COMPARISON OF SPECTROPHOTOMETRIC AND FLUORIMETRIC METHODS IN EVALUATION OF BIOTINIDASE DEFICIENCY

POREĐENJE SPEKTROMETRIJSKOG I FLUORIMETRIJSKOG METODA U PROCENI NEDOSTATKA BIOTINIDAZE

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Summary

Background: Biotin, a water-soluble vitamin, is used as a co-factor by enzymes involved in carboxylation reactions. Biotinidase (BTD) catalyzes the recycling of biotin from endogenous and dietary sources. Biotinidase deficiency (BD) is an autosomal recessively inherited disorder of biotin recycling that is associated with neurologic and cutaneous consequences when untreated. The aim of the study was to compare the results of spectrophotometric and fluorimetric methods, as well as to evaluate the advantages and disadvantages of both methods in current research practices.
Methods: Study group was chosen among the BD suspected newborn, children and parents (n=52) who applied to Hacettepe University Pediatric Metabolism Unit.
Results: BTD activity is stable for 2 hours at room temperature and at 4 °C, and for 4 months at –20 °C and –80 °C. Genetic and clinical results showed that 25% of the total number of patients had complete BD which was treated with 10 mg/day biotin, while 15.38% of the patients had partial BD, and they were prescribed biotin 5 mg/day. The area under the ROC curve was 0.960±0.25 and 0.927± 0.41 for the fluorimetric and spectrophotometric method, respectively. Fluorimetric method showed 100% sensitivity and 97% specificity, whereas spectrophotometric method showed 90.5% sensitivity and 93.7% specificity.
Conclusions: Fluorimetric method is superior to the spectrophotometric method due to higher sensitivity and specificity.

Keywords: biotinidase, enzyme deficiency, spectrophotometry, fluorimetry

Kratak sadržaj

Uvod: Enzimi uključeni u reakcije karboksilacije koriste biotin, vitamin rastvorljiv u vodi, kao kofaktor. Biotinidaza (BTD) katališe recikliranje biotina iz endogenih izvora i iz ishrane. Nedostatak biotinidaze (NB) predstavlja autozomni recesivni nasledni poremećaj recikliranja biotina koji se u slučaju nelečenja povezuje s neurološkim i kožnim posledicama. Cilj ove studije bio je da se uporede rezultati spektrofotometrijskog i fluorimetrijskog metoda, kao i da se procene prednosti i mane oba ova metoda u današnjoj istraživačkoj praksi.
Metode: Studijsku grupu smo izabrali između novorođenčadi, dece i roditelja (n=52) sa sumnjom na NB koji su se javili Pedijatrijskom odeljenju za metabolizam Univerziteta Hacettepe.
Rezultati: Aktivnost BTD stabilna je 2 sata na sobnoj temperaturi i na 4 °C, a 4 meseca na –20 °C i –80 °C. Genetski i klinički rezultati pokazuju da 25% ukupnog broja obolelih ima potpuni NB, koji je tretiran sa 10 mg biotina dnevno, dok 15,38% obolelih ima delimični NB, tretiran sa 5 mg biotina dnevno. Oblast ispod ROC krive iznosila je 0,960± 0,25 za fluorimetrijski, odnosno 0,927±0,41 za spektrofotometrijski metod. Fluorimetrijski metod pokazao je osetljivost od 100% i specifičnost od 97%, dok je spektrofotometrijski metod pokazao osetljivost od 90,5% i specifičnost od 93,7%.
Zaključak: Fluorimetrijski metod je superioran u odnosu na spektrofotometrijski zbog svoje veće osetljivosti i specifičnosti.

Ključne reči: biotinidaza, nedostatak enzima, spektrofotometrija, fluorimetrija

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Introduction

In many countries, newborn screening tests are mandatory public health programs to check for the presence of particular genetic or metabolic disorders which provides early detection and treatment of some serious conditions (1). In Turkey, the national newborn screening program has progressively improved; the screening rate of the live born babies was 4.7% in 1987, reached to 95% by 2008 and above 95% by the end of 2012. Phenylketonuria and congenital hypothyroidism screening have been a part of the national child health program in our country since 1994 and 2006, respectively (2, 3). The first published scientific study of biotinidase deficiency (BD) was done by Tokatli et al. in 1992. Since then, many cases have been reported in various clinical laboratories (4) and nowadays nearly all newborns are being screened for phenylketonuria, congenital hypothyroidism and BD (1).

