Asian Journal of Medicine and Biomedicine
eISNN: 2600-8173
https://journal.unisza.edu.my/ajmb

PENERBIT Universiti Sultan Zainal Abidin

Asian Journal of Medicine and Biomedicine, Vol 3:2.

Original Article

Open Access

A Reliable Method Using In-house Prepared Reagents For Total Galactose Measurement As A Screening Tool For Galactosaemia

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Abstract

Galactosaemia is an autosomal recessive inherited disorder of galactose metabolism. Classical galactosaemia (CG), a disorder due to galactose-1-phosphate uridyl transferase (GALT) deficiency, is the most common and severe form in Caucasian population. In Malaysia, the incidence of Galactosaemia is still unknown. Currently, the diagnosis of galactosaemia in Malaysia is using commercialized kit which is expensive. The purpose of this studies is to establish and evaluate the method for total galactose (TG) assay utilising in-house prepared reagents and to diagnosis of classical galactosemia among high risk neonates and infants in Malaysia. The TG assay was based on the enzymatic end point assay using fluorometer measurement. Performance characteristics were determined for this method and reference range were established using dried blood spot samples from normal healthy babies. The validated TG assay was then used as a screening marker in 5313 neonates and infants with prolonged jaundice to make diagnosis of galactosaemia. The TG calibrators showed linearity up to 10000 µM while the precision coefficient variation (CV) was less than 10%. Limit of detection and quantitation were 56 and 176 µmol/L respectively. Glucose and ascorbic acids were found to have interference with TG assay. Comparison study of the TG assay with commercial kits showed a significant relationship ($p<0.05$). From this screening, two patients were diagnosed as CG, galactokinase or galactose-4-epimerase deficiency (n=5) and citrin deficiency (n=10). In conclusion, TG assay method was successfully established and validated in Malaysia and also serves as a confirmatory test for diagnosis of citrin deficiency diagnosis.

Keywords: Galactosaemia, Newborn screening, Galactose-1-phosphate uridyl transferase, Total Galactose, Fluorometry

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Received (April 6th, 2019), Accepted (Oct 2nd, 2019) & Published (Oct 31st, 2019)
Cite as: Affindi, O., Zakiah, Z., Zabedah, M.Y.,& Julaina, A.J.(2019). A Reliable Method Using In-house Prepared Galactose Measurement ass a Screening Tool for Galactosemia. *Asian Journal of Medicine and Biomedicine , 3(2): 52-58*

Introduction

Galactosaemia is an autosomal recessive inherited disorder where the human body is unable to use or metabolise galactose due to enzyme deficiency in galactose metabolism. There are three types of enzyme deficiency; (i) Classical galactosaemia due to deficiency of galactose-1 phosphate uridyl transferase (GALT), (ii) galactokinase deficiency and (iii) uridin diphosphogalactose-4'-epimerase deficiency (3, 4). Classical galactosaemia is the most common and severe form with incidence of 1:20,000 among Caucasian population (2). Deficiency of GALT leads to unable to convert galactose-1-phosphate to galactose which it will accumulate in the blood and damage to the liver, brain, kidneys and eyes due to toxicity of galactose-1-phosphate (3). Infants with galactosaemia can develop symptoms in the first few days of life once they are fed with milk. The symptoms may begin with poor feeding, vomiting, lethargy and jaundice and may proceed to failure to thrive, hepatomegaly, liver dysfunction and septicaemia if feeding with the milk is continued (3). Early diagnosis and treatment by introducing lactose-free diet during neonatal period helps to prevent clinical manifestation and allow normal growth development in affected neonates (3, 4). Therefore, it is important that all newborns are screened for galactosaemia through newborn screening as early as before any ingestion of galactose-containing formula or breast milk (1).

The incidence of classical galactosaemia varies with ethnicity. For example, the risk in Japan is 1:73,700 (1) as compared to Ireland 1:10,000 – 20,000 (2). However, the incidence in Malaysia is still unknown. The risk of neonatal death is very high (almost 75%) if left untreated. Affected children can have serious, irreversible effects or even die within few days after birth. The combination of total galactose (TG) level and GALT assays are useful for diagnosis of classical galactosaemia. There are other established methods for determination of TG such as qualitative Beutler test (9), using fluorescence nicotinamide adenine denucleotide NAD^+ (5, 6, 7) and tandem mass spectrometry (TMS) (8). However, the use of high technology equipments such as Technicon autoanalyser or TMS are unlikely to be utilised for routine screening test because of the high operational cost, expensive equipment, reagent and the requirement for skilled personnel (6, 8). Currently, the laboratory diagnosis of galactosaemia in Malaysia utilises either urine reducing sugar, qualitative TG or GALT assay using dried blood spot and commercialized kit for affected neonates with symptoms such as prolonged jaundice and hepatomegaly. These tests are neither specific nor sensitive and expensive to be used as a screening method for the detection of galactosaemia in neonates (19). The purpose of this study is to evaluate and establish spectrofluorometry method for TG assay using in-house prepared reagents as screening tool for galactosaemia in Malaysia.

