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Dipstick based immunochemiluminescence biosensor for the analysis of vitamin B_{12} in energy drinks: A novel approach

L.S. Selvakumar, M.S. Thakur*

Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute (a constituent laboratory of the Council of Scientific and Industrial Research, CSIR), Mysore 570020, Karnataka, India

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ABSTRACT

In this article, we describe a dipstick based immunochemiluminescence (immuno-CL) biosensor for the detection of vitamin B_{12} in energy drinks. The method is a direct competitive type format involving the immobilization of vitamin B_{12} antibody on nitrocellulose membrane (NC) followed by treatment with vitamin B_{12} and vitamin B_{12}–alkaline phosphatase conjugate to facilitate the competitive binding. The dipstick was further treated with substrate disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2¢-(5¢-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)-1-phenyl phosphate (CDP-Star) to generate chemiluminescence (CL). The number of photons generated was inversely proportional to the vitamin B_{12} concentration. After systematic optimization, the limit of detection was 1 ng mL^{-1}. The coefficient of variation was below 0.2% for both intra- and inter-assay precision. Vitamin B_{12} was extracted from energy drinks with recovery ranged from 90 to 99.4%. Two different energy drinks samples were analyzed, and a good correlation was observed when the data were compared with a reference enzyme linked immuno sorbent assay (ELISA) method. The developed method is suitable for an accurate, sensitive, and high-throughput screening of vitamin B_{12} in energy drinks samples. The dipstick technique based on immuno-CL is suitable for the detection of several analyte in food and environmental samples.

1. Introduction

An immunological method is one of the promising tools for the development of easy-handling biosensors. Besides, immunoassay methods are sensitive, cost-effective, easy to perform, and require a small sample volume [1]. However, such techniques often require long reaction times and involve multiple steps [2]. The utilization of these immunoassays has been confined to laboratories equipped with tools and devices for analysis. Therefore, the convenience and speed of the test have been achieved by a novel concept of immunodipstick that depends on the transportation of a reactant to its binding partner immobilized on a membrane surface [3]. It combines several benefits including a user-friendly format, short assay time, long-term stability over a wide range of climates, and cost-effectiveness. These characteristics make it ideally suited for on-site screening by people who are not skilled analysts [4].

Vitamin B_{12} is one such analyte needs urgency for the detection. Vitamin B_{12} belongs to the B vitamin group and prevents pernicious anemia, which is caused by vitamin B_{12} deficiency. Vegetarians and people who do not eat meat need to supplement their diet by taking multivitamin tablets and beverages supplemented with vitamin B_{12} as plant products contain very little amount of vitamin B_{12}. As the excessive consumption of vitamin B_{12} may cause asthma and folic acid deficiency, therefore, typically only a low level of vitamin B_{12} (e.g., ng g^{-1}) is added to products, thus making direct analysis difficult. The most common requirement for the analysis of vitamin B_{12} is in the quality control of pharmaceuticals (tablets or injections), blood plasma serum, milk products for infants, and fermentation products which involves complicated sample preparation [5]. The daily requirements of vitamin B_{12} are very low when compared with other vitamins [6], and deficiencies are reported to be at the nanogram level [7]. Extracting vitamin B_{12} from a larger amount of sample is simple and effective for some relatively large and solid samples, such as Algae [8]. However, this strategy is not always suitable for liquid samples (e.g., beverages). At the onset of these challenges, it is very important to diagnose vitamin B_{12} at a sensitive level.

The conventional methods for the detection of this compound for analytical purposes are including a microbiological assay that uses