BD, caused by impaired function of the biotinidase (BTD) enzyme (EC 3.5.1.12), is an autosomal recessive metabolic disorder. BTD cleaves biocytin, the degradation product of holocarboxylases, and plays a role in releasing biotin from dietary protein-bound sources. The main symptoms of untreated BD patients are neurological impairment, hypotonia, seizures, respiratory abnormalities, ataxia, hearing and vision problems, skin rash, alopecia, developmental delay, lactic acidosis and ketosis, organic aciduria and hyperammonaemia (5, 6). Developmental delay, vision and hearing problems are usually irreversible. The disorder can be treated effectively and inexpensively with pharmacological doses of biotin when diagnosed timely (7).

Patients are classified based on serum/plasma biotinidase (BTD) activity as having either profound BD (serum/plasma BTD activity < 10%) or partial BD (serum/plasma BTD activity 10–30%). Most of the partial BD patients without treatment stay asymptomatic, while profound BD patients exhibit symptoms in early childhood and, if left untreated, symptoms usually progressively worsen and may lead to coma and death (8, 9). Research results revealed that profound BD patients may not develop any symptoms until adolescence (6) and may even remain asymptomatic into adulthood (8, 10, 11). Each of the BD patients may exhibit variable clinical symptoms and age at onset varies (9, 12–15). Therefore, correct measurement of BTD enzyme activity in newborns is crucial.

In this study, we have measured BTD activity both with fluorimetric and spectrophotometric methods and compared the results with clinical and genetic findings.

Materials and Methods

Patients

Study group was chosen among the BD suspected newborns, children and parents (n=52) among 145 patients who applied to Hacettepe University Pediatric Metabolism Unit for the BTD activity checkup between 2013 and 2014. Ages of those patients were from 6 days to 9 years and the parents' ages were between 25 and 40 years.

Ethics and Dissemination

Ethical approval was taken from the Hacettepe University Medical Faculty Non-interventional Clinical Research Ethics Committee (date: 23/01/2013 and number: LUT 12/178-12).

Blood samples

Heparinized tubes and serum separating tube were used to collect blood samples from each patient including newborns, children and parents. One mL blood was drawn from each patient into heparinized tubes containing lithium heparin for preparation of plasma samples, whereas 2 mL blood was drawn from each patient into a serum separating tube for the preparation of serum samples. The samples were transferred to the laboratory in 4 to 6 hours maintaining the cold chain. One mL heparinized blood samples were centrifuged at 2000 × g for 5 minutes and 3×75 µL plasma samples were prepared from each patient for measurements. Serum separating tubes containing 2 mL blood were centrifuged at 2000 × g for 5 minutes to obtain 3×100 µL serum samples from each patient. Serum and plasma samples were stored at –80 °C until the assay or BTD activity was measured immediately. Plasma was used for fluorimetric measurement while serum was used for spectrophotometric measurement according to the literature (9, 16).

Research results showed that it was not possible to analyse undialised serum samples with the fluorimetric assay because of the interference of small peptides and amino acids in the serum. Therefore, fluorimetric measurements could be done in dialysed serum samples or in plasma samples (17). Because of the fact that dialysis of the serum in routine analyses is time consuming, we prefer to analyse plasma samples for fluorimetric analyses.

Spectrophotometric assay

BTD activity was determined spectrophotometrically by a UV-1800 Shimatzu Model spectrophotometer according to the method proposed by Wolf et al. (19) by measuring p-aminobenzoate liberation from N-biotinyl-p-aminobenzoate. Fasting human serum

