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The Rapid and Sensitive Quantitative Determination of Galactose by Combined Enzymatic and Colorimetric Method: Application in Neonatal Screening

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Abstract The quantitative measurement of galactose in blood is essential for the early diagnosis, treatment, and dietary monitoring of galactosemia patients. In this communication, we aimed to develop a rapid, sensitive, and cost-effective combined method for galactose determination in dry blood spots. This procedure was based on the combination of enzymatic reactions of galactose dehydrogenase (GalDH), dihydrolipoyl dehydrogenase (DLD), and alkaline phosphates with a colorimetric system. The incubation time and the concentration of enzymes used in new method were also optimized. The analytical performance was studied by the precision, recovery, linearity, and sensitivity parameters. Statistical analysis was applied to method comparison experiment. The regression equation and correlation coefficient (R^2) were $Y = 0.0085x + 0.032$ and $R^2 = 0.998$, respectively. This assay exhibited a recovery in the

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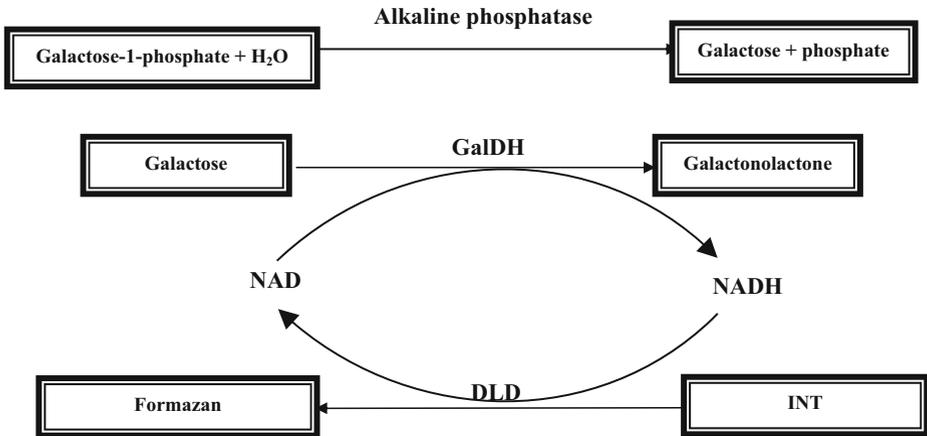[6] Department of Biology, Bandar Jask Branch, Islamic Azad University, Bandar Jask, Iran

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range of 91.7–114.3 % and had the limit detection of 0.5 mg/dl for galactose. The between-run coefficient of variation (CV) was between 2.6 and 11.1 %. The within-run CV was between 4.9 and 9.2 %. Our results indicated that the new and reference methods were in agreement because no significant biases exist between them. Briefly, a quick and reliable combined enzymatic and colorimetric assay was presented for application in newborn mass screening and monitoring of galactosemia patients.

Introduction

Galactosemia is an inherited metabolic disorder transmitted in an autosomal recessive manner. Three enzymes are involved in galactose metabolism: D-galactose-1-phosphotransferase (kinase), alpha-D-galactose-1-phosphate uridyl-transferase (GALT), and UDP-glucose-4-epimerase (epimerase). GALT deficiency is the most common cause of galactosemia. It is usually referred as classical galactosemia, and its incidence is about 1 per 62,000 births with different rates among geographic populations [1–3]. For example, the incidence of this disease in Fars province of Iran is 1/28,000 [4]. Galactosemia is the inability to metabolize galactose, leading to accumulation of toxic levels of galactose-1-phosphate in body, which can result in renal failure, cataracts, vomiting, seizure, hypoglycemia, lethargy, brain damage, and ovarian failure. Without treatment, mortality and morbidity rate in infants with galactosemia is high. However, when the disease is identified soon and proper treatment is begun immediately, the symptoms can be prevented. Hence, there is a need for improved screening and diagnosis of patients at risk for galactosemia [5, 6]. For this reason, galactose measurement is necessary for the early diagnosis, treatment, and determination of galactosemia type [7]. The first test used for screening of classical galactosemia is the Beutler-Baluda fluorescent spot method. This test indicates the presence or absence of transferase activity in blood. Transferase is heat labile and may lose activity, even when stored at 4 °C. Therefore, this test has a high false-positive rate, especially in warm weather [8]. Other techniques including bacterial micro-assay [9], fluorometric [10], high performance liquid chromatography (HPLC), and tandem mass spectrometry (MS/MS) [11] have also been used for measuring of galactose concentration. However, these methods are time-consuming or they require specialized instruments and not available in every laboratory. Nonetheless, enzymatic methods are of particular interest because they are specific, rapid, and affordable in most clinical laboratories [12]. In this study, we presented an enzymatic assay using three enzymes, galactose dehydrogenase (GalDH), dihydrolipoyl dehydrogenase (DLD), and alkaline phosphates coupled with an electron acceptor system for the rapid and accurate quantitative determination of galactose in dry blood spots. The principle of this procedure was as follows. Firstly, alkaline phosphates catalyze the conversion of galactose-1-phosphate to galactose and phosphate (Fig. 1). Next, GalDH catalyzes galactose to galactonolactone in the presence of nicotinamide adenine dinucleotide (NAD^+). In the last step, DLD enzyme converts the NADH to NAD^+ and simultaneously H^+ is transferred to iodonitrotetrazolium chloride (INT) as a reduction indicator, which leads to the generation of formazan with an absorbance maximum at 490 nm. This is the first report for a combined enzymatic and colorimetric assay to measure galactose concentration in dry blood spots (DBS).

