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Accurate determination of Biotinidase activity in serum by HPLC and its utilization as second tier test for the confirmation of initial positive newborn screening results

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ABSTRACT

Diagnosis of Biotinidase deficiency (BTD) is extremely important to avoid several neurodevelopmental problems in early childhood. Colorimetric and fluorometric methods lack specificity and selectivity due to several interferences resulting in a high number of false positive results. We developed an HPLC method for BTD activity in serum with fluorescent detection. In colorimetric assays, biotinidase attacks the amide linkage of the artificial substrate biotinidyl-4-aminobenzoic acid (B-PABA) and releases *p*-aminobenzoic acid (PABA), which is converted to a purple dye by diazotization reaction. The newly developed method injects the reaction mixture directly into the HPLC column and quantifies using a six-point calibration curve without coupling and diazotization reaction. The method is linear over the 5–1000 μmol/L range. The detection and quantitation limits were 2.5 μmol/L and 5.0 μmol/L, respectively. When compared with colorimetric assay, the correlation coefficient (R^2) was 0.9963. The within-assay and between-assay precision was <10.0% for four levels of quality control samples. No significant variation in BTD activity was detected due to hemolysis, icteric, and lipemic samples. The newly developed method eliminates the potential interference due to the presence of aromatic amines and significantly reduces the false positive results observed with the colorimetric method. It is simple, specific, sensitive, faster in sample preparation, and requires a small sample volume. The newly developed HPLC method was used in our laboratory as a secondary tier test for initial positive BTD samples from newborn screening programs. To our knowledge, no similar HPLC method has been reported to date.

1. Introduction

Biotinidase deficiency (BTD) is an autosomal recessive inherited metabolic disease. BTD cleaves biocytin to produce a water-soluble vitamin called biotin [1]. Biotin acts as a coenzyme for four important carboxylases involved in gluconeogenesis, amino acid catabolism, and fatty acid synthesis [2,3]. In biotinidase deficiency, all carboxylase enzyme activities are impaired, resulting in the accumulation of different molecules, including lactic acid and alanine due to pyruvate carboxylase deficiency; 3-hydroxy propionic acid and methyl citrate due to propionyl-CoA carboxylase deficiency; 3-methylcrotonylglycine and 3-hydoxyisolerate due to 3-methylcrotonyl-CoA carboxylase deficiency and different derivatives of hexanoic acid in acetyl CoA carboxylase deficiency [4]. All patients with multiple carboxylase deficiencies have low biotinidase activity. Biotinidase deficiency occurs in various diseases such as ataxia, seizures, mental retardation, hypotonia, dermatitis, hair loss, lactic acidosis, organic aciduria, and fetal malformations [5–8].

Early diagnosis of BTD deficiency is essential in untreated patients to prevent physical and neurological abnormalities in early childhood and adolescence [9–11].

Biotinidase deficiency is diagnosed by measuring its activity in

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serum samples. Several analytical methods have been used to quantify biotinidase activity in serum. In a microbiological method, biotinidase activity is determined by measuring biotin cleaved from biocytin. This method is very laborious, requires a more extended sample preparation, and is not robust [12]. The most common colorimetric procedure was reported by Heard et al. In this method, biotinidase hydrolyzes the artificial substrate biotinyl-*p*-aminobenzoate (B-PABA) to *p*-aminobenzoic acid, which undergoes a diazotization reaction with *N*-1-naphthylethylenediamine dihydrochloride. Interference due to aromatic amines, high chloride concentration, and azo dye instability are the main drawbacks of colorimetric assays [13].

In another report, a sensitive fluorometric rate assay is developed with a new substrate called biotinyl-6-aminoquinoline. The problems with this method are the longer incubation time (2 h), higher inter-assay precision (> 10%), and the use of a separate blank for each serum sample [14,15].

Several liquid chromatographic methods combined with mass spectrometry (LCMS & LCMSMS) have also been developed for the activity measurement of biotinidase in dry blood spots and serum samples with high accuracy, precision, sensitivity, and selectivity. This technique is unavailable in most laboratories due to its high cost, lack of expertise, and complexity [16,17]. Hayakawa et al. reported two HPLC methods using B-PABA and biotinyl-6-aminoquinoline as a substrate in milk and serum [18,19]. The method required the synthesis of B-PABA, which is difficult to perform in a routine clinical laboratory. Other disadvantages include the larger sample volume (1 mL serum), the longer run time (26 min), the dialysis of a serum sample, and the lack of interference, selectivity, and linearity studies. The boiling of the substrate and enzyme mixture to stop the reaction enhances the production of PABA and reduces enzyme activity. Overall, these two methods are too complicated for routine analysis. Therefore, a faster, more straightforward, and specific method is required that should be free from all the above problems.

