Annals of Medical Research
DOI: 10.5455/annalsmedres.2019.07.415 2019;26(10):2306-11

Original Article

Specificity and sensitivity of biotinidase activity measured from dried blood spot by colorimetric method

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Abstract

Aim: A variety of methods are used to determine biotinidase activity (BA), such as different substrates, biological samples, and analytical techniques. In this study, we aimed to discuss the specificity and sensitivity of the fluorometric method used in the measurement of biotinidase activity in the newborn screening program (NSP) in Turkey.

Material and Methods: Medical records of 164 patients who were referred to our clinic with the diagnosis of biotinidase deficiency (BD) from primary health care institutions were evaluated retrospectively. According to this classification, those with normal BA were included in the negative group and those with partial or profound BD were included in the positive group.

Results: Four patients had profound and 66 patients had partial BD, whereas 94 patients had normal BA. ROC analysis was performed to determine the specificity and sensitivity of BA from dry blood spot. Analysis showed 41% sensitivity and 39% specificity for BA≥56.91 MRU (AUC: 0.339, p <0.001).

Conclusion: TIt was thought that low efficacy of fluorometric method used for the diagnosis of BD in the NSP may cause diagnostic delay. It is thought that a more effective neonatal screening program can be applied by using tandem mass spectrometer method.

Keywords: Biotinidase deficiency; fluorescence; inborn errors of metabolism; neonatal screening.

INTRODUCTION

Biotin is a water-soluble vitamin that is frequently found bound to proteins in small amounts in natural foods. The classic role of biotin is to function as the coenzyme of four important carboxylases playing a role in gluconeogenesis, fatty acid synthesis and catabolism of various amino acids (1,2). Covalent binding of biotin to the four inactive apocarboxylases catalyzed by holocarboxylase synthetase is required for the production of active holocarboxylases. These inactive apocarboxylases are acetyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase and propionyl-CoA carboxylase (1,2). When these apocarboxylases are examined, it is seen that they have various functions in the body. In biotinidase deficiency (BD), various diseases such as seizures, hypotonia, ataxia, dermatitis, hair loss, mental retardation, lactic acidosis, organic aciduria and also fetal malformations can be seen (3-6). Early diagnosis of the disease is extremely important in patients with BD, who are untreated and/or diagnosed late, as various neurodevelopmental problems may occur both in early childhood and adolescence (7-11).

Various methods such as different substrates, biological samples and analytical techniques are used to determine biotinidase activity (BA) (12). Samples obtained from dried blood spot specimens in newborn screening programs (NSP) can be measured using different methods. The most common of these methods is the use of different substrates. The natural substrate of biotinidase (BTD) is biocytin, but since BTD is also capable of hydrolysis of amide bonds between biotin and different structures, artificial substrates have been developed to measure the activity of this enzyme. In the colorimetric measurement method, biotinyl-p-aminobenzoic acid is used as a substrate (12). There is also an alternative method for determining biotinidase activity using biotinyl-

Received: 18.07.2019 **Accepted:** 18.09.2019 **Available online:** 22.10.2019
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6-aminoquinoline as the substrate. This method is based on a semi-quantitative fluorometric method (12,13). Although, today, more precise measurements can be made with liquid chromatography (LC) by the combined mass spectrometry (MS) method compared to colorimetric and fluorometric methods, this method is not widely used yet (12). Within the scope of NSP in Turkey, BA is measured from dried blood spot specimens by means of fluorometric method (14). Today, more accurate and precise measurements are made by using methods called tandem mass spectrometry (MS-MS) in NSP (15). However, since many different diseases can be detected at the same time with the MS/MS method, it is now used as a more cost-effective method in NSP in many developed countries (16,17).

The aim of this study was to investigate the specificity and sensitivity of the fluorometric method used in BA measurement in NSP in Turkey.

