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Quantitative Bacterial Micro-Assay for Rapid Diagnosis of Galactosemia: Application in Galactosemia Neonatal Screening

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Abstract

In the present study a new economic and rapid bacterial micro-assay for simultaneous detection and quantitative measurement of serum galactose was developed. Analysis of the standard curve showed a linearity range for galactose from 2 mg/dL to 180 mg/dL with a regression equation of $Y = 0.013X - 0.083$; $R^2 = 0.962$. The advantage of the method is its ability to measure serum galactose quantitatively. The cost per sample is about 20-50 cents, which is much less than HPLC and enzymatic commercial kits. The method can be automated, which is suitable for galactosemia neonatal and mass screening especially in developing countries in which funding is a limiting factor.

Keywords: Galactose; Galactosemia; Bacterial micro-assay; Neonatal Screening

Introduction

Galactosemia (OMIM 230400), is caused by deficiency in the enzyme galactose 1-phosphate uridyl transferase (GALT3; EC 2.7.7.12), which is an important key enzyme in the Leloir pathway of galactose metabolism [1, 2, 3]. The disease is inherited in an autosomal recessive manner [1]. The affected infants usually born normal, but symptoms start developing after milk feeding [3]. In this disorder, ingestion of milk causes accumulation of galactose in the blood and urine, leading to high intracellular concentrations of galactose 1-phosphate (gal-1-P). Gal-1-P is considered toxic for several tissues, especially the liver, brain, and renal tubules [1]. Clinical manifest-tations of the disease appear shortly after ingestion of milk, predominantly as gastrointestinal, hepatic, neurological symptoms, and lethargy. If treatment is not initiated early, patients become comatose, and death often occurs during the first week of life as a result of hepatic or renal failure [4, 5 and 6]. Early treatment with a dietary of galactose/lactose restriction causes regression of symptoms and signs within 1 or 2 weeks [7-10]. Rapid and in time diagnosis of galactosemia in affected infants can help preventing the progression of mental and developmental disorders associated with the disease. There are currently a number of methods for the quantitative and qualitative determination of galactose in the blood [11-13]. The most common methods used are enzymatic colorimetric, Paigen, a biological inhibition assay, and Beutler, a fluorescent method [14-16, and 20]. Prospective screening of newborns with galactosemia is widely accepted procedure throughout the world. However in most of the developing countries, especially those with limited health budget, neonatal mass screening programs such as galactosemia are

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basically delayed or ignored.

Quantitative measurement of galactose is necessary in positive cases to confirm the disease and to omit the false positive results as well as to determine the type of galactosemia (Classic form or Duarte form) [17-18]. This is very important for both molecular analysis of the disease and selection of the therapy procedures. HPLC and enzymatic assays which are usually used after thin layer chromatography, TLC, or Gatheri bacterial inhibition assay, GBIA, for quantitative measurement of galactose, are excellent approaches, but are very expensive and not suitable for mass population screening [12-14]. Therefore, always an economic, simple, and rapid method is favored. GBIA has been a widely accepted inexpensive method for analysis of serum galactose. However, usually a second method is required for quantitative measurement of galactose and to rule out the false positive results, which naturally occur using the GBIA. Taking advantage of the GBIA method, we have developed a new economic, rapid and inexpensive bacterial micro-assay for simultaneous detection and quantitative measurement of serum galactose.

Materials and Methods

Bacterial Strains, Media and Reagents

Bacillus cereus and *Proteous mirabilis*, facultative anaerobic bacteria were used as the bacterial strains in the present study. These strains were obtained from Microbiology Department of University of Isfahan, Isfahan, I.R. Iran. Both bacterial strains were negative for galactose utilization which was confirmed using the galactose utilization test as described previously [18].

The culture medium for maintaining the bacteria contained yeast extract (3g/L), NaCl (5g/L) and Na_2HPO_4 (1g/L). Agar plates were prepared by the addition of 1.5% Bacto agar (Difco, Detroit, MI, USA) to the culture medium before autoclaving. To prepare MSB (Mineral Base Salt) Medium , KH_2PO_4 (1g/L), $KHPO_4$ (1g/L) and $(NH_4)_2SO_4$ (1g/L) were autoclaved, then added to autoclaved stock solutions of $FeCl_3$ (0.001g/L) and $MgSO_4$ (0.002g/L).