2. Experimental

2.1. Chemical and reagents

Biotinidyl-*p*-aminobenzoic acid (B-PABA), *p*-aminobenzoic acid (PABA), dithiothreitol, ammonium acetate, sodium hydroxide, and trichloroacetic acid were purchased from Sigma Aldrich. HPLC-grade methanol and acetic acid were purchased from Fisher Scientific. The 150 mm × 5 mm with 5 μm particle size HPLC column was obtained from Atlantis. HPLC-grade water was collected from the Millipore unit.

Ammonium acetate buffer (10 mM, pH 5.0) and HPLC grade methanol were used as mobile A and B, respectively. HPLC column was equilibrated with a 70% mixture of A & B. Gradient elution started at 0.6 mL/min with 70% mobile phase A, which changed to 20% after 6 min for the next 2 min. The gradient was brought to the initial condition with 70% mobile phase A for 2 min before the next injection.

Trichloroacetic acid (3%) was used as a precipitation reagent. The substrate solution of 1.5 mM was prepared by dissolving 5 mg of B-PABA in 9 mL of HPLC grade water and 1 mL of 0.1 N sodium hydroxide. Acetate buffer (400 mM) of pH 6.0 and dithiothreitol solution (100 mM) were prepared in water.

A stock solution of *p*-aminobenzoic acid (2500 μmol/L) was prepared in water. Six points calibration standards (5, 25, 100, 250, 500, and 1000 μmol/L) were made by diluting stock solution in HPLC grade water and stored at 2–4 °C until consumed. A pool serum sample was used as normal quality control. Two abnormal quality controls were prepared by heating normal pool serum for several hours at 37 °C. The quality controls were aliquoted (25 μL each) and stored at −20 °C for one year.

2.2. Sample collection

Blood samples in red top tubes were collected from all newborn patients with positive BTD screening results tested in our laboratory using dry blood spots. Serum separated by centrifugation and stored at −20 °C until analysis.

2.3. Extraction procedure

25 μL of water blank, calibration standard, quality control, and patient serum samples were transferred into separate Eppendorf tubes. 100 μL of acetate buffer and 25 μL of dithiothreitol solution were added into each tube. 250 μL of water was added in blank and all calibration standard solutions, whereas 200 μL of water and 50 μL substrate solution were added into each quality control and patient sample. A serum blank was also prepared for each patient sample similarly without adding substrate. All samples were vortexed for 5–10 s and incubated at 37 °C for 1 h, followed by the addition of 400 μL of trichloroacetic acid to stop the reaction. After leaving 10 min at room temperature, the mixture was centrifuged at 15000 RPM, and the supernatant was transferred into HPLC vials. A batch of 20–30 patient samples can be easily prepared within two hours including one hour incubation time.

2.4. HPLC equipment

A high-performance liquid chromatography system was purchased from Waters, USA. The Alliance HPLC 2790 separation module consisted of a binary pump and an auto-sampler. 2475 fluorescent detector and Waters Empower 2 software were used to detect and process the *p*-aminobenzoic acid peak (Em = 276, Ex = 340). A volume of 25 μL from calibration, quality controls, and patient samples was loaded into the HPLC system.

2.5. Method validation

2.5.1. Selectivity and specificity

The method's selectivity was assessed by analyzing ten serum samples collected from five hospitals and incubating them with and without substrate (B-PABA) at 37 °C for 1 h. The retention time of PABA in pure standard solution was compared with the chromatogram obtained with the serum sample.

In addition, the effect of most common interferences was also evaluated by spiking serum pool samples with three increasing concentrations of hemolysate, bilirubin, and triglyceride. The mixtures were mixed and analyzed by the developed HPLC method.

2.5.2. Linearity and range

Six calibration standards of PABA were prepared in water at a concentration of 5, 25, 100, 250, 500, and 1000 μmol/L. These standards were injected into the HPLC column after the addition and mixing with all reagents except the substrate solution. A linear response was obtained by plotting concentration against the corresponding peak area. Regression analysis was performed.

