Application of Thiamine-Biotin Conjugate for Serum Thiamine Levels Measurement

Penggunaan Konjugat Tiamin-Biotin pada Pengukuran Kadar Tiamin dalam Serum

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ABSTRAK

Tiamin berfungsi sebagai koenzim untuk beberapa enzim yang terlibat dalam metabolisme karbohidrat. Mengingat pentingnya peran tiamin, maka dilakukan pengembangan teknik pengukuran tiamin yang analog dengan enzyme linked immunosorbent assay *(ELISA), dimana antibodi diganti dengan protein pengikat spesifik yaitu protein ikat tiamin kacang hijau (PITKH). Teknik pengukuran ini dilakukan secara kompetitif, kompetitor akan dikompetisikan dengan tiamin bebas yang akan diukur. Kompetitor tersebut berupa konjugat antara tiamin-biotin. Tiamin murni diikatkan dengan biotin menggunakan senyawa perangkai yaitu glutaraldehid. Pada analisis* liquid chromatography-mass spectrometry *(LC-MS) ditemukan 3 puncak. Puncak ke-3 merupakan konjugat tiamin-biotin. Dibuat kurva standar dan diperoleh persamaan garis lurus dengan nilai R^2 = 0,9986. Uji validasi menggunakan konjugat tiamin-biotin menunjukan nilai* coefficient of variation *(CV) = 3,81%, nilai ini lebih kecil dari CV Horwitz = 8,12%, akurasi dengan nilai* Recovery *(R) = 94% - 98%. Hasil ini menunjukkan syarat pengukuran dengan teknik* enzyme-labeled protein ligands assay *(ELPLA) sudah terpenuhi, dengan presisi dan akurasi yang baik. Aplikasi pengukuran kadar tiamin pada serum normal sebanyak 15 sampel didapatkan kadar tiamin berkisar 6,97 – 8,68 µg/mL. Dengan demikian, teknik ELPLA dengan konjugat tiamin-biotin sebagai kompetitor dapat digunakan pada pengukuran kadar tiamin dalam serum.*

Kata kunci: *tiamin, biotin, protein ikat tiamin kacang hijau, serum,* enzyme-labeled protein-ligand assay

ABSTRACT

Thiamine has a coenzyme function in several enzymes involved in carbohydrate metabolism. Considering the important role of thiamine, a thiamine measurement technique analogous to the enzyme-linked immunosorbent assay (ELISA) was developed, and the antibody was replaced by a specific binding protein named mung bean thiamine binding protein (MBTBP). The measurement technique was carried out competitively in which competitors would be competed with free thiamine to be measured. The competitor is a thiamine-biotin bond. Pure thiamine was bound to biotin using a coupling compound called glutaraldehyde. In the liquid chromatography-mass spectrometry (LC-MS) analysis we found 3 peaks. The third peak was the thiamine-biotin conjugate. A standard curve was made and the value of its straight-line equation was obtained R^2 = 0.9986. The validation test using thiamine-biotin conjugate showed a coefficient of variation (CV) value = 3,81% which was smaller than Horwitz CV = 8.12%, with the accuracy of the Recovery (R) value = 94% – 98%. These results indicated that the measurement requirements for the enzyme-labeled protein ligands assay (ELPLA) technique had been met with good precision and accuracy. The application of the serum measurements to 15 samples showed thiamine levels ranging from 6.97 – 8.68 µg/mL. Thus, the ELPLA technique with thiamine-biotin conjugate as a competitor could be used in the measurement of serum thiamine levels.

Keywords: thiamine, biotin, mung bean thiamine binding protein (MBTBP), enzyme-labeled protein-ligand assay

Introduction

Vitamins are essential organic molecules. Vitamin B1 or thiamine was known as aneurine or 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-5-(2-hydroxyethyl)-4-methylthiazolium has an important function as a coenzyme in several enzymatic reactions. Thiamine is an organosulfur compound with a molecular weight of 265 D/mol. The chemical structure of thiamine is a combination of pyrimidine and thiazole, which are a couple by a methylene bridge.[1,2]

