obtained using Fisher's Z-transformation.

kg per day of natural protein.

Figures 3 and 4 show the scatter plot of the 35 and 18 pairs of samples respectively. The correlation coefficient obtained from the 18 pairs was 0.76 [95% CI is 0.462 to 0.907]. This represents a strong association between the two methods.

This was an important finding since dried blood spot BCAA levels determined by thin layer chromatography could serve as an alternative and reliable method for diagnosing MSUD patients in a developing country. Ideally, for the subsequent monitoring of the branched chain amino acid levels of MSUD patients, quantitative plasma amino acid analysis by HPLC stays as the gold standard since it is essential to evaluate the complete amino acid profile for possible deficiencies needing amino acid supplementation and proper dietary adjustments. However, in a developing country like the Philippines, where most patients cannot afford to have plasma amino acid analysis done on a regular basis, monitoring of BCAA using TLC may be a useful guide for the dietary and medical management of these patients.

It is recommended that further studies be done on a greater number of samples to test the sensitivity and specificity of TLC in order to fully discover its diagnostic potential. Likewise, 2,4-dinitrophenylhydrazine (DNPH) testing, an easy and cheap tool for determining the presence of branched chain ketoacids in the urine and which is a useful indicator of a metabolic illness in MSUD patients, may be correlated simultaneously with TLC and HPLC for its monitoring potential in the local setting

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