Alkaline phosphatase

Galactose-1-phosphate + H$_2$O $\longrightarrow$ Galactose + phosphate

Galactose $\xrightarrow{\text{GalDH}}$ Galactonolactone

NAD   NADH

Formazan $\xleftarrow{\text{DLD}}$ INT

Fig. 1 Combined enzymatic and colorimetric test for the quantitative determination of galactose

Materials and Methods

Chemicals and Reagents

NAD$^+$ and alkaline phosphates were from Sigma-Aldrich (St. Louis, MO, USA). Galactose and galactose-1-phosphate were obtained from Merck (Darmstadt, Germany). INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The specimen collection paper used for blood spot samples was Schleicher and Schuell grade 903 (S&S 903). All other chemicals and biochemical reagents were of laboratory grade, and double-distilled water was used throughout the experiments.

Production of Recombinant GalDH

The coding gene *gdh* was amplified by PCR with the forward primer GDHFw (5′-TGGATCCATGCAACCGATTCGTCTCG-′3) and the reverse primer GDHRev (5′-GCGAAGCTT TTAATCGTAGAACGGC-′3), which contained the restriction sites for *Bam*HI and *Hind*III, respectively (underlined). PCR amplification was performed under condition: preincubation at 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 61 °C, and 1 min at 72 °C with a final elongation step at 72 °C for 10 min. The PCR reaction product was cut with *Bam*HI and *Hind*III and ligated into the pET-28a (+) expression vector. A recombinant strain of *Escherichia coli* BL21 (DE3) was grown overnight in Luria-Bertani (LB) medium containing 40 μg/ml of kanamycin at 37 °C and 150 rpm until an cell density of OD$_{600}$ = 0.6–0.8 was reached. GalDH expression was induced by the addition of 0.7 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG). After 5 h induction at 37 °C, cells were harvested and stored at −20 °C for further use. Pelleted *E. coli* cells were suspended in lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0), mechanically broken by sonication using a pulse sequence of 15 s on and 10 s off, and clarified by centrifugation at 4000 rpm for 1 h at 4 °C. The supernatants were employed as a crude enzyme in purification experiments. Purification of recombinant enzyme was carried out as described in our preceding paper [13–15].

Production of Recombinant DLD

To amplify the DLD gene, forward and reverse primers were designed using DNASIS MAX software (DNASIS version 3.0, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The primers used were DLDFw (5′-CCCGGATCCATGGCTTATAAATATGATCTGATTG -′3) and DLDRev (5′- CCCGTCGACTTACTCCCTCATCAATTCCC-′3), which contained the restriction sites for *Bam*HI and *Sal*I, respectively (underlined). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 70 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 7 min. The resultant PCR product was double digested with *Bam*HI and *Sal*I, gel purified, and then ligated into the pET28a (+) expression vector. *E. coli* strain BL21 (DE3) cells bearing pET28aDLD were cultivated overnight in LB medium containing 40 μg/ml of kanamycin at 37 °C and 180 rpm. Preculture broth (100 ml) was transferred into 1 L of LB medium in culture flasks and incubated at 37 °C and 180 rpm. When cell density reached an OD_{600} of 0.6–0.8, DLD enzyme was expressed by the addition of 1 mM sterile IPTG. After 5 h induction at 37 °C, cells were harvested, washed twice with 0.9 % NaCl solution, and stored at −20 °C for further uses. The harvested cells were washed twice with 0.9 % NaCl solution, suspended in 50 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4), and disrupted by ultrasonic oscillator for 20 min. Cells and insoluble materials were removed by centrifugation at 4000 rpm for 1 h at 4 °C. The supernatant solution was used for enzyme purification. Recombinant enzyme was purified as described elsewhere [16, 17].