Thiamine has an important role in energy metabolism, especially in the brain as a central nervous system. Thiamine was carried to the brain as thiamine diphosphate (TDP), which acts as the main coenzyme for the pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and transketolase. In the thiamine deficiency state, the activity of those enzymes will decrease due to changes in several intracellular or extracellular neurotransmitters.[1,3]

Various techniques had been developed for the measurement of thiamine levels in the human body, such as microbiological methods, thiochromes, high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA). The thiochrome technique had disadvantages in that the test required a relatively large amount of purified material and therefore, a longer examination time.[4] Thiamine measurement using the HPLC technique was very specific but required expensive reagents and equipment, higher cost, and specially trained personnel.[5] The common approach for the measurement of a small number of substances with relatively high precision and accuracy was the immunochemical technique such as enzyme-linked immunosorbent assay (ELISA) which used specific antibodies to the sample to be measured.[6] Referring to the ELISA technique, a specific thiamine binding protein that acted as an anti-thiamine-specific antibody was developed. Gunarti's research showed that thiamine binding protein (MBTBP) could be used as an anti-thiamine-specific antibody using ELISA analogs technique called enzyme-labeled protein ligands assay (ELPLA).[7]

This research was done to develop thiamine levels measuring technique in serum, which utilizes TBP from mung bean and thiamine-biotin conjugate in ELPLA method based on principles analogous to ELISA competitive. Because the molecular size of thiamine-biotin is not much different from free thiamine, it was expected that this conjugate in competitive technique could facilitate a faster reaction, equal chances, and more accurate results.

Methods

This study is an experimental study using mung bean thiamine binding protein as an anti-thiamine specific antibody, and a thiamine-biotin conjugate as competitors. This study was conducted in the Center of Hypoxia and Oxidative Stress Study, Departement of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. It was approved by the Ethics Committee of Faculty Medicine, Universitas Indonesia (No. KET-877/UN2.F1/ETIK/PPM.00.02/2020).

Materials

Mung Bean Thiamine Binding Protein (MBTBP) used was the result of post-affinity chromatography with levels of 1216 µg/mL, KH_2PO_4, Na_2HPO_4, Bovine Serum Albumin (BSA), biotin, avidin, thiamine, glutaraldehyde, buffer sulfate pH 6.0 and pH 7.4, buffer sulfate-tween, biotin peroxidase, tetramethylbenzidine, 2 N of sulfuric acid, ammonium format 20 Mm, human blood serums, and microplate 96 wells.

Protein Assay

The concentration of MBTBP was determined by the method of Warburg-Christian using bovine serum albumin as a

standard. Determined at the wavelength of 280 nm with BSA standard for measuring protein assay. The soluble MBTBP concentration was 1.216 mg/mL.[2,7]

Preparation of Thiamine-biotin Conjugate

The conjugate was prepared by mixing 2 mL of each thiamine and glutaraldehyde solution, homogenizing it using a rotator mixer for 15 minutes. 2 mL of the biotin solution was then added to the thiamine and glutaraldehyde solution mixture. The process of homogenizing a solution of thiamine, glutaraldehyde, and biotin utilized a rotator for 24 hours at room temperature and a darkroom to form a thiamine-biotin conjugate. The homogenized mixture was then stored in a cellophane bag and then dialyzed for 24 hours with the aqua-bidest exchange.[7]

Identification of Thiamine-biotin Conjugate by Liquid Chromatography-Mass Spectrometry (LC-MS)

Prepared 1 mL of thiamine-biotin conjugate, 20 mM ammonium formate solution as the mobile phase. The first step was the separation of the thiamine-biotin conjugate on liquid chromatography occurred by spraying 10 µL of the thiamine-biotin conjugate from the spray needle tip by Taylor cone. The conjugate and solvent (eluent) were then sprayed through the Taylor cone to form droplets that would undergo solvent evaporation The droplets were then transferred through the capillary opening for analysis using mass spectrometry. Thus, the mass spectrophotometry is to be detected based on the mass to charge ratio of each molecule. The detector would create peaks of the analyte being measured.[8]