Materials and Methods

Subjects

Normal healthy subjects were recruited from few healthy staffs from our laboratory whom had given their consent that the blood taking to use for this study. The blood will be used for calibrator and internal quality control (IQC) preparation as well as for method validation. 30 ml of whole blood was collected in ethylene diaminetetraacetic acid (EDTA) tube from normal subject (n=6) and pooled together before divided into three parts of 10 mL each respectively. The first part of pool blood was used for the preparation of calibrator and linearity study. The second part was used for the preparation of internal quality control and study of precision, accuracy and detection limits and the last part was used for as samples for interference study. For the preparation of dried blood spot (DBS), about 70 µL of blood was spotted on each circle plot on Whatman 903 filter paper and left dried overnight at room temperature. Over the period of this study, a total of 5313 DBS samples were collected from babies from all over Malaysia for screening of inherited metabolic disorders with clinical history of jaundice and/or hepatosplenomegaly and/or with juvenile cataract (confirmed by ophthalmologist). Babies whom were confirmed diagnosis of glucose-6-phosphate dehydrogenase deficiency (G6PD) and fructose dehydrogenase deficiency were excluded. Samples of DBS from 156 asymptomatic neonates aged less than 18 days, were selected for the establishment of reference ranges for TG. Informed consents (written form) were obtained for each individual.

Samples collection

A volume of 2 ml of blood was collected from each subject, mixed appropriately in EDTA tube and 70 µL were then spotted on Whatman 903 filter paper (Whatman Inc, USA). Blood spot cards were left at room temperature until fully dried and sent to lab for further analysis. Consent was obtained from parent or guardian prior using the samples for this study. The DBS samples were analysed in duplicate. This study was approved by the Medical Research and Ethics Committee (MREC), Ministry of Health (Ref: (5)dlm.KKM/NIH-SEC/08/0804/P06-29) which complies with the Declaration of Helsinki.

Reagents

Galactose, galactose-1-phosphate, human serum albumin, human gamma globulin, trizma hydrochloride, nicotinamide adenine denucleotide (NAD) used in this study were manufactured by Sigma (United States) while alkaline phosphatase and galactose dehydrogenase were manufactured by Roche Diagnostic GmbH (Germany). Sodium chloride, methanol and acetone were manufactured by Merck (Germany). All materials, reagents and chemicals were obtained from stated suppliers and used directly unless mentioned otherwise. Commercial Total Galactose kit was purchased from Perkin Elmer (Turku, Finland)

Preparation of calibrators in DBS

This study was carried out at the Biochemical Genetics Laboratory, Biochemistry Unit, Specialised Diagnostic Centre, Institute for Medical Research (IMR). Erythrocytes from normal subjects were washed three times with saline solution and the haematocrit level was

adjusted to 55% by using diluent buffer containing of human serum albumin (40 g/L), human gamma globulin (25 g/L), Tris hydrochloride (20 mM) and sodium chloride (150 mM). A series of concentrations of total galactose standard containing galactose and galactose-1-phosphate between 0 and 6000 µM were prepared. A volume of 2.5 ml of each concentration of TG standard was spiked into 5 ml of washed erythrocytes. About 70 µL of these calibrators was spotted on each circle of Whatman 903 filter paper and was dried overnight at room temperature then stored in a zippered plastic bag at -30°C.

TG assays

The method used in this study was based on the published method by Yamaguchi et al (7). A spot of 3 mm in diameter of the DBS sample was punched into a well of clear 96-well microtitre plate. A volume of 20 µl of methanol:acetone solution (1:1 v/v) was added to each well. The plate was incubated at 37°C for 30 minutes and later 200 µl of buffer containing trizma hydrochloride 77 mmol/L, pH 8.6 with NAD 17.6 µmol/L with 991 U/ml of alkaline phosphatase and 0.308 U/mL of galactose dehydrogenase was added to each well. The plate was covered with plate-sealer and incubated for 60 minutes at 37°C. The upper layer was then transferred into a black microtiter plate. Fluorescence product was measured at excitation of 355 nm and at emission of 460 nm using Victor 1420 spectrofluorometer (Turku, Finland). A calibration curve was constructed using calibrator with series of concentration from 0 to 3000 µmol/L.