Dried Blood Controls and Standards

Standard solutions containing 0, 2.5, 5, 10, 25, and 50 mg/dl of galactose were prepared by diluting the stock solution with aqueous solution of phosphate buffer saline (PBS). These samples were directly spotted on filter paper and dried at room temperature for at least 24 h.

Design and Optimization of New Combined Method

The principle of this technique was the quantitative determination of galactose based on the use of GalDH, DLD, and alkaline phosphate enzymes and its colorimetric measurement. In the assay procedure, 3-mm (diameter) DBS from calibrators, controls, and patient specimens were punched into a 96-well microplate and left for 15 min at 95 °C in a bain-marie. At the end of this step, 150 μL of 0.1 M Tris–HCl (pH 8.0) was added to each well and the plate was shaken for 60 min at 25 °C. The extracts were transferred to the corresponding test microplate, and the enzymes, coenzyme, and INT reagent were then added to each well. After 30 min shaking, the absorbance was read bichromatically at 490/630 nm. Calibration curve was plotted, and the experimental data were fitted by linear regression analyze. For better performance of new assay, the incubation time and the concentration of enzymes were also optimized. The absorbance of reaction was measured every 10 min after the time of incubation for 1 h.

Method Validation Tests

The analytical performance of the presented enzymatic method was studied by the precision, recovery, linearity, and sensitivity parameters [18]. The intra-run precision was determined using data obtained from 20 replicates from three samples. The inter-run precision involved

analysis of the above samples in 10 different assays. Recovery was measured by adding 100 μL of stock galactose standard to 900 μL normal whole blood specimens. Linearity was analyzed by making several dilutions of a plasma pool with added galactose (50 mg/ml). The detection limit was determined with different concentrations of galactose from 0 to 5 mg/dl after 10 experiments.

## Method Comparison Experiment

The comparison of test and reference method was based on paired data obtained from several independent subjects. Approximately 50 newborn blood spot specimens were selected randomly from the newborn screening program at the Division of Neonatal Screening, Shahid Beheshti University of Medical Science, Tehran, Iran. The DBS samples were also kept in sealed plastic bags at −20 °C until used. These specimens were tested in this study using the designed method and HPLC assay. HPLC is called the reference method. The results were expressed as mean ± SD. All statistical analysis including paired samples t test, regression analysis, and difference plot was performed using MedCalc statistical software version 15.11.4 (MedCalc Software bvba, Ostend, Belgium).

Results and Discussion

Performance of the Designed Method

The linear calibration curve was determined by using standard solutions of galactose in the concentration range from 0 to 50 mg/dl with the DBS filter papers (Fig. 2). Each plot was displayed as an average value of triplicate measurement. As seen in Fig. 2, the regression equation was $Y = 0.0085x + 0.032$. The correlation of coefficient (R^2) was 0.998, indicating a good degree of correlation between measured values. Analysis of standard curve showed a nearly linear curve which confirmed the linearity of the method in this range. The optimum time of assay in respect to the speed of galactosemia screening was investigated as well. As shown in Fig. 3, the best result was achieved after 45 min which was favorably comparable

Fig. 2 Calibration curve for the combined enzymatic determination of galactose in DBS. Experiments for each concentration were performed in triplicate run