was obtained from children and their parents. After preincubation at 37 °C for 15 min, the enzyme assay was initiated by the addition of 0.1 mL serum to 1.9 mL of a mixture containing 200 µmol potassium phosphate buffer, pH 6.0, 20 µmol EDTA, 0.5 mg serum albumin, and 0.3 µmol N-biotinyl-p-aminobenzoate bringing the final volume to 2 mL. The mixture was incubated for 30 min at 37 °C and the reaction was terminated by the addition of 0.2 mL of 30% trichloroacetic acid. The mixture was centrifuged at 2000 × g for 10 min. Then, 1.5 mL of the supernatant was added to 0.5 mL water. At room temperature, and at 3-min intervals, 0.2 mL of 0.1% sodium nitrite (prepared fresh daily), 0.2 mL of 0.5% ammonium sulfamate and 0.2 mL of 0.1% N-1-naphthyl ethylenediamine hydrochloride were added in succession and incubated for 10 min, and then the spectrophometric absorbance was measured at 546 nm. BTD activity is expressed as nmol of p-aminobenzoate liberated per min per mL serum (9).

Fluorimetric assay

The fluorimetric assay of BTD activity was carried out using a Shimatzu Model fluorimeter by using biotin 6-amidoquinoline (BAQ). The reaction was started by the addition of plasma (75 µL) to a reaction mixture containing potassium phosphate (0.15 mol/L, pH 6.5), dithiothreitol (0.1 mmol/L), and BAQ (0.05 mM) in a total volume of 2.55 mL at 37 °C. Reaction was started by the addition of substrate and incubated for 120 min. at 37 °C. The excitation wavelength was 350 nm and the emission wavelength was 550 nm. Fluorescence units were converted to nanomoles product formed by reference to a standard solution of 6-amidoquinoline (16).

Statistical analysis

Results were given as mean ± SD. Chi-square test was used to compare the differences between groups. P<0.05 was accepted as statistically significant. Differences of the methods applied and the diagnostic power were evaluated by ROC (Receiver Operating Characteristic) analysis. Statistical values were given as 95% confidence interval. IBM SPSS Statistic programme 21 was used for statistical analysis.

Results

BTD enzyme activity was measured in serial pH values between pH 5.5 and pH 7.5 using a spectrophotometric method and the optimum pH value was detected at pH 5.5–6.0; the enzyme activities at those pH values were 7.7 nmol/min/mL and 7.6 nmol/min/mL respectively. Our results were similar to the optimum pH results found by Wolf et al. (9). The
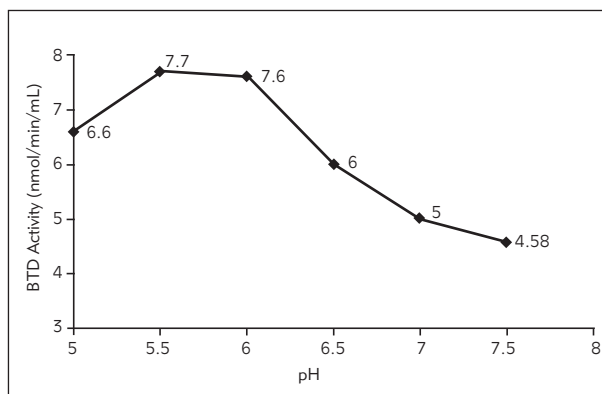

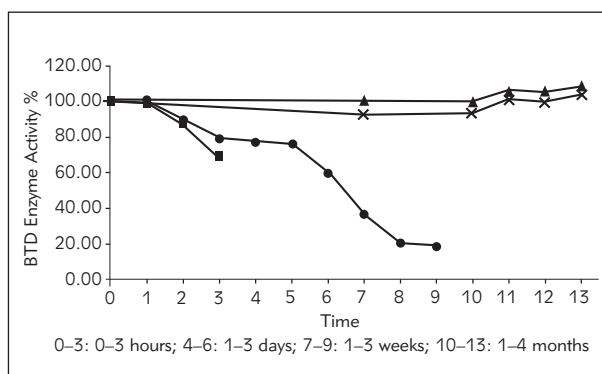**Figure 1** pH dependent changes in serum BTD activity.

Figure 2 Changes in BTD activity (%) in serum samples stored at room temperature, 4 °C, –20 °C and –80 °C. ●— BTD activity % in 4 °C; ▲— BTD activity % in –20 °C; ■— BTD activity in room temperature; ✕— BTD activity % in –80 °C.

increase in pH values decreased the enzyme activity; as shown in *Figure 1*, the enzyme activity was 6 nmol/min/mL, 5 nmol/min/mL and 4.58 nmol/min/mL at pH 6.5, pH 7.0 and pH 7.5 respectively.