Di-Nitro Salicilic Acid (DNS) reagent at 1% concentration was made up DNS (10 g/L), NaOH (16g/L) and KNaC4H4O6 (sodium potassium tartarate) (250 g/L). For preparing of the 1% galactose medium, 1g Galactase were added to 100 mL MSB medium and then autoclaved. All chemical were prepared from analytical grade which were purchased from Merck, Germany.

Bacterial Culture

The bacteria were spread onto agar plates containing the culture medium and incubated at 37 °C overnight to produce single colonies. A single colony was inoculated into 2 ml MSB medium and incubated at 37 °C and 150 ×g in a thermo-mixture (Eppendorf, Hamburg, Germany) for 2 hours. This bacteria suspension was then used to assay serum galactose.

Serum Samples Preparation

Control healthy blood was obtained from blood transfusion centre of Isfahan. After incubating for 30 min at 25 °C, the blood samples were centrifuged at 1800 ×g for 5 min to get clear solution of serum. Working galactose standard solutions containing 2, 4, 8, 10, 16, 20, 32, 40, 60, 120 and 180 mg/dL were prepared immediately before the assay by diluting a stock solution of 600 mg/dL galactose. The galactose concentrations were then added to normal serum samples, to prepare different serums with galactose.

Experimental Design

B .cereus, *P. mirabilis* and a mixture of the suspension of the bacteria in 1:1, V/V, were applied for assaying the galactose in the serum samples. Different concentrations of the galactose in MSB medium were prepared as mentioned above in 100µL MSB medium, then 100µL of normal serum was added. Finally, 50 µL of the suspension of each bacterial culture and the mixture of suspension of both bacteria were added separately into 1.5 mL eppendorf tubes. The tubes were incubated for 5 h at 37 ºC in 150 × g shaking incubators (Eppendorf, Hamburg, Germany). After incubation, the content of the tubes were centrifuged at 3000 × g for 5 min to sediment the bacterial cells. Then, 200 µL of the clear supernatant was transferred into new micro-tubes containing 50 µL fresh DNS 1% reagent. The contents of the tubes were briefly mixed by flicking, and were then placed for 5 min at 100 ºC water bath. The presence of the galactose in the medium causes the color to change to brown, which can be measured spectrophotometrically at 540 nm. The different concentrations of galactose samples were plotted against the corresponding optical density and the linearity of the standard curve was analyzed. The standard curve was calculated by a linear regression or a weighted linear regression function.

Optimization of Bacterial Incubation Time

100 µL of *B.cereus* suspension culture was added to serum sample with 180 mg/dL galactose concentration.

Then the tubes were immediately incubated at 37 °C with 150 ×g shaking in a thermo mixture. After different incubation times, the OD_{540nm} of the medium was measured as described above.

Precision and Sensitivity

The precision of the assay was determined by analysis of intra- and inter-assay variations. Intra-assay variation was determined using 10 replicate of three samples of 4, 60 and 180 mg/dL galactose concentration. The inter-assay variation study involved analysis of the above samples in 10 different assays. The detection limit of the assay was determined on serum samples with different concentrations of galactose from 2-180 mg/dL.

Statistical Analysis

The results were analyzed using Students T-test, and reported as mean ± SD. Regression lines were plotted for the data from three separate assays using SPSS 14.5 software (SPSS Inc., Chicago, IL, USA).

Results

As presented in Figure 1, the linearity of standard curve for the serum galactose ranging from 2-180 mg/dL was analyzed. According to the Figure, the regression equations and R squares were Y=0.013X-0.083; R^2 =0.964, Y=0.01X- 0.014; R^2=0.898, Y= 0.009X – 0.155; R^2=0.859 for *B.cereus, P.mirabillis* and a mixture of both bacteria, respectively. As shown in Figure 1A, R^2 for *B.cereus* is about 0.962, which is higher than that for *P.mirabillis* and/or a mixture of both bacteria, resulting in a more linear curve. Therefore, *B.cereus* was selected for the micro-assay and used thought this study.

The precision and sensitivity of this assay was determined by analysis of intra- and inter-assay variations. Table 1 presents precision data for the galactose micro-assay test. As shown in this Table, the micro-assay introduced in the present study is capable of measuring a relatively wide spectrum of possible concentrations of serum galactose, from normal to highly elevated levels, with suitable accuracy and precision.