$y = 0.008x + 0.032$
$R^2 = 0.998$
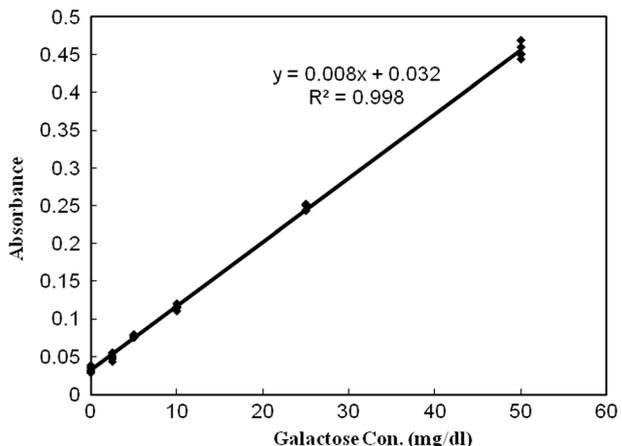
Fig. 3 Optimization of analysis time for the combined enzymatic determination of galactose
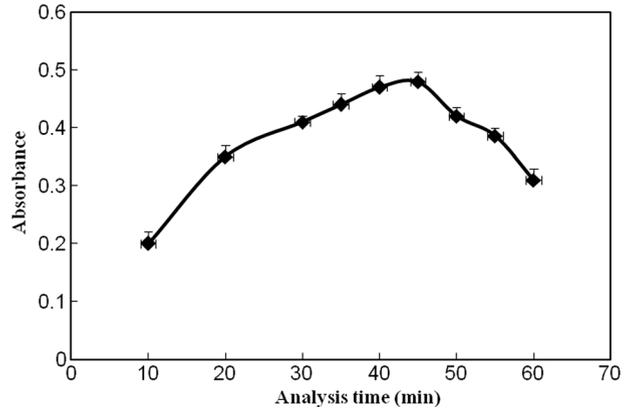
than other methods (Table 4) [6, 11]. Another important feature in method optimization was the concentrations of used enzymes. The optimized concentrations for GalDH, DLD, and alkaline phosphates were 1, 3.0, and 2.5 U/ml, respectively.

Analytical Precision, Recovery, and Sensitivity

Analytical precision was defined by replicate analysis of intra- and inter-assay variations. As shown in the Table 1, the between-run coefficient of variation (CV) mean was between 2.6 and 11.1 %. The within-run CV was between 4.9 and 9.2 %. Also, it can be found that the presented method was capable of measuring a relatively wide spectrum of possible concentrations of serum galactose from normal to highly elevated levels with suitable accuracy. The analytical recoveries for galactose added to blood spots were depicted in Table 2. The enzymatic determination of galactose in DBS showed a recovery in the range of 91.7–114.3 %, thus fulfilling the requirement for a screening method. The sensitivity was 0.5 mg/dl for galactose, highlighting the ability of the method in the lower range. Concentration differences of 0.5 and 1.0 mg/dl galactose were detectable at the 91.6 % confidence level.

Comparison of Designed Assay with Reference Method

Table 3 shows the results obtained from the neonatal dried blood samples by our designed assay and HPLC. As can be found, the results indicated that two methods for measuring

Table 1 The intra-run and inter-run precision of the combined enzymatic assay method

Standard	Concentration (mg/dl)	Within-run ($n = 20$)		Between-run ($n = 10$)	
		(mean ± SD)	CV%	(mean ± SD)	CV%
Galactose	1	5.7 ± 0.3	4.9	37.4 ± 8.1	2.6
	8	19.4 ± 1.9	9.2	111.4 ± 9.0	9.7
	15	221.5 ± 9.8	5.8	234.8 ± 20.9	10.3
	44	410.4 ± 17.4	6.1	397.3 ± 52.1	11.1

SD standard deviation, *CV* coefficient of variation

Table 2 Recover of galactose added to blood samples

Galactose concentration (μmol/L)			Recovery (%)
Added	Expected	Observed	
0	–	12.3	–
43.7	56.0	64.0	114.3
80.1	92.4	88.3	95.6
110.9	123.2	118.6	96.3
136.9	149.2	136.9	91.7

galactose were very similar because no significant biases exist between them. The average difference between two methods (0.01) is not statistically different from zero at 95 % confidence. P value was 0.3018 indicating that the two methods agreed with 95 % confidence.

Table 3 Determination of galactose concentration in neonate samples by designed method and HPLC