Standard curve determination by competition test

The standard curve was determined by using free thiamine prepared in various concentrations. After the microplate surface was fixed with MBTBP and saturated with BSA, 50 µL of free thiamine was then pipetted into each of the microplate wells and incubated for 1 hour at 37°C. The washing stage was then carried out using Tween 20 PBS pH 7.4. The next reaction occurred by the addition of thiamine-biotin conjugate as a competitor. Enzymatic staining was then performed and read at 450 nm wavelength. Absorption with the best linearity would be used for the determination of the thiamine standard curve during the measurement of thiamine levels in serum.[7]

Sample Preparation

Every 15 samples were 3 mL of human blood without anticoagulant from respective 15 patients. Then all samples were centrifuged at a rate of 1600 rpm at 4°C for 20 minutes to separate serum from red blood cells. The serum sample immediately separated into an aliquot, stored at -20°C, and shielded from light.[9]

Validity test on thiamine measurement in Enzyme-labelled protein-ligand assay (ELPLA) method

Precision Tests

Precision tests were carried out in each sample in Duplo under the same samples and temperature. Each sample was prepared and then measured for thiamine ten times within the same day.[10]

Accuracy Tests

Accuracy tests on thiamine measurement in ELPLA method were carried out by the addition of known thiamine concentrations which consist of 2,5 µg/mL, 5 µg/mL, and 10 µg/mL into different samples.[10]

Application of MBTBP in ELPLA method on thiamine measurement with thiamine-biotin conjugate as a competitor

This is consists of ten steps: A total of 100 µL of MBTBP 250 µg/mL was conjugated on the surface of polystyrene

microplates for 24 hours at 4ºC. After that, the MBTBP was discarded and the well was washed thrice with 200 µL of PBS-Tween 20 pH 7.4. A total of 150 µL BSA 1% was added to each well for 24 hours at 4°C to saturate the surface. The well was washed thrice again with the same washing buffer. Serum (50 µL) was added to the well and incubated for 1 hour at 37ºC before washing it. A total of 50 µL of thiamine-biotin conjugate and 50 µL avidin 1 µmol were added to each well and incubated again. The well was washed thrice and dried for 1-2 minutes before adding 50 µL of biotin peroxidase. The reaction was performed for 1 hour at 37ºC. Before the staining step, the well was washed thrice again. The enzymatic staining was carried out by 100 µL of tetramethylbenzidine (TMB) solution as peroxidase's substrate. This reaction was incubated for 15 minutes at room temperature in a dark place and stopped with 100 µL of sulfuric acid 2/3 N solution. The optical density (OD) of 450 nm was measured to detect the thiamine concentration by comparing the value with the thiamine standard curve. The same procedure was also used for the thiamine-avidin conjugate.[7]

Result

Identification Conjugate Thiamine-biotin with LCMS

The chromatogram result showed the peaks of the measurement sample. The first peak is biotin, the second peak is thiamine. The highest peak that is the peak of thiamine-biotin conjugate had an Mr=608 (Figure 1).

Standard curve determination by competition test

The best standard curve results for thiamine-biotin conjugate in a competition test is by incubating at the temperature of 37°C, using 250 µg/mL MBTBP.

Precision & accuracy of the enzyme-labeled protein-ligand specific binding assay on serum thiamine measurements

The result of precision analysis (Table 1). The accuracy test of ELPLA was conducted by adding a known concentration of thiamine to the sample (recovery test) (Table 2).

The precision test was measured based on the repeatability of processing and measuring the samples. The measurement was repeated 10 times as a precision test with the same sample and procedure.