Given the high precision and sensitivity of the micro-assay, the long term application of bacteria was investigated. To investigate the capability of the micro-assay to be used for long-term studies, ambient storage, and automation, the bacterial strains were lyophilized and then the lyophilized powder was utilized for assaying the serum galactose. The data demonstrated that the lyophilized bacteria also produced similar results as compared to the bacterial suspensions.

The optimum time for bacterial incubation with respect to the speed of diagnosis of galactosemia by the micro-assay was examined. The OD_{540nm} of the culture media containing 180 mg/dL serum galactose was

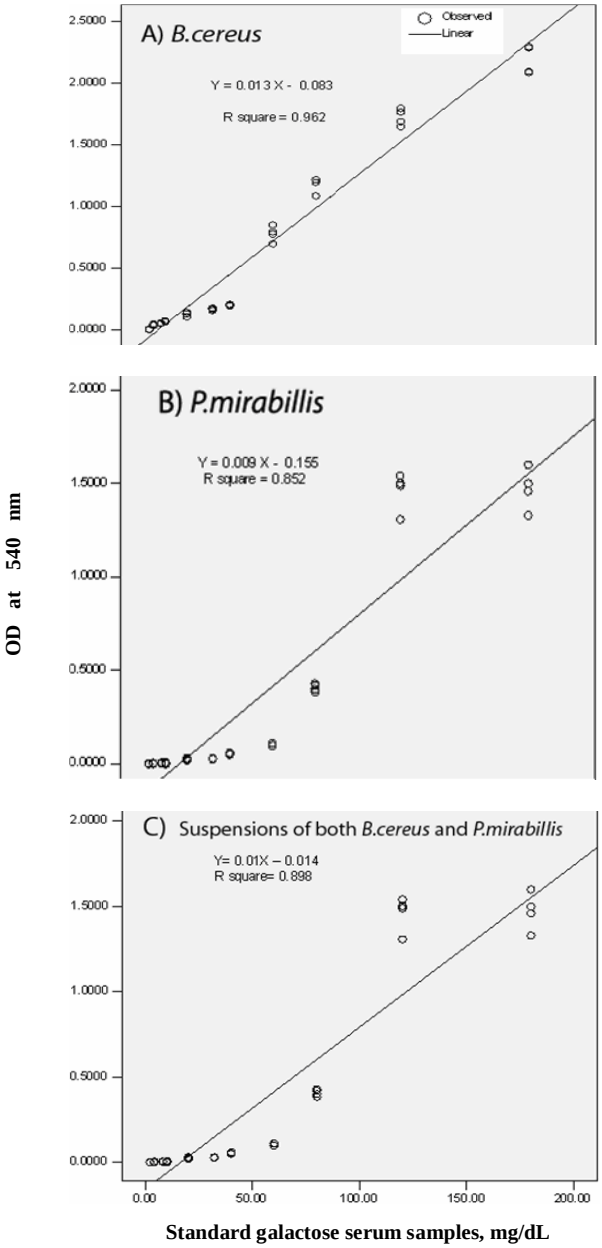

Figure 1. Standard curves for quantitative measurement of serum galactose. A) *B.cereus*; B) *P.mirabillis* and C) Suspensions of both *B.cereus* and *P.mirabillis*. The curves were plotted based on spectrophotometeric measurements of bacterial cultures at 540 nm.

measured every one hour after the time of bacterial incubation for 12 hours (Fig. 2). Using the micro-assay, it was possible to determine the exact concentrations of the galactose in the serum samples. This could facilitate the classification of the samples as normal, Duarte and classical forms of galactosemia.

Discussion

All current methods such as HPLC and TLC, require special reagents with limited stability, involving multi-step sample preparation procedures, and are expensive and time-consuming. Thus, an accurate, inexpensive and economical system for rapid diagnosis of galactosemia with least false positive results is favored in newborn screening programs. In the present study, a novel method of screening for galactosemia is introduced. This is the first study that introduces a new, rapid and inexpensive bacterial micro-assay test for simultaneous detection and quantitative measurement of galactose in the serum. The linearity, precision and sensitivity of the micro-assay were analyzed. In this study bacterial strains named *B.cereus* and *P.mirabillis* and a mixture of the suspension of both were used separately. According to the standard curves, the micro-assay is linear in 2-180mg/dL serum galactose. Since the normal concentration of the serum galactose in newborns is 0-20 mg/dL, concentrations above 20 mg/dL are considered to represent Duarte form or classical form of galactosemia, depending on the concentration of serum galactose. This micro-assay is capable of measuring normal and increased concentrations of the serum galactose, making it possible to classify the patients.