MATERIAL and METHODS

This retrospective study was conducted at the pediatric metabolic diseases clinic in Turkey between 01.03.2017 and 01.04.2019. After the approval of the local ethics committee, written informed consent was obtained from the parents of the patients who participated in this study. This study was carried out in accordance with the principles of the Declaration of Helsinki, 2008, and was approved by the local ethics committee in Turkey (Approval date and number: 07.01.2019, Session 1, 190103). Patients with suspected BD, who were referred to our clinic for the first time from primary health care institutions within the scope of NSP applied in Turkey were included in the study. Physical examination findings, height and body weight information of the patients included in the study, BA values measured by fluorometric method from dried blood spot specimens in primary health care institution and BA values measured by colorimetric method in our clinic were recorded. According to BA values measured from venous blood by colorimetric method, those with BA <10% were defined as profound biotinidase deficiency (PFBD), while those with BA of 10-30% were defined as partial biotinidase deficiency (PBD), and those with BA ≥ 30% were defined as normal biotinidase activity (NBA) (1,11). According to this classification, those with NBA were included in the negative group and those with PFBD or PBD were included in the positive group. Patients who had previously been diagnosed with BD and those whose serum BA measurements differed during clinical follow-up after being diagnosed with BD (those with NBA in the second measurement, or those whose second measurement is PBD while their first measurement is PBD or those whose second measurement is PFBD) were excluded from the study.

Determination of Serum Biotinidase Enzyme Activity

Two-to five mL venous blood sample was taken into the gelose tube (Vacutainer SST II advance - Becton, Dickinson and Company Franklin Lakes, NJ, USA). The blood sample was centrifuged at 10000 rpm for five minutes. After adding 100 µl of biotinidase to the wells, 10 µl of the supernatant formed by centrifugation was added to the wells to determine BA by colorimetric method (ODAK Neonatal Biotinidase Assay). Samples were incubated in a drying-oven at 37 °C for two hours. After 100 µl of trichloroacetic acid reagent was added to the well, the samples were centrifuged at 4000 rpm for five minutes. 100 µl of the supernatant formed by centrifugation was (taken and) added to new wells. After 30 µl of Reagent-1 was added to the wells, they were incubated at room temperature for five minutes, during which they were stirred gently by hand for 30 seconds. After this process, 30 µl of Reagent-2 was added and they were incubated again for five minutes, during which they were stirred slowly by hand for 30 seconds. Finally, after 30 µl of Reagent-3 was added to the wells, they were incubated at room temperature for five minutes, during which they were stirred gently by hand for 30 seconds. The resulting absorbances were read by Thermo Scientific Multiskan GO spectrophotometer (Thermo Fisher Scientific) at 570 nm wavelength.

Determination of Biotinidase Enzyme Activity from Dried Blood Spot Specimens

In this program, it was stated that blood samples should ideally be transferred from heel sticks to a filter paper in accordance with the manufacturer's instructions after adequate feeding between 48th and 72nd hours after birth. Dried blood spots from heel sticks were placed on filter papers in accordance with manufacturer's instructions for BA study by colorimetric method in all primary health care institutions in Sanliurfa (Whatman 903). The samples were stored in the refrigerator at 4 °C during the period from drying at room temperature to laboratory analysis. Samples were transferred to the central laboratories under the authority of the Public Health Institution of Turkey under room temperature conditions twice a week. The measurement result was determined centrally within 72 hours. The sample with 3 mm diameter taken from the blood spot on blotting paper was incubated for five hours at 37°C in a dark colored micro well (Trimaris fluorometric biotinidase kit) with 50 µl of reaction mixture [0.15 M potassium phosphate buffer (pH 6.5), 1.5 mM dithioerythritol and 0.075 mM biotinidase substrate]. After 250 µl of ethanol was added, samples in the well were incubated for another hour at room temperature. After the samples were centrifuged for five minutes at 3000 rpm, fluorescence measurements were carried out (Wallac Victor2 1420 Multilabel Counte) at excitation wavelength of 360 nm and emission wavelength of 460 nm to determine the BA from the dried blood spot specimens. According to this conclusion, those with a BA value of 65 Motion Reference Unit (MRU) and above were assessed as normal, while those with a BA value of 65 MRU (suspected BD) were re-sent (14,18). Patients with a BA value below 65 MRU in the repeat sample were referred to our clinic.

Statistical Analysis

Statistical analysis of the data was performed using

SPSS 24.0 software (SPSS Inc. Chicago, IL, USA). Visual (histogram and probability plots) and analytical techniques (Kolmogorov-Smirnov, Shapiro-Wilk tests) were used to evaluate whether the variables follow normal distribution. Continous variables were analyzed with either Student t test or Mann-Whitney U test depending on distribution and homogeneity of the data. Categorical variables were analyzed using Pearson's chi-square or Fisher's exact test (When any of the theoretical values observed in the 2x2 table is <5). Specificity and sensitivity analysis was performed using receiver operating characteristic (ROC) curve analysis technique. As a result of the analysis, the area under the curve (AUC) values were examined. Statistical significance level was accepted as p <0.05 in all statistical analyses.