2.5.3. Accuracy and precision

Within the run, accuracy, and precision were determined by analyzing five replicates of recovery samples prepared by the spiking stock standard solution of PABA into pool serum at four different concentrations (50, 100, 200, and 300 μmol/L). Between runs, accuracy and precision were tested by analyzing five replicates of the same samples for three consecutive days.

2.5.4. Method comparison

For method comparison, twenty patient samples of varying concentrations were analyzed by colorimetric and HPLC methods to check method accuracy. The slope, intercept, bias, and correlation coefficient were calculated.

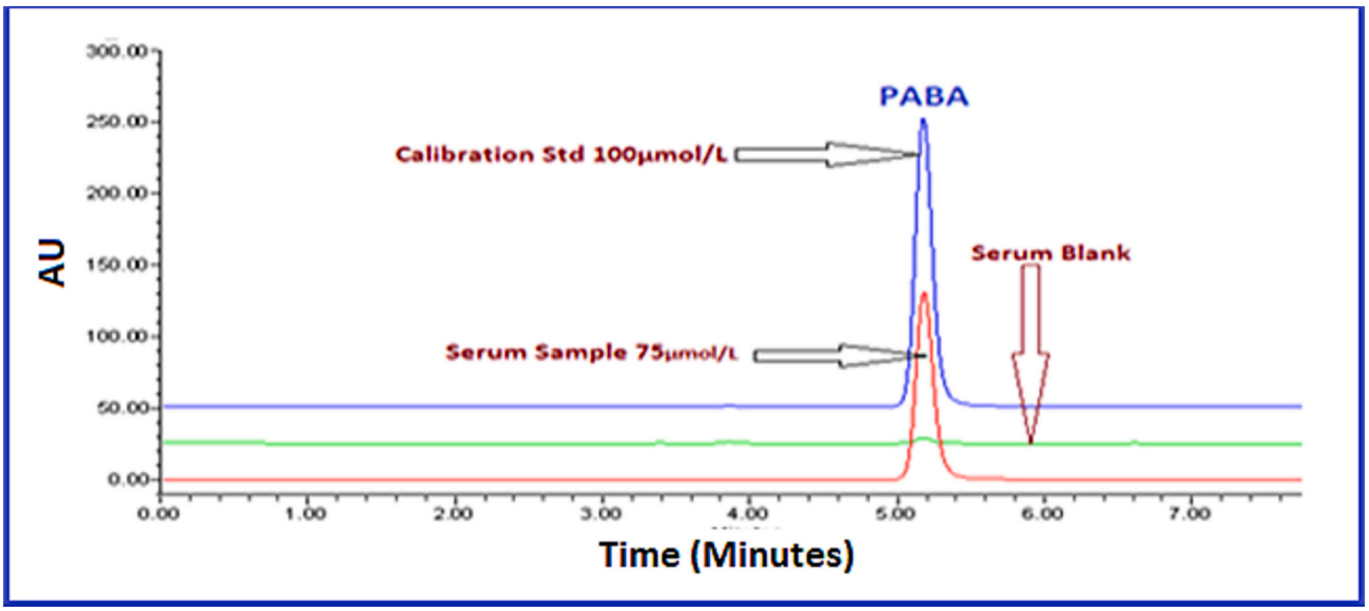

Fig. 1. Chromatograms of Blank, Standard and Serum Sample.

2.5.5. Limit of detection and quantitation (LOD and LOQ)

The method's sensitivity is determined by analyzing low-concentration serum samples prepared by spiking PABA at 2.5 and 5.0 µmol/L. The spiked samples were mixed and analyzed by the procedure ten times to evaluate the limit of detection and quantification.

2.5.6. Carryover and system suitability parameters

Carry over study was conducted according to College of American Pathology common check-list of clinical chemistry tests. A low-concentration sample was injected five times to calculate the mean value. The same sample was tested five times after high samples, and another mean of low samples was calculated. The percentage carryover was obtained from the two mean values. System suitability, such as theoretical plates and tailing factor, was automatically calculated by empower software for all samples.

2.5.7. Reaction parameters and substrate concentration

Six samples containing 0, 10, 20, 30, 40, and 50 µL of serum were prepared with the same incubation time and substrate concentration to evaluate the effect of BTD on the PABA formation from the substrate. In another experiment, six serum samples were made and incubated with the same substrate concentration for 15, 30, 45, 60, 75, and 90 min to determine the effect of reaction time on the amount of PABA released. Similarly, serum samples were incubated with 10, 25, 50, 75, 100, and 125 of 1.5 µM substrate (B- PABA) for one hour at 37 °C to investigate the effect of substrate amount on the formation of PABA.