Method validation

Limit of detection and quantitation

Determination of the limit of detection and quantitation was conducted by assaying 10 blank samples and 10 low level value samples in duplicate in a single run. Both levels of samples were taken from calibrator with estimated 0 µM and 188 µM respectively

Linearity

Linearity curves were constructed by performed a serial dilution of the calibrator with concentration of 20000 µM and plotted using the measured values against the expected values of each dilution. All levels were tested in duplicate and the average of mean was taken.

Precision

Repeatability and reproducibility were determined at four levels of concentration: baseline (~150 µM), low (~500 µM), medium (~1000 µM) and high (~2000 µM). Each level was assayed in duplicate for 10 days over a period of one month.

Accuracy

An accuracy was determined by doing assay on DBS samples with known concentration obtained from Centre for Disease Control (CDC) Atlanta. The percentage of bias was calculated.

Inter Laboratory Comparison

The inter laboratory comparison was carried out through participation in the proficiency testing program conducted by CDC. Five DBS samples were received and were analysed using our in-house method in the laboratory.

Method comparison with a commercial Perkin Elmer's kit for TG

The commercial kit measures TG using a fluorescent galactose oxidase method. The fluorescence was measured using excitation wavelength of 340 nm and emission wavelength of 405 nm. The lowest and highest calibrators for the kit were 0 and 2220 µM respectively. The commercial assay was performed according to the manufacturer's instructions. A total of 154 DBS specimens with various concentration of TG from low, medium and high, were assayed in parallel using both in-house reagents and Perkin Elmer's kit. Linear regression analysis was used to determine the relationship between the conventional method and commercial kit. The strength of linear relationship was determined by using the value of Spearmen's rank correlation.

Interference study

Various concentration levels (100 – 6000 mg/dL) of glucose, raffinose, mannose, fructose, maltose, glutathione, sucrose, ascorbic acids and xylulose standards were spiked into the whole blood containing galactose before spotted onto the filter paper and analyzed using in-house method. Each interfering substance was grouped according to the concentration level.

Statistical analysis

The assessment of normality or parametric data was based on Kolmogorov-Smirnov or Shapiro-Wilk test, skewness, kurtosis and population's histogram. For interference study, ANOVA (parametric) with post hoc test (Tukey or Dunnett) or the Mann-Whitney U-test (non-parametric) was used to compare variables between groups. For the reference range determination, 5^{th} to 95^{th} percentile was used for non-parametric data. An upper cut off value for TG assay was calculated from 99^{th} percentiles (non-parametric). Statistical analysis for the comparison of two methods using Bland-Altman plot & Passing Bablock regression analysis were conducted using the Statistical Program Social Sciences (SPSS) (Chicago, IL).

Results

Method Validation

Table 1 showed results for validation of TG assay. The TG assay was observed to be linear up to ~ 10000 micromole (µM). TG assay was performed a good precision for both repeatability and reproducibility, CV 8.3% and 6.6% respectively). The limit of detection was 53 µmol/L and the limit of quantitation was 176 µmol/L. The mean bias was +8.9% and the recovery was between 95.4% - 108.3% with a mean value of 100.7% ± 6.8%. The participation in the inter laboratory comparison showed satisfactory performance. The sensitivity and specificity of TG assay were 100% and 99.9% respectively.

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eISNN: 2600-8173
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Table 1: Validation results for TG assay

Parameter	Total galactose assay				
Working linearity operating range	Non linear				
Precision (CV%)	Base line	Low	Medium	High	Average
-Repeatability	13.3	8.2	7.0	4.6	8.27
-Reproducibility	11.4	5.4	3.7	6.0	6.61
Limit of detection	56 µmol/L				
Limit of quantitation	176 µmol/L				
Accuracy (%)	Base line	Low	Medium	High	Average
-Bias	10.2	3.4	13.5	8.5	8.9
-Recovery	-	95.4	98.4	108.3	100.7
Inter laboratory comparison	CDC 10 (C3 and C4)			Results and interpretation given were 100% satisfactory	
	CDC 11 (C1, C3 and C4)				
	CDC 12 (C1, C3 and C4)				

Method comparison for TG assay

Passing-Bablock regression analysis are shown in Figure 1. The Spearmen correlation coefficient is 0.63 ($p < 0.05$), [95% CI 0.53 - 0.72]. Bland-Altman plot showed mean bias of -71.2 (95% CI: -135.9 to -6.4). (Figure 2).