We have evaluated the enzyme stability via measuring the enzyme activity with a spectrophotometric method in several conditions: serum samples prepared from blood drawn from healthy adults and stored at room temperature for 3 hours, +4 °C for 3 weeks and at –20 °C and –80 °C for 4 months, before we compared the results with initial enzyme activity. Our results revealed that initial BTD activity decreased by 12% and 30.8% respectively at the 2nd hour and 3rd hour when serum samples were stored at room temperature (*Figure 2*). Initial serum BTD activity was compared to the BTD activity of the serum stored at 4 °C and it was found that initial BTD activity decreased by 10.4% at the 2nd hour, 22.9% at the 1st day and at the end of the 3rd week serum BTD activity decreased by 81.3% (*Figure 2*).

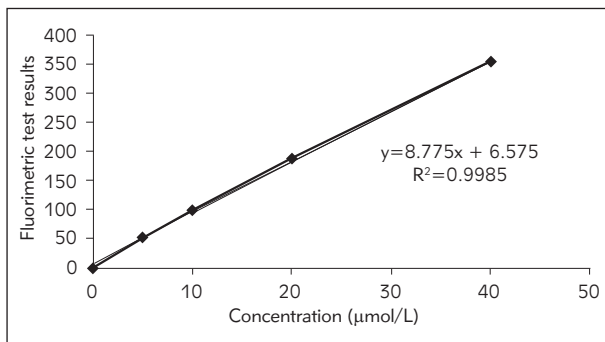We have performed serial BTD activity measurements weekly and monthly and recorded that initial

Figure 3 Linear calibration curve of 6-aminoquinoline used for fluorimetry.

BTD activity did not change in serum samples stored at –80 °C and –20 °C for up to 4 months. As given in *Figure 2*, BTD activity was stable for 2 hours at room temperature and 4 °C, 4 months at –20 °C and –80 °C. No differences in BTD activity were noted between serum samples frozen immediately or stored at –20 °C and –80 °C for up to 4 months.

Fluorimetric test results were calculated using 6-Aminoquinolin standard curve (*Figure 3*). Accuracy of the results of fluorimetric and spectrophotometric methods was evaluated via ROC analysis. Both of the test results were compared to the clinical and genetic test findings of patients (*Figure 4*). Area under the curve was found to be 0.960±0.025 with the fluorimetric method and 0.927±0.041 with the spectrophotometric method (*Figure 4*). According to those findings, we conclude that the diagnostic value of fluorimetric measurement is higher than the diagnostic value of the spectrophotometric method, but both test results statistically have no probability.

BTD activities of 145 patients including newborns, children and parents were measured with the spectrophotometric method in the Metabolism Laboratory over 12 months. Results of the patients were evaluated by age to see the distribution of mean enzyme activity by age. Mean BTD activity of 27 clinically healthy parents with the mean age of 30.4±5.4 was found to be 4.26±1.45 U/L. Three of the parents were diagnosed with Total BD with mean enzyme activity 0.19±0.34 U/L and 2 of the parents were diagnosed with partial BD with mean enzyme activity 0.83±0.06 U/L. Mean BTD activity of 28 children (12 girls, 16 boys) with the mean age of 4.7±3.7 was found to be 4.9±3.7 U/L. Mean BTD activity of 85 newborns was found to be 3.9±2.8 U/L. Fifty-two of those newborns (23 girls, 29 boys) had normal BTD activity (mean BTD activity: 5.53±2.27 U/L). Nine of the newborns (3 girls and 6 boys) were diagnosed with total BD with the mean BTD activity of 0.34±0.25 U/L and 24 of the newborns (9 girls, 15 boys) were diagnosed with partial BD (mean BTD activity: 1.58±0.40 U/L). The mean enzyme activity measured in asymptomatic and healthy individuals was not changed statistically by age.
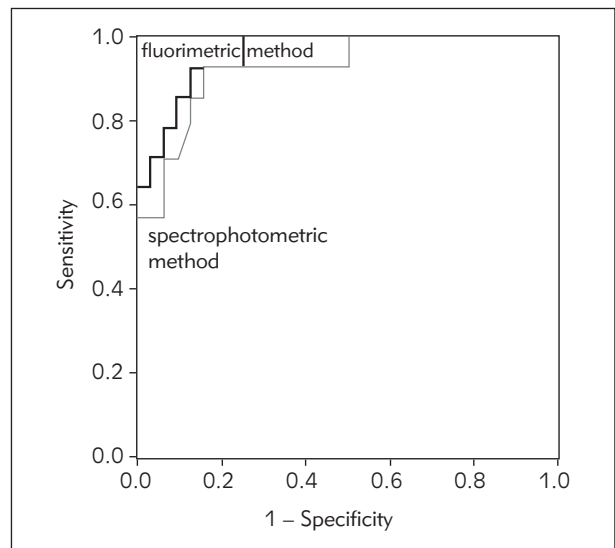