RESULTS

Of the 164 patients included in the study, 73 were male (44.5%) and 91 (55.5%) were female. Four patients (2.4%) had PFBD, 66 patients (40.2%) had PBD, and 94 patients (57.3%) had NBA. The mean age of the patients at the time of diagnosis was 96.30±138.30 (min-max, 2-828) days. Seventy (42.7%) of the patients referred to us with a preliminary diagnosis of BD from primary health care institutions were in the positive group and 94 (57.3%) were in the negative group. Gender, height and body weight distributions of the positive and negative groups were similar (Table 1). The median BA measured from dried blood spots was significantly lower in the positive group than in the negative group (Table 2). Similarly, the mean serum BA was significantly lower in the positive group than in the negative group (Table 2). Family history of BD in siblings and frequency of consanguineous marriages were significantly higher in the positive group than in the negative group (Table 1). Physical examination findings were normal in most of the patients. When pathological physical examination findings were examined, four patients had diffuse seborrheic dermatitis and alopecia. All of the patients with pathological physical examination

Table 1. Sociodemographic and clinical evaluations of cases

	Positive group (n=70) BTD activity < % 30	Negative group(n=96) BTD activity ≥ % 30	p value
Gender (n) Male / Female	28/42	45/49	[a]0.34
Age/Day Median (min-max)	32.50(3-828)	42(2-630)	[c]**<0.001**
Consanguineous marriage among the parents (n), Yes / No	27/43	13/81	[a]**<0.001**
BD story in his brother (n), Yes / No	11/59	1/93	[a]**<0.001**
Lenght percentile (n)			
<3 p	**<3 p**	-	
3-10 p	**3-10 p**	6	
10-25 p	**10-25 p**	11	[b]0.85
25-50 p	**25-50 p**	22	
50-75 p	**50-75 p**	27	
75-90 p	**75-90 p**	17	
90-97 p	**90-97 p**	5	
>97 p	**>97 p**	6	
Weight percentile (n)			
<3 p	5	5	
3-10 p	5	8	
10-25 p	11	19	
25-50 p	16	23	[b]0.98
50-75 p	14	18	
75-90 p	9	10	
90-97 p	5	6	
>97 p	5	5	

[a]: Pearson chi square test; [b]: Fisher's exact test; BTD: Biotidinase; SD: standart deviation; BD: Biotidinase deficiency [c]: Mann whitney U test

findings were in the PFBD group.
ROC analysis was performed to determine the specificity and sensitivity of BA measured from dried blood spots of 164 patients with biotinidase deficiency preliminary diagnosis.
As a result of the analysis, AUC:0.339, p<0.001, was found and 41% sensitivity and 39% specificity were determined for the MRU value of ≥56.91 (Figure 1). There was a weak positive correlation between BA in dried blood spots and serum BA in Pearson correlation analysis (r=0.205, p=0.009).

Table 2. Comparison of BTD activity measurements of groups

	Positive group (n=70) BTD activity < % 30	Negative group(n=96) BTD activity ≥ % 30	p value
Dry blood spot BTD,(MRU) Median(min-max)	53.75(6.32-65)	59.14(2.1-79.7)	[a]**<0.001**
Serum BTD, (%) mean±SD	22.15±5.53	49.05±16.83	[b]**<0.001**

[a]: Mann whitney U test; [b]: Independent samples t test; BTD: Biotidinase; SD: standart deviation; BD: Biotidinase deficiency; MRU: Motion Reference Unit