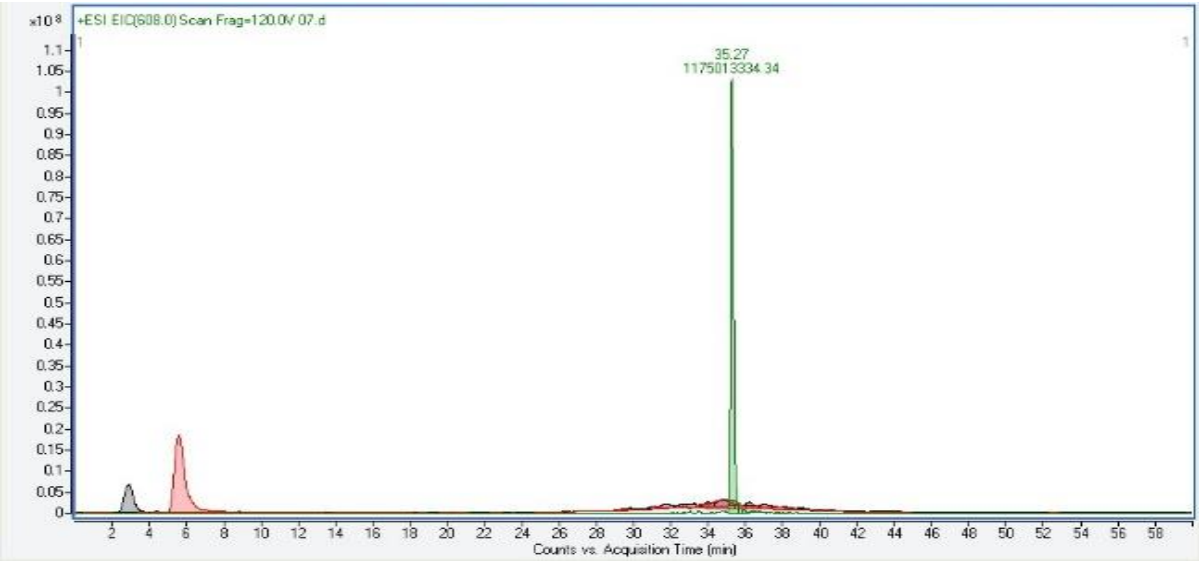

Figure 1. Chromatogram of thiamine-biotin conjugate

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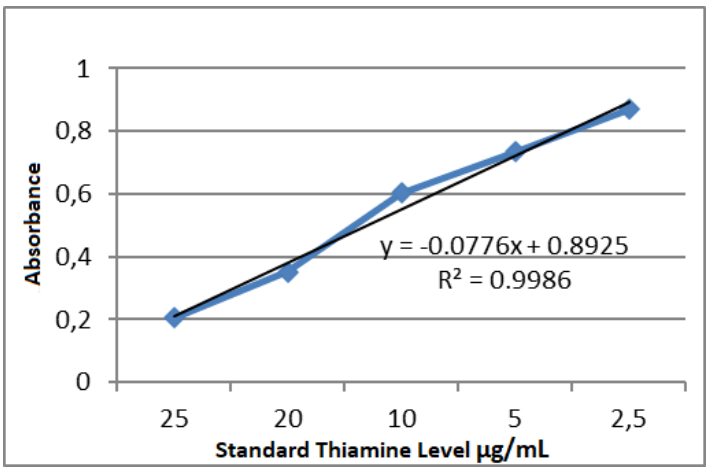

Figure 2. Standard curve of thiamine measurement

Table 1. Precision test of ELPLA

Sample Repetition	Absorbance	Thiamine Level µg/mL
1	0.483	5.27
2	0.460	5.57
3	0.462	5.54
4	0.455	5.63
5	0.447	5.74
6	0.435	5.88
7	0.432	5.93
8	0.430	5.96
9	0.434	5.90
10	0.445	5.76

Average	= 5,718	Log C	= -5,2428
Standar Deviasi (SD)	= 0,2179	0,5 log C	= -2,6214
Varian	= 0,0474	1-0,5 log C	= 3,6214
CV	= 3,81%	$2^{1-0,5 \log C}$	= 12,307
C	= 0,000005718	CV Horwitz	= 0,66 x $2^{1-0,5 \log C}$ = 8,12%

Sample (N=10)	Thiamine Level µg/mL Mean	SD	CV (%)	CV (Horwitz)
Serum	5.72	0.218	3.81%	8.12%

Table 2. Accuracy test of ELPLA

Number of Samples	Thiamine	Thiamine Level		R
	Spike µg/mL	Before Spike µg/mL	After Spike µg/mL	%
9	10	2.62	7.48	96
10	5	4.42	9.33	98
1	2,5	7.75	9.80	94

Application of MBTBP in ELPLA method on thiamine measurement with thiamine-biotin conjugate as a competitor

The resulting method on thiamine measurement with thiamine-biotin conjugate as a competitor of 15 samples (Table 3).