| Subjects	HPLC technique (mg/dl)	New method (mg/dl)	D	Subjects	HPLC technique (mg/dl)	New method (mg/dl)	D
N1	2.1	2.0	0.1	N26	3.1	3.1	0
N2	2.3	2.3	0	N27	4.4	4.3	0.1
N3	3.0	3.1	−0.1	N28	4.1	3.9	0.2
N4	3.3	3.2	0.1	N29	4.4	4.6	−0.2
N5	4.0	3.8	0.2	N30	1.3	1.4	−0.1
N6	4.2	4.1	0.1	N31	1.9	1.9	0
N7	5.0	5.0	0	N32	2.9	2.9	0
N8	4.3	4.4	−0.1	N33	3.2	3.2	0
N9	4.1	4.1	0	N34	3.9	4.0	−0.1
N10	4.0	4.1	−0.1	N35	5.2	5.2	0
N11	1.7	1.8	−0.1	N36	7.1	7.0	0.1
N12	2.7	2.5	0.2	N37	3.3	3.1	0.2
N13	3.0	2.9	0.1	N38	2.0	2.0	0
N14	3.3	3.2	0.1	N39	1.7	1.6	0.1
N15	3.5	3.3	0.2	N40	3.1	3.0	0.1
N16	3.6	3.5	0.1	N41	3.4	3.3	0.1
N17	3.8	3.7	0.1	N42	2.0	2.0	0
N18	4.0	4.1	−0.1	N43	3.2	3.2	0
N19	3.3	3.2	0.1	N44	4.4	4.4	0
N20	4.8	4.7	0.1	N45	5.1	5.0	0.1
N21	1.4	1.3	0.1	N46	3.1	3.0	0.1
N22	2.3	2.2	0.1	N47	2.2	2.2	0
N23	2.9	2.8	0.1	N48	3.1	3.3	−0.2
N24	3.2	3.1	0.1	N49	1.9	2.0	−0.1
N25	3.6	3.5	0.1	N50	2.4	2.4	0

D difference between methods

Also, only specimen N36 was identified as galactosemia patient with both methods. This child had been diagnosed as classical galactosemia by the screening laboratory. For neonatal screening, a cutoff of 5.0 mg/dl was defined for galactose concentration. Figure 4 represents a linear regression analysis using the data from Table 3. Linear regression analysis provides useful information about constant error and proportional error via, respectively, intercept and slope. As shown, there was a high degree of correlation ($R^2 = 0.996$) between the concentration of galactose as determined by the new method and reference technique. Ordinary linear regression analysis revealed intercept $= −0.007553$ (95 % confidence interval5 $= −0.06904$ to 0.05393) and slope $= 1.0053$ (95 % confidence interval $= 0.9876$ to 1.0230). The intercept was not statistically significantly different from 0, and hence no constant error was present. Also, proportional error did not exist, since the slope was not statistically significantly different from 1.0. The concordance correlation coefficient was 0.9981, which was considered as indicating almost perfect performance. To judge acceptability of agreement between methods, a difference plot (also known as a Bland-Altman plot) with the mean value of the methods on the x-axis and the difference between methods on the y-axis was constructed (Fig. 5). The differences were distributed around 0, and 95 % of the differences were within the lines. Two lines representing, respectively, $0 + 1.96$ and $0 − 1.96$ were also inserted in the plot. The combined CV of both methods is 1.45 %. From these result, it was evident that the new method and reference method were in agreement.

Application of New Method in Galactosemia Neonatal Screening

Galactosemia is a disorder of galactose metabolism, and its classic form results from a severe deficiency of GALT enzyme. Therefore, measuring of blood galactose concentration is very important for diagnosis and treatment [19]. Different methods have been utilized for this task including fluorometric, spectrophotometric, chromatographic, and enzymatic assay [20]. HPLC and tandem mass spectrometry (MS/MS) are not yet widely available in every laboratory as enzymatic techniques for the routine screening and monitoring of galactosemia. The extremely high cost of equipment and the need for sample derivatization are their main disadvantages [19]. Therefore, new methods that are selective, simple, and inexpensive while offering rapid and quantitative determination of galactose are needed. Considering the improvement in sensitivity and specificity of enzymatic tests and the quality of care for galactosemia patients, we aimed to find a new technology, which is suitable for accurate assay of galactose. Our presented procedure

Fig. 4 Comparison of new method with reference method (HPLC)