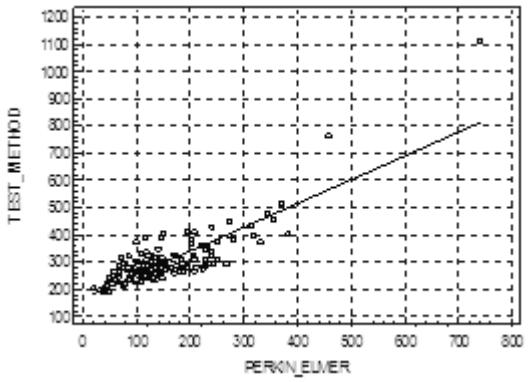

Fig 1: Correlation graph between our method and commercial kit by Perkin Elmer

y axis: Our method
x-xis: Perkin Elmer's method

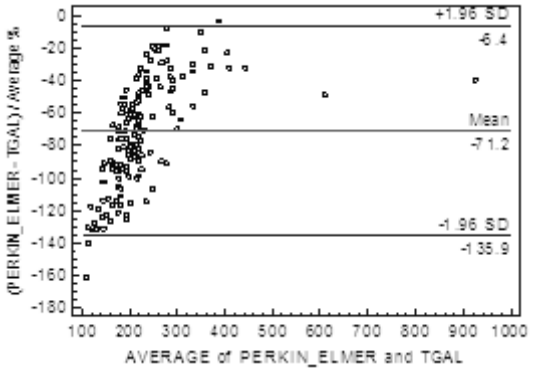

Fig 2: Bland-Altman Plot

y axis: Difference between our method and Perkin Elmer's method
x axis: Average of our method and Perkin Elmer's method

Interference study

Glutathione, sucrose, fructose, xylulose, maltose, raffinose and mannose were not found to interfere with the assay at ranges of concentration 88 – 200 µmol/L. High concentration of glucose was noted to cause reduction in measured TG concentration especially at the concentration of 555 µmol/L which significantly reduced TG level to 62% ($p=0.001$). Meanwhile ascorbic acid at a concentration of 570 µmol/L ($p<0.05$) was noted to increase TG level up to 14% and at concentration of 11360 µmol/L increased TG level up to 838%.

Reference ranges and cut off

The distribution of TG levels among the normal neonates was observed to be non-Gaussian pattern. The reference range for TG assay taken at 5^{th} – 95^{th} percentile was 13 – 331 µmol/L. The upper cut off was established at 528 µmol/L at 99^{th} percentile.

Screening of jaundiced babies using TG

The finding results obtained from the screening of jaundiced babies are shown in Table 2. The mean value for TG in these babies was 123.36 µmol/L (95% CI 115.67 – 131.06 µmol/L). Apart from 5313 samples received during study period, only 17 patients showed abnormal elevation of TG.

Table 2: Patients with increase total galactose and their final diagnosis

Patient	Age	Symptoms	TG[1]	GALT[2]	Newborn Screening (Citruline)[3]	Diagnosis
#1	Day 19	Jaundice, Hepatosplenomegaly	8387	2.1	-	Galactosemia (GALT deficiency)
#2	4 months	Jaundice, Seizures, Dystonia, Hypotonia, Kernicterus	2253	2.2	48	
#3	4 months	Jaundice, Hepatosplenomegaly	3087	8.8	34	Liver dysfunction/ Galactosemia (Epimerase deficiency)
#4	5 months	Jaundice, Hepatosplenomegaly, Ascites	1868	8.2	29	
#5	7 months	Jaundice, Hepatosplenomegaly, Abdominal distension, Pallor	1819	4.0	29	
#6	3 days	Asymptomatic	1291	9.8	11	
#7	Day 4	Hypotonia, Dysmorphic features, Optical atrophy	955	3.0	5	
#8	2 months	Jaundice, Hepatosplenomegaly	2530	7.0	403	Citrin deficiency
#9	2 months	Jaundice, Hepatosplenomegaly, Neonatal hepatitis	632	6.1	93	
#10	5 months	Prolonged jaundice, Hepatomegaly	1953	6.6	135	
#11	2 months	Jaundice, Hepatosplenomegaly	8151	9.3	394	
#12	3 months	Jaundice, Hepatomegaly	7050	8.3	129	
#13	2 months	Jaundice, Hepatomegaly, Failure to thrive	7184	10.7	281	
#14	2 months	Jaundice	9362	4.2	89	
#15	4 months	Jaundice, Hepatomegaly, Dysmorphic features	3331	4.2	123	
#16	1 month	Prolonged jaundice	7060	2.2	179	
#17	Day 34	Fever, Jaundice, Smelly urine, Septicemia	3778	4.8	60	