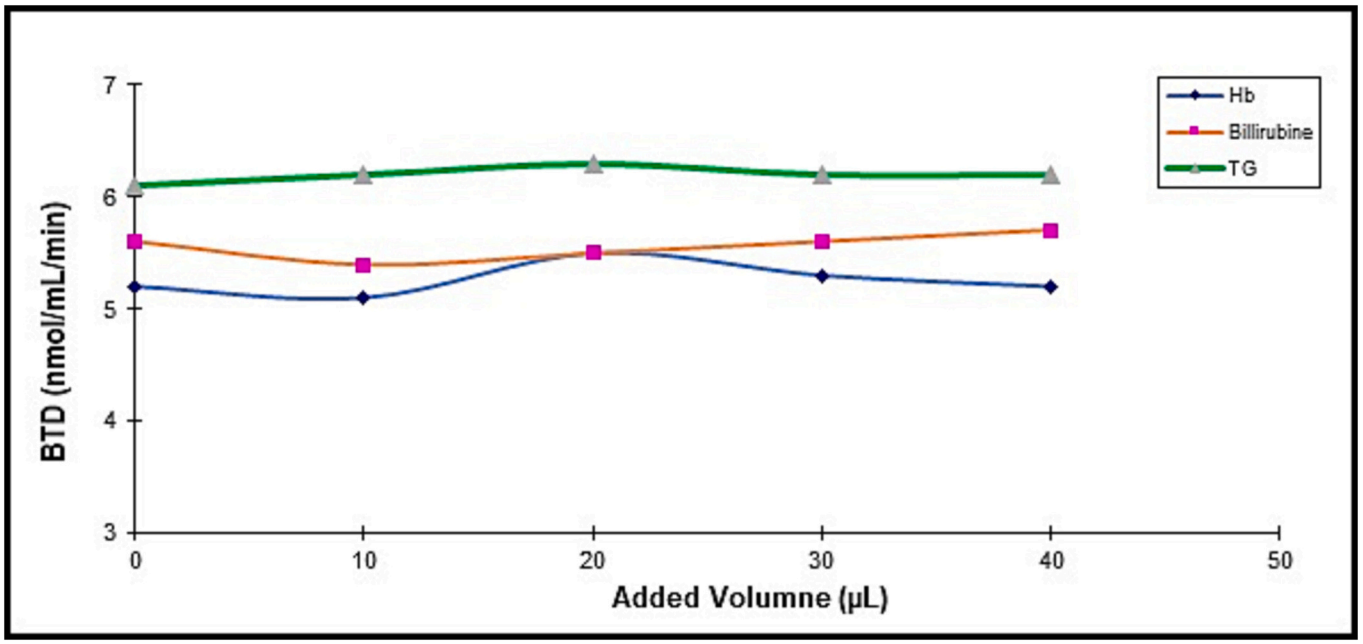

Fig. 2. Effect of interferences on BTD activity

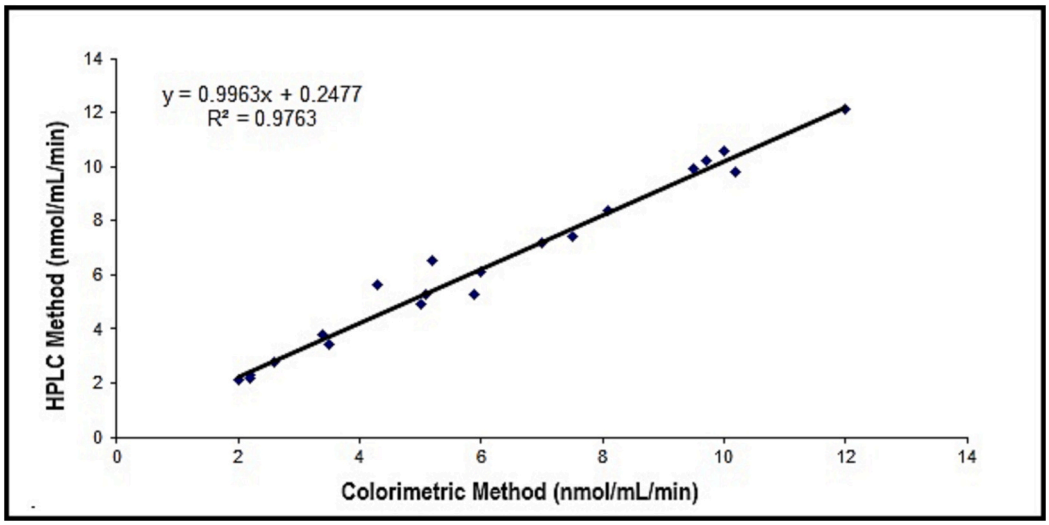

Fig. 3. Comparison of HPLC method with colorimetric method

3. Results and discussions

3.1. Second-tier test for positive newborn screening samples

The proposed HPLC method has been utilized as a second-tier test for initial positive samples of BTD from newborn screening programs performed in dry blood spots in our laboratory using a Perkin Elmer genetic screening processor. Four initial positive samples of BTD deficiency were identified. Serum samples were collected from these patients and analyzed using both the proposed HPLC method and the colorimetric method. Three of the four samples were confirmed as BTD deficiency by both methods, with the fourth sample being false positive. By using the HPLC method, the number of false-positive results from newborn screening, which were due to the high humidity and temperature in the region, was significantly reduced.. The method provided excellent results compared to the colorimetric method with 100% positive predictive value and negative predictive value (Table 3). Different reaction parameters of colorimetric method from Nenad Blau and HPLC parameters were also compared (Table 4).

3.2. Routine analysis

The current method has been used in our lab since 2021. Our routine analysis utilizes three levels of quality controls. Pooled serum was used as normal quality control (BTD > 5 nmol/mL/min), and two abnormal controls were prepared (BTD 2–4 nmol/mL/min) by heating the same pooled sample at 37 °C for several hours. These controls were aliquoted and stored at −20 °C for one year.

The published reference value of Biotinidase activity has been verified by analyzing 30 normal serum samples. However, a broader study is still required to differentiate between partial and profound deficiency by the HPLC method.

3.3. Specificity and selectivity