Table 3. Thiamine Measurement

Number of Samples	Abs	Thiamine Levels
1	0.291	7.75
2	0.216	8.68
3	0.243	8.35
4	0.275	7.91
5	0.256	8.16
6	0.288	7.78
7	0.257	8.18
8	0.298	7.62
9	0.292	7.70
10	0.225	8.60
11	0.226	8.58
12	0.292	7.73
13	0.224	8.57
14	0.295	7.69
15	0.351	6.97

Discussion

Isolation and purification of thiamine binding protein from mung beans were carried out using standard procedures to obtain pure MBTBP. The bioinformatics analysis of protein showed that the MBTBP resulting from affinity chromatography could bind 4 thiamine molecules.[2]

The MBTBP concentration resulting from affinity chromatography was 1.216 mg/mL and measured by using BSA standard curve. Improper storage and temperature would accelerate the decrease of protein concentration.[11]

In this study, a smaller competitor, the thiamine-biotin conjugate was used. It was estimated that the competition between free thiamine and thiamine – biotin would be more evenly spread. For this reason, thiamine was bound to biotin with the help of a bifunctional reagent,

glutaraldehyde. The compound had two aldehyde groups binding to -NH_2 or = NH. Through amidation reaction, these two vitamins could be linked to one another. The binding formed a new compound, as shown by the appearance of the third peak in the results of the LC-MS chromatogram at the retention time of 30 minutes and the peak intensity reached $5x10^7$ which was the peak of thiamine biotin conjugate had a reactive molecular mass/Mr = 608 (Figure 1). Thus, it was thought that the thiamine-biotin conjugate could also be used, in addition to the avidin-thiamine conjugate. LC-MS technique which uses several ion sources, in this study used an ion source named electrospray ionization (ESI). electrospray ionization (ESI) could be used for small or large molecules thus applied to thiamine-biotin conjugate. One of the disadvantages of ESI as an ion source was that a complex mixture could reduce the sensitivity.[8,12]

A competition test was performed to determine the standard curve for measuring thiamine levels in serum. The principle of this competition test was that the more free thiamine the MBTBP could bind with, the fewer thiamine competitors the MBTBP could bind to, thus producing lower absorbance and color intensity. A competition test was conducted to determine the best standard curve and working temperature. Figure 2 showed that MBTBP with 250 µg/mL concentration and 37 °C working temperature gave the best linearity results while using thiamine biotin conjugate, with the value of R^2= 0,9986.[7]

The validation involves the entire analytical procedure. Accuracy and precision test are included among several parameters that must be validated on laboratory tests to ensure said procedure met a certain laboratory fit requirements.[10]

The precision test was measured based on the repeatability of processing and measuring the samples. The measurement was repeated 10 times as a precision test with the same sample and procedure. Table 1 showed that the coefficient of variation (CV) for repeatability of sample preparation is 3.81 % is below CV Horwitz (8.12%). This result shows that ELPLA method was precise.[10]

The accuracy test was performed by adding the measured concentration of thiamine into unknown samples. The value was measured before and after the addition of measured thiamine. The results of measurement would be shown as recovery value. Table 2 showed that the mean recoveries percentages of samples were found to be 94-98% respectively which are within the acceptance limit.[10]

The results showed that the use of the thiamine-biotin conjugate as a competitor could be applied in the measurement of thiamine levels in serum (Table 4).

As a suggestion are applied other samples such as erythrocytes, leukocytes, urine, or saliva use thiamine- with biotin conjugate for measurement of thiamine levels. Applied biotin-thiamine conjugate for measurement of thiamine levels in serum of diabetes mellitus patients, cardiovascular disease patients, and Wernickle-Korsakoff syndrome.

Conclusion

Based on the discussion analysis results, it can be concluded, in the LC-MS analysis showed that thiamine can be bonded chemically. The ability of MBTBP for the ELPLA technique had been met with good precision and accuracy. The ELPLA technique with thiamine-biotin conjugate as a competitor could be applied in the measurement of serum thiamine levels.

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