Figure 4 Area under the ROC curve for fluorimetric and spectrophotometric methods.

The range of normal BTD activity in serum and plasma was measured with the blood samples of healthy individuals. The range of normal serum BTD activity obtained from the spectrophotometric assay with the number of 10 samples was found to be 4.7–13 U/L and the range of normal plasma BTD activity obtained from the fluorimetric assay with 24 samples was found to be 1.1–5.24. BD was determined when BTD activity was <0.71 U/L measured by the spectrophotometric method or BTD activity was <0.23 U/L measured by the fluorimetric method. Serum and plasma samples were taken and BTD enzyme activities were measured using both spectrophotometry and fluorimetry in 52 patients out of 145 patients who visited the metabolism unit during one year. Twenty-five of those patients (10 newborns and children; 3 parents) were diagnosed with complete BD, 15.4% (8 children) with partial BD and 59.6% (17 children, 14 parents) were healthy according to clinical examination, laboratory and genetic test results.

In our study, 3 out of 13 of the patients who were clinically diagnosed as complete BD and received 10 mg biotin/day were genetically tested and confirmed as complete BD with a compound heterozygous mutation (2 patients) and a homozygous mutation (1 patient). According to spectrophotometric test results, only 6 of them were diagnosed as complete BD and 7 of them as partial BD. According to fluorimetric test results, 12 of them were confirmed as complete BD whereas only 1 of them was partial BD. Serum samples of 8 patients, who were clinically diagnosed as partial BD and treated with 5 mg biotin/day, were evaluated both with the spectrophotometric and fluorimetric method. According to the spectrophotometric method, only 1 of them was

healthy and the rest of them were confirmed as partial BD while 4 had complete BD and 4 were confirmed as partial BD with the fluorimetric method. Two of the 4 patients who were diagnosed as complete BD according to the fluorimetric assay were investigated genetically and confirmed as complete BD with compound heterozygous and homozygous mutations. Patients whose Guthrie test results revealed trace enzyme activity and who were suspected of BD were genetically tested. It was found that 2 out of 8 patients had compound heterozygous mutations, 3 had homozygous mutations while 3 of them were healthy. Among the genetically healthy carrier patients, only one had shown partial BD both by spectrophotometric and fluorimetric methods. Consequently, the patient was clinically diagnosed as partial BD and 5 mg biotin/day treatment was prescribed.

According to the data of the patients and the test results, we have concluded that the fluorimetric method had 100% sensitivity, 97% specificity, 95.5% positive predictivity and 100% negative predictivity that means when there is a true disease, probability of the fluorimetric assay results to become negative is 0%. Spectrophotometric method had 90.5% sensitivity, 93.7% specificity and those values are lower than the fluorimetric method values.

Discussion

Newborn screening tests have vital importance for the timely detection of numerous congenital genetic and metabolic disorders to prevent morbidity, mortality, and disability (18, 19). In developed countries, the routine screening panels may include 20 or more newborn screening parameters as a routine part of the care. However, many countries already have screening programmes for PKU and hypothyroidism (19). It is very important to find out the most specific, sensitive, accurate reproducible biomarkers for modern laboratory medicine, to examine normal biological processes, pathogenic processes, or pharmacologic responses (18–26). There are various types of enzyme deficiencies, which differ in severity and treatment. For instance, glucose-6-phosphate dehydrogenase deficiency is the most common human congenital defect of metabolism. It affects a part of the population, and usually shows no signs or symptoms until exposed to certain medications, foods or infections (21). Deficiency of this enzyme does abide by the above criteria to some extent albeit neonatal screening for this enzyme deficiency has been suggested in few researches (27).