Serum samples were collected from five different sources in duplicate and incubated with and without substrate solution. The released PABA's retention time was compared with the pure standard solution injected directly without incubation with substrate. In all serum samples (blank) incubated without substrate solution, no peak was found at the retention time of PABA. In contrast, in all serum samples incubated with substrate solution, a significant peak was found at the same retention time as the pure standard solution of PABA, proving the specificity of the method. These results show that the method is free from interfering substances such as aromatic amines and serum components.

The chromatograms of serum blank (without substrate), serum sample (with substrate), and pure standard solution of PABA are shown in Fig. 1.

For the interference study three different pooled serum samples from normal patients were separately spiked with four increasing concentrations of hemolysate, bilirubin and triglyceride. These samples were mixed and analyzed once according to the proposed method. No interfering peak was found in the retention time of PABA, and the results were not significantly different from those of the non-spiked sample. The entire study was performed only once. The data are summarized in Fig. 2.

Table 1
Method validation data.

Parameter	Results of validation parameters			
Linearity	Range (µmol/L)	Slope	Intercept	R^2
	5–1000	0.9998	0.0	0.9999
Precision	% CV at 50 µmol/L	% CV at 100 µmol/L	% CV at 200 µmol/L	% CV at 300 µmol/L
Within Run ($N = 5$)	5.37	7.40	2.90	4.55
Between Run ($N = 15$)	9.50	6.31	5.15	4.35
Comparison ($N = 20$)	Slope	Intercept	Bias	R^2
	0.9963	0.2477	−0.23	0.9763
Sensitivity	Limit of Detection (µmol/L)		Limit of Quantitation (µmol/L)	
	2.5		5.0	
% Carry Over	0.20			
System Suitability	Retention Time (Min)	Theoretical Plate	Tailing Factor	Resolution Factor
	5.2	> 2000	< 1.2	NA

3.4. Method comparison

For method comparison, twenty patient samples of varying concentrations were sent to King Faisal Hospital for colorimetric analysis. The results were compared with the proposed HPLC method, and no significant bias was found, as shown in Fig. 3. The slope, intercept, bias, and correlation coefficient were measured (Table 1).

Table 2
Method recovery data.