$y = 0.991x + 0.019$
$R^2 = 0.996$
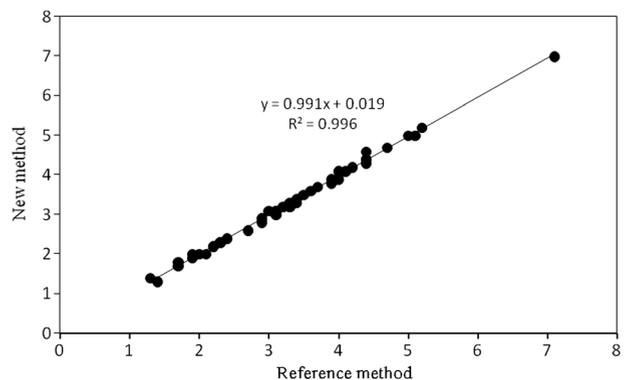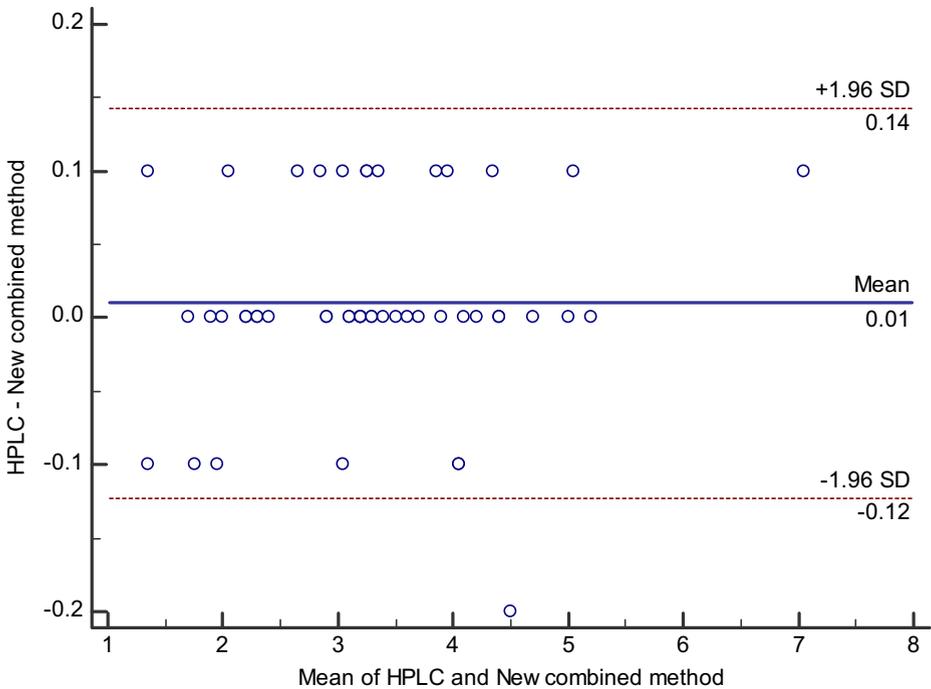
Fig. 5 Difference plot to show the difference between new method and reference method (HPLC) against the average measurement. The *dotted lines* represent 0 ± 1.96

involves the combination of enzymatic reactions of alkaline phosphates, GalDH, and DLD with colorimetric system of INT. An important feature of this test was the use of recombinant GalDH which has the narrowest board specificity toward galactose. In fact, with this galactose-specific enzyme, it makes it possible to increase the specificity and the sensitivity of detection [18]. The procedure was capable of measuring galactose in the low range which could be useful in screening program. Thus, it can be said that the presented enzymatic assay differed from the previously reported techniques. First, our assay was carried out in microplate reader instead of a conventional spectrophotometer, thus allowing the simultaneous analysis of a large number of samples. Second, we increased the sensitivity and selectivity by using recombinant GalDH. Third, we changed the assay to an endpoint assay at 490 nm wavelength which was conventional for all routine clinical laboratories. It had a wider linear range and good precision that correlate with those of the previously used technique [20, 21]. The material cost of the assay was estimated to be about 1$ per sample in triplicate, which was economical to use rather than the enzymatic commercial kits [6]. To verify the validity of the described test, we compared the performance parameters of our

Table 4 Comparison of presented combined enzymatic test with the two used methods for measurement of galactose

Method	Sensitivity (mg/dl)	Analysis time (min)	R^2
Combined enzymatic and colorimetric presented here	0.5	45	0.998
Beutler-Baluda fluorescent spot test	1.0	240	0.894
Bacterial micro-assay	2.0	300	0.962

method with the two used methods to determine galactose concentrations (Table 4). Time required for each assay was 90 min which was favorably comparable than other enzymatic methods [6, 11].

Conclusions

In conclusion, we developed a simple, reliable, and quick combined enzymatic and colorimetric assay based on GalDH for galactose determination in DBS. Our results indicated that the new and reference methods agreed with each other because no significant biases exist between them. The new method could serve for both screening and monitoring of galactose concentrations in galactosemia patients.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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