Remarks

(1) TG Cutoff: <528 µmol/L

(2) GALT Cutoff: >2.4 U/g Hb

(3) Citruline Cutoff: <41 µmol/L

Discussion

Evaluation of TG assays

Overall performance showed that our in-house method is precise with CV of less than 10 % at all levels of concentration. The bias was less than 10% and recovery was within 90 – 110%. TG assay results showed 100% score in the participation of inter laboratory comparison scheme. TG assay shown a higher specificity and significant higher sensitivity as these two parameters are important to assess the accuracy of a diagnostic test.

Interference study for TG assay

From the study, we found that most of the analytes did not interfere with the assays including glutathione which has been shown to interfere with the TG method (10, 11). However, glucose and ascorbic acid have a significant interference in the assay. This could be explained by similarity of chemical structure between glucose and galactose except for the hydrogen molecule in carbon number 4. Glucose and galactose compete for the active site of galactose dehydrogenase enzyme during the reaction process which leads to decrease in TG concentration, as less NADH was produced in the assay. (12). This finding was also reported by William *et. al* (12). In this study, ascorbic acid was found to interfere with the assay by increasing the level of TG. This may be due to the role of ascorbic acid as a reducing agent and strong antioxidant. This assay is measuring the concentration of NADH as an indirect measurement of TG, therefore, increase NADH would mean increase in TG concentration.

Method comparison for TG assay

Study on method comparison was done to identify and estimate systematic errors in that particular method. In this study, we compared in house TG assay with commercial kit. Spearmen coefficient showed non-parametric population in normality test of Kolmogorov-Smirnov. The value of Spearmen coefficient showed that a moderate correlation between the two methods. Moreover, Deming regression demonstrated there was a constant systematic error of 165.4 µmol/L for our method compared with the kit. The value of coefficient of determination, R^2 = 0.685 elucidated that at least 68.5% of measurement made by our method is explained by measurement made with the kit. Good correlation between the two methods were noted in low and medium concentration while poor correlation was detected in the high concentration of TG. Bland-Altman plot was used to assess this agreement and gave confident results which is not being explained well by the correlation graph. Higher correlation does not mean both methods agreed with each other therefore coefficient of determination was used to explain the strength of correlation of both methods and not the agreement between them (13). Bland-Altman plot between two methods showed that most of the data were distributed equally within range. However, the mean difference between two methods showed a bias of -71.2 µM which required establishment of our own range from local population Reference ranges and cut off

Our data of TG assay among normal population were found to be non-parametric distribution. Therefore, the percentile approach was taken to establish our reference range and upper cut-off levels. When compared with reports in Germany and United States (14), our cut-off was established at a higher level. This could be likely because classical galactosaemia is more common among the Caucasian population compared with Asian population (2). Higher cut off would simply reduce the possibility of false positive when screening within the Malaysian population.

Screening for jaundiced babies

Among 17 abnormal cases identified during screening for jaundiced babies, classical galactosaemia was diagnosed in two patients, epimerase deficiency (n=5) and citrin deficiency (n=10). Classical galactosaemia is characterised by low activity of GALT and elevation of TG. One patient has a homozygous mutation at intron 4 (c377+2dup) and located close to the donor splice site of intron 4 which influenced the splicing process that resulting in inactive enzymes (15). The other patient is still unknown or unidentified (lost to follow-up). In practice, suspected patients are treated with lactose restricted diet (17). There is a high number of cases being reported to have associated citrin deficiency. TG in patients with citrin deficiency was elevated whereas the GALT activity was within the normal limits. This phenomenon is described by Naito et al (16), Berry (17) and Timson (18) as hypergalactosaemia. The galactose level will usually return normal once the patient is being treated.

Conclusions

In conclusion, the in-house prepared reagent method for TG assay developed in our lab were successfully being established and validated. It was proven to be effective and reliable to be used for galactosemia screening especially to differentiate classical galactosaemia with other types of galactosaemia and as a supportive test to confirm citrin deficiency. In addition, it is relatively cost effective (40 sen compared RM 3.65 per sample), provides fast results and comparable among other labs with high accuracy. We also revealed that glucose and ascorbic acids could interfere with the TG assay. Therefore, it is highly recommended that patients should be restricted from taking any vitamin C drugs prior to sample collection. We have strongly proven that this method can be used for screening of classical galactosaemia in Malaysia.