Days	Replicates	Level 1 (50 μmol/L)		Level 2 (100 μmol/L)		Level 3 (200 μmol/L)		Level 4 (300 μmol/L)	
		Found (μmol/L)	Recovery (%)	Found (μmol/L)	Recovery (%)	Found (μmol/L)	Recovery (%)	Found (μmol/L)	Recovery (%)
	1	51.9	103.8	102.7	102.7	213.9	107.0	218.1	72.7
	2	44.5	89.0	97.4	97.4	216.3	108.2	271.4	90.5
Day 1	3	61.4	122.7	100.6	100.6	233.5	116.8	269.6	89.9
	4	52.0	103.9	117.2	117.2	205.9	103.0	272.0	90.7
	5	57.5	115.0	107.8	107.8	218.1	109.1	273.9	91.3
	1	58.3	116.6	104.2	104.2	213.4	106.7	282.5	94.2
	2	57.0	114.0	107.2	107.2	210.9	105.5	271.9	90.6
Day 2	3	66.8	133.6	103.4	103.4	225.5	112.8	276.2	92.1
	4	46.9	93.8	105.2	105.2	225.8	112.9	280.4	93.5
	5	62.1	124.2	108.3	108.3	220.6	110.3	268.1	89.4
	1	49.4	98.8	91.5	91.5	202.3	101.2	258.0	86.0
	2	50.6	101.2	95.1	95.1	204.4	102.2	258.3	86.1
Day 3	3	56.1	112.2	110.5	110.5	191.3	95.7	258.6	86.2
	4	49.5	99.0	102.2	102.2	205.0	102.5	283.9	94.6
	5	51.8	103.6	97.3	97.3	204.9	102.5	260.9	87.0
Mean Recovery		108.8		103.4		106.4		89.0	
Standard Deviation		12.3		6.5		5.5		5.3	
% CV		11.3		6.3		5.1		6.0	

Table 3
Analysis of Initial Positive Samples by HPLC and colorimetric Methods.

Samples	BTD in DBS	Serum BTD -HPLC (nmol/mL/min)	Serum BTD -Colorimetric (nmol/mL/min)	Reference (nmol/mL/min)
1	Remarkable	3.8	2.0	4.0–9.57
2	Remarkable	1.5	3.0	
3	Remarkable	2.8	3.3	
4	Remarkable	6.5	4.5	

Table 4
Colorimetric and HPLC parameters comparison.

Parameter	Colorimetric method	HPLC method
Sample Volume (μL)	50 μL × 4 = 200 μL	25 μL
Preparation Time (Min)	4 h	2 h
Incubation Time (Min)	60 min	60 min
Incubation Temperature	37 °C	37 °C
Diazotization Reaction	Required $NaNO_2$	Not Required
Coupling Reaction	Required	Not Required
Aromatic amine interferences	Yes (due to Diazotization)	No
False Positive Results	Yes	No
Calibration Point	1	6
Quality Controls	2	3

3.5. Calibration curve

Six calibration standards of PABA were prepared in water at 5, 25, 100, 250, 500, and 1000 μmol/L concentrations. These standards were treated as the patient sample; all reagents except the substrate solution were added. The resulting mixtures were injected into the HPLC system, and a linear calibration curve was constructed by plotting the peak area against the target concentrations. The regression analysis performed and results are given in Table 1. Serum-based calibration standards have to be studied instead of aqueous calibration standards.

3.6. Accuracy and precision

Within- and between-run accuracy and precision were determined according to FDA guidance for validating bioanalytical methods. Pool serum samples were spiked with the pure standard PABA at concentrations of 50, 100, 200, and 300 μmol/L and analyzed by the assay procedure without adding substrate. The analysis was repeated five times on three different days. The percent coefficient of variance for within and between run precision was <7.5% and 10.0%, respectively (Table 1).

The accuracy was determined by measuring the percentage recovery of PABA from the serum samples. The recovery test was repeated five times for three days with four different concentrations. The recovery range of PABA was 88–109% for all spiked serum samples (Table 2).

3.7. Sensitivity

Low concentrations of PABA samples (2.5 and 5.0 μmol/L) were prepared by adding a pure standard solution to the pooled serum. Each sample was analyzed ten times. The coefficient of variance (% CV) was <10 at both concentrations, and accuracy was within 100–110% with a good signal-to-noise ratio, so 2.5 μmol/L was considered the limit of detection and 5.0 μmol/L the limit of quantification (Table 1).