Moreover, analysis of the lyophilized form of the bacteria resulted in a similar result as fresh bacteria. This indicated that the bacteria can be stored at ambient condition, and be used with no limitation for the micro-assay. Therefore, the assay has the advantage of having the potential to be easily automated as a quantitative micro-assay kit.

The rapid nature of the micro-assay could provide the chance for early diagnosis of the galactosemia in emergency cases to avoid subsequent clinical problems. Often patients will be diagnosed at a stage of medical emergency that may lead to sequel or death, and some cases will be misinterpreted as gram-negative sepsis, a frequent complication of galactosemia [11 and 19]. Therefore, always a rapid and careful diagnosis of galactosemic newborns could be incredibly helpful. Moreover, prospective screening of newborns for galactosemia is a widely accepted procedure throughout the world. In many laboratories screening utilizes the Guthrie test to assay for total galactose (galactose plus glactose-1-phosphate) and/or activity of the GALT enzyme using the Beutler screening test [17]. False positive results in newborn screening for galactosemia are frequent and represent a substantial problem for screening programs. A common observation is the adverse affects that environmental factors and sample handling procedures (practiced at the site of specimen collection or during specimen transport) may have on the GALT assay. This could result in low activity and false positives. The most notable environmental influences are heat and especially humidity. Specimens collected during hot, humid summer seasons or in climates where such conditions are persistent, often present with reduced GALT activity. Also, since the activity of the GALT enzyme in a dried blood specimen deteriorates over time at room temperature, the practice of batching, where dried blood specimens are permitted to accumulate at the hospital before being mailed to the screening laboratory, may also adversely affect GALT activity.

The micro-assay method uses inexpensive materials and reagents and could measure serum total galactose in a relatively short period of time with high accuracy and precision. One of the main advantages of the method is the use of both galactose and galactose-1 phosphate as

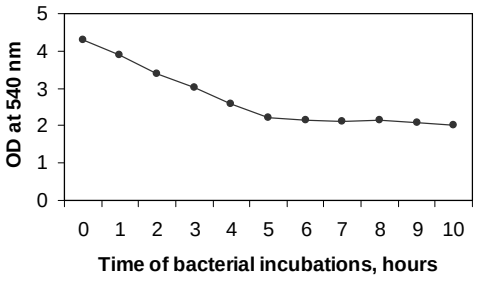

Figure 2. Optimization of incubation time of bacterial culture for measurement of serum galactose.

Table 1. Analytical precision of the micro-assay

	n	Galactose ($mgdL^{-1}$)	Mean± SD	CV %
Within-	10	4	0.045±0.005	10.40
run	10	60	0.780±0.065	8.08
	10	180	2.196±0.105	4.80
Between-	10	4	0.043 ±0.007	15.60
Run	10	60	0.730±0.083	11.40
	10	180	2.170±0.089	4.10

n: number of samples; SD: Standard Deviation; CV: Coefficient of variance

the target sugars in the serum samples. Therefore, total galactose is measured by this method. Moreover, due to the persistent nature of these sugars in the serum samples, old samples (up to one month) could be used.

The speed and easy setup of the method as well as the lack of false positive (i.e. a known normal serum Galactose level detected wrongly as an abnormal level) and false negative results (i.e. a known abnormal serum galactose level detected as a normal one) could make the method as an attractive option in situations where a unlimited number of samples are to be analyzed. This can be the case with selective screening procedures and in confirmatory testing of samples from galactosemia screening, where low GALT activity has been found. Also, the method lends itself to rapid follow-up analysis of routine neonatal screening samples with increased concentrations of galactose.

In conclusion, the micro-assay could provides suitable mean for mass screening galactosemia in developing countries, which have low financial funding for performing this kind of screening programs. We previously developed a new quantitative bacterial micro-assay for our PKU screening program [21], and now with conjunction of this method, a dual micro-assay kit could be produced for simultaneous detection of serum phenylalanine and galactose.