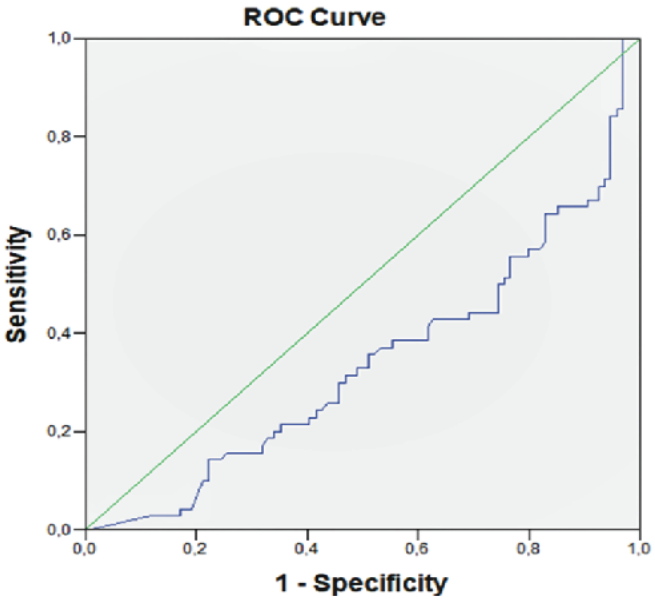

Figure 1.ROC analysis of dry blood spot biotidinase measurements

DISCUSSION

Biotinidase deficiency is a metabolic disease that can be seen with various neurological and dermatological complications (1,4,11). This autosomal recessive disease can be seen more often in regions such as Turkey where consanguineous marriages are common (19,20). Similarly, we found that rates of consanguineous marriages and family history of BD in siblings were more frequent in the positive group than in the negative group. Physical examination findings were found to be mostly normal in the positive group, and this was thought to be due to the presence of PBD in the majority of patients. As a matter of fact, all of the pathological physical examination findings were observed in patients with PFBD. In this study, the incidence of combined PFBD and PBD was 1:1177 in Şanlıurfa province where 63570 live births occurred in 2018. In a study conducted earlier in Turkey by Baykal et al. (21), this ratio was found as 1:11614. While the incidence of combined BD in the literature is around 1:60000, the fact that the combined BD incidence is more common in Şanlıurfa, located in Southeastern Anatolia, may be due to the prevalence of consanguineous marriages in this province.

Early diagnosis of BD is extremely important, as delays in the diagnostic process of the disease may lead to serious mental problems (22-24). As a matter of fact, when the reasons for delay in NSP were examined in the past, the most common reason was reported as not having an effective NSP (25,26). In our study, the median age of diagnosis was 42 days in the positive group and 32.5 days in the negative group. Although patients in the positive group were diagnosed significantly earlier than those in the negative group, the median age of diagnosis in the positive group was over one month. However, when the minimum and maximum diagnostic ages were examined in this study, diagnosed patients older than two years of age were also identified.

In this study, BD was detected in 40% of the BD suspected patients referred to our clinic within the scope of NSP. In addition, the fluorometric method used in NSP was found to have a sensitivity of 41% and a specificity of 39% for an MRU value of ≥56.91. The fact that the specificity and sensitivity of the fluorometric method used for BD screening under the current NSP being not sufficient is thought to contribute to possible delays in the diagnostic process. However, Erten et al. (27) conducted in Turkey by fluorometric method has been found to be more successful. In this study, samples of patients of various age groups were obtained under the same laboratory conditions. This suggests that the samples obtained in primary health care institutions may not have been kept under appropriate conditions until the time of operation. Furthermore, we believe that the effectiveness of the method used in NSP may not be fully demonstrated due to the inclusion of participants from various age groups (27). In a study conducted by Heideri et al. (26) in Iran, the cause of diagnostic delays within the scope of NSP for phenylketonuria was investigated. As a result of the study, it was reported that low socioeconomic status, insufficient awareness and limited access to screening

program could lead to diagnostic delay. Although the NSP is effectively implemented in Turkey, the MS/MS method is not yet used within the scope of the national NSP (14). Today, NSPs are performed effectively with the MS/MS method in many developed countries (16,17). With the use of MS/MS method in NSP in Turkey in the near future, many diseases can be diagnosed simultaneously in a shorter time and with greater precision.

Limitations of the study

In this study, it was thought that the difference between the methods used in sampling and storage of NSP in primary care institutions could not be fully illustrated so the other reasons that could be effective in the diagnostic delay and effectiveness of methods could not be revealed. In addition, only the incidence of BD in 2018 was given due to the lack of data regarding 2017 and 2019. The frequency of the disease in this geography and used diagnostic methods needs to be revealed more clearly with multi-centred studies in Southeast Anatolia.

CONCLUSION

This study is important in terms of being conducted in one of the regions with the highest prevalence of BD worldwide when the literature data is examined. In addition, this study is also important for examining the specificity and sensitivity of current national NSP for BD. It is thought that low efficacy of fluorometric method used for diagnosis of BD in NSP in Turkey may lead to diagnostic delay, and that NSP can be performed more effectively with the use of the MS/MS method in the near future.

Competing interests: The authors declare that they have no competing interest.
Financial Disclosure: There are no financial supports.
Ethical approval: This study was approved by the Institutional Ethics Committee and conducted in compliance with the ethical principles according to the Declaration of Helsinki.

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