References

1. Wada Y, Nakajima H, Irie M, Hirayama M, Suwa S. Mass Newborn Screening in Japan – 1984. *Japan Journal of Human Genetics* 1984, 29:277 – 286.
2. Honeyman MM, Green A, Holton JB, Leonard JV. Galactosemia: Results of the British pediatric surveillance unit study, 1988 – 1990. *Archive of Disorder Child* 1993, 69: 339 – 341.
3. Berry GT, Segal S. Disorders of Galactose Metabolism. In *The Metabolic & Molecular*

Bases of Inherited Disease. *Volume 1*. 7th edition. Edited by Schiver CR, Beaudet AL, Sly WS, Valle D. New York, McGraw –Hill Medical Publishing; 1995: 967 – 1000

4. Bosch AM. Classical galactosaemia revisited. *J. Inherit. Metab. Dis*. 2006, 29: 516 – 525
5. Rhode H, Elei E, Taube I, Podskarbi T, Horn A. Newborn screening for galactosemia: Ultramicro assay for galactose-1-phosphate-uridyltransferase activity. *Clinica Chimica Acta* 1998, 274: 71 – 87.
6. Fujimura Y, Ishii S, Kawamura M, Naruse H. Microdetermination of galactose and galactose-1-phosphate in dried blood spots. *Analytical Biochemistry* 1981, 117: 187 – 195.
7. Yamaguchi A, Fukushi M, Mizushima Y, Shimizu Y, Takasugi N, Arashima SI, et al. Microassay for Screening Newborns for Galactosemia with use of a fluorometric microplate reader. *Clinical Chemistry* 1989, 35(9): 1962 – 1964.
8. Simonsen H, Jensen UG, Brandt NJ, Christensen E, Skovby F, Norgaard-Pedersen B. Neonatal Screening for Galactosemia by Quantitative Analysis of Hexose Monophosphates Using Tandem Mass Spectrometry: A Retrospective Study. *Clinical Chemistry*. 2001, 47 (8): 1364 – 1372.
9. Beutler E, Baluda MC. A simple spot screening test for galactosemia. *J. Lab. & Clin. Med*. 1966, 68 (1): 137 – 141.
10. Kawamura M, Fujimura Y, Naruse H. Simultaneous Quantitative Estimation of Galactose-1-Phosphate and Galactose in Blood for the Diagnosis of Galactosemia. *Tohoku J. Exp. Med*. 1982, 137: 289 – 295.
11. Misuma H, Wada H, Kawakami M, Ninomiya H, Shohmori T. Galactose and galactose-1-phosphate spot test for galactosemia screening. *Clinica Chimica Acta* 1981, 111: 27 – 32.
12. Williams CA, Phillips T, Macdonald I. The influence of Glucose on Serum Galactose Levels in Man. *Metabolism* 1983, 32 (3): 250 – 256.
13. Bland JM, Altman DG. Statistical Methods for Assessing Agreement Between Two Methods of Clinical Measurement. *Lancet* 1986, i: 307 – 310.
14. Freer DE, Ficicioglu C, Finegold D. Newborn Screening for Galactosemia: A Review of 5 Years of Data and Audit of a Revised Reporting Approach. *Clinical Chemistry* 2010, 56 (3): 437 – 444.
15. Choy YS, Breda Klobus A, Zabedah MY, Endo F, Rolfs A. A new variant of classical galactosemia due to a novel mutation in the GALT gene. *J. Inherit Metab* 2012, 35 (Suppl 1): S79
16. Naito E, Ito M, Matsura S, Yokoya I, Saijo T, Ogawa Y, et al. Type II citrulinaemia (citrin deficiency) in neonate with hypergalactosemia detected by mass screening. *Journal of Inherited Metabolism Disorder* 2002, 25: 71 – 76.
17. Berry GT. Galactosemia: When is it a newborn screening emergency? *Molecular Genetics and Metabolism* 2012, 106: 7-11
18. Timson DJ. The molecular basis of galacrosemia – Past, present and future. *Gene* 2016, 589: 133-141.
19. Coelho A.I., Rubio-Gozalbo M.E., Vicente J.B. & Rivera I. Sweet and sour: an update on classic galactosemia. J Inherit Metab Dis. 2017, 40:325-342