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References

1. Wang BB, Xu YK, Ng WG, Wong LJ. Molecular and biochemical basis of galactosemia. *Mol Genet Metab*, **63**: 263–269 (1998).
2. Holdena, H. M. Thodena, J. B. Timsonb D. J and Reecec, R. J. . Galactokinase: structure, function and role in type II galactosemia .*Cell. Mol. Life Sci*, **61**: 2471–2484 (2004).
3. Elsas LJ and Lai K: The molecular biology of galactosemia. *Genet Med*, **1**:40–48 (1998).
4. Segal S, Berry GT. Disorders of galactose metabolism. In "The Metabolic and Molecular Bases of Inherited Disease" Scriver CR, Beaudet AL, Sly WS, Valle D, Eds., 7th ed., Vol. 1. New York: McGraw-Hill, pp 967–1000 (1995).
5. Segal S, Blair A, Roth H. The metabolism of galactose by patients with congential galactosemia. *Am J Med*, **38**: 62–70 (1965).
6. Gitzelmann R, Steinmann B. Galactosemia: how does long-term treatment change the outcome? *Enzyme*, **32**:37–46 (1984).
7. Waisbren SE, Norman TR, Schnell RR, Levy HL: Speech and language deficits in early-treated children with galactosemia. *J Pediatr*, **102**: 75-77 (1983).
8. Novelli G, Reichardt JK. Molecular basis of disorders of human galactose metabolism: past, present, and future. *Mol Genet Metab*, **71**: 62-65 (2000).
9. Jan JE, Wilson RA. Unusual late neurological sequelae in galactosaemia. *Dev Med Child Neurol*, **15**: 72–74 (1973).
10. Waggoner DD, Buist NR, Donnell GN. Long-term prognosis in galactosaemia: results of a survey of 350 cases. *J Inherit Metab Dis*, **13**: 802–818 (1990).
11. Jensen U. G., Brandt N. J., Christensen E., Skovby F., Nørgaard-Pedersen,B and Simonsen H. Neonatal Screening for Galactosemia by Quantitative Analysis of Hexose Monophosphates Using Tandem Mass Spectrometry: A Retrospective Study. *Clinical Chemistry*, **47**(8): 1364–1372 (2001).
12. Baker L, Mellman WJ, Tedesco TA, Segal S. Galactosemia: symptomatic and asymptomatic homozygotes in one. Negro sibship. *J Pediatr*, **68**: 551–558 (1966).
13. 13-Hongl S. P. Yoon H. R. Kim M. K.Development of a New HPLC Diagnostic Method for Galactosemia Using 8-Amino-2- naphthalenesulfonic acid .*Chromatographia*, **54**:83-86 (2001).
14. Berry G. T. Nissim I. Gibson J. B. Mazur A. T. Lin. Z. Elsas L. J. Singh · R. H. Klein · P. D Segal · S. Quantitative assessment of whole body galactose metabolism in galactosemic patients. *Eur J Pediatr*, **156** [Suppl 1]: S43–S49 (1997).
15. Watanbe N, Kawasaki S. Determination of galactose in human plasma by HPLC with electrochemical detection. *Biomed. Chromatogr*, **2**: 95–98 (1987).
16. Jeong JS, Kwon HJ, Lee YM, Yoon HR, Hong SP.Galactosemia screening by simultaneous blood spot quantification of galactose and galactose 1-phosphate. *Clin Chem*, **54**(12): 2080-2 (2008).
17. 17-Beutler E, Mitchell M: New rapid method for the estimation of red cell galactose-1-phosphate uridyltransferase activity. *Lab Clin Med*, **72**: 527-532 (1968).
18. Boone RD and Castenholz RW. Bergey's manuals of systematic bacteriology. Second ed., Vol (1), Springer, Baltimore.2000.
19. Walter JH, Collins JE, Leonard JV . Recommendations for the management of galactosaemia. UK Galactosaemia Steering Group. Arch Dis Child, **80**(1): 93-6 (1999).
20. Paigen K, Pacholec F, Levy HL. A new method of screening for inherited disorders of galactose metabolism. *J Lab Clin Med*, **99**: 895–907 (1982).
21. Vallian, S., Moeini, H. Quantitative bacterial micro-assay for rapid detection of serum phenylalanine in dry blood-spots: Application in phenylketonuria screening .*Clin Chem Lab Med*, **44**(1): 76-79 (2006).