3.8. Mobile phase pH

Ammonium acetate buffer (10 mM, pH = 5) and HPLC methanol were used as mobile phases A and B. The PABA was separated from the substrate and other serum components on the Atlantic C_{18} HPLC column within 10 min using a gradient of 70% mobile phase A and 30% mobile phase B. The pH of mobile phase A was adjusted to 5.0 ± 0.1 to separate the PABA peak from other components **and** the unreacted substrate (Fig. 1). Decreasing the pH also shortens the retention time of PABA due to protonation of the amino group in the molecule, making it more polar and less retained on the C_{18} HPLC column.

3.9. Reaction parameters and substrate concentration

Different substrate concentrations (0.5, 1.0, 1.5, 2.0 mmol/L) were tested for 25 μL serum. The PABA concentration initially increases and becomes constant after 1.0 mmol/L. We chose 1.5 mmol/L as the optimal substrate concentration for routine analysis because BTD activity is consumed at this concentration as reported by Eszter Szabó et al. [16].

The amount of PABA increases linearly with increasing incubation time and serum volume, which is due to the hydrolysis of a larger amount of B-PABA to PABA. At the same time, the PABA concentration does not change significantly with increasing substrate amount (1.5 μM), as no enzyme remains in the serum sample for the reaction (Fig. 4).

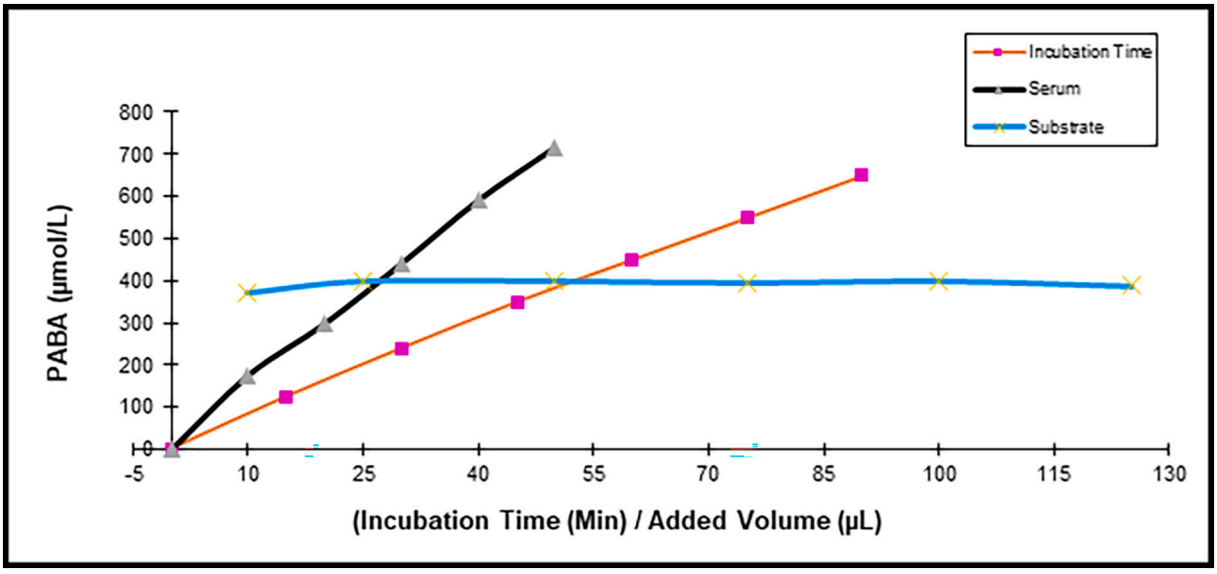

Fig. 4. Effect of incubation time, serum, and substrate on PABA Formation.

4. Conclusion

To our knowledge, the newly developed method has not been reported before. It has several advantages over colorimetric assay, such as reduction in false positive rate, use of six-point calibration, easier and faster sample preparation, low sample volume, and free from interferences due to aromatic amines.

Use of Artificial Intelligence

We used instatex.io for the appropriate description of our research in English. After use, the text was reviewed and edited before submission for publication in the Journal. Authors have full responsibility for the publication.

CRediT authorship contribution statement

Abdul Rafiq Khan: Writing – original draft, Validation, Methodology, Investigation. **Souad Al-Enazi:** Formal analysis. **Areej Al-Gahtani:** Formal analysis. **Saleh Al-Zahrani:** Formal analysis. **Syed Muhammad Saad:** Writing – review & editing. **Khalid Mohammed Khan:** Writing – review & editing. **Ali Alothaim:** Writing – review & editing, Supervision.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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