Autosomal recessive metabolic diseases including BD are very common in Turkey (28). Baykal et al. (29) diagnosed 66 complete BD and 36 partial BD patients with newborn screening between 1991 and 2005. We have screened 52 patients in one year and our sample included 13 complete BD and 8 partial

BD patients. However, this high BD patient number is not surprising, as our data do not present the population based BD screening. Our hospital collects patient samples which are already analysed in the Laboratories of the Turkish National Health Ministry with Gutrie screening test and diagnosed as »BD suspected«.

There are numerous methods for the determination of biotinidase activity using different protocols and some of these studies are reporting the advantageous properties of fluorimetric methods over the other ones (30, 31). A novel assay to quantify biotinidase concentration in dried blood spots was developed and optimized on the digital microfluidic platform that correctly identifies normal and affected samples from newborn dried blood spots (32). Researchers reported patients at the age of 5, 10, and 11 admitted first time to the hospital for acute visual loss and suspected of BD (33, 34). In other studies, the patients who reported to the hospital with rash, ataxia, paraparesia symptoms at the age of 3, 15, 1.5 and 2 were diagnosed with BD (35–38). Considering the clinical research, BD cases could be asymptomatic or late symptomatic; additionally, BD patients should be treated and followed up even if they are asymptomatic. It is clear that laboratory specialists need diagnostic tests with high sensitivity to identify asymptomatic BD patients. Patients with BD could only be treated with pharmacological doses of a biotin supplement (5 mg biotin/day for partial BD, 10 mg biotin/day for complete BD patient treatment dose). BTD enzyme activity should be correctly measured to plan treatment dose for each patient and to follow up each patient's treatment.

Human serum BTD activity was measured with the spectrophotometric method by different researchers using N-biotinyl PABA as a substrate and the healthy human serum BTD activity range was found to be 4.68–6.75 U/L (12), 4.30–7.54 U/L (9) and 5.70–11.2 U/L (35). Healthy human plasma BTD activity range measured by the fluorimetric assay using BAQ as a substrate was found to be 2.17–5.53 U/L (11). Our findings of healthy human serum and plasma BTD activity range are compatible with the earlier research results.

Our study results show that the fluorimetric method could diagnose true patients wihout missing cases, but the spectrophotometric method has a risk of misdiagnosis; 9.5% of the true patients may be accepted as healthy persons. The risk could seem not so high but BD could be easily and completely treated without complications if it is diagnosed in the early phase. If there is a delay in diagnosis, complications and death might occur. Therefore, it is important to choose the method with high sensitivity to diagnose BD correctly on time.

Spectrophotometric method is a cost effective test and thus the most frequently used diagnostic assay for BTD measurement even if its sensitivity is

lower than the one of the fluorimetric method. Spectrophotometric method is time consuming with a high failure rate during the assay. The incubation time of the fluorimetric method is longer than that of the spectrophotometric method, its substrate used during the enzyme activity assay is expensive, dissolves only at room temperature in 5 hours and can be stored at room temperature for only 5 days. When these problems are solved, the fluorimetric method may be preferred for BTD measureatr with a lower failure ratio and higher sensitivity compared to the spectrophotometric method.

Conclusions

In summary, we may conclude that serum BTD enzyme keeps its stability for 2 hours at room temperature, one day at 4 °C, four months at –20 °C and –80 °C. During sample collection and transportation, we considered the stability conditions. In our study, the sensitivity was 100% for the fluorimetric method and 90.5% for the spectrophotometric method. Although the differences between the sensitivity of the two methods are not statistically significant, the method with higher sensitivity should be preferred for the early and right diagnosis of BD. Considering the high frequency of BD in our country, the fluorimetric method with high sensitivity should be used to diagnose BD but further research is needed to improve some factors such as high substrate costs and storage/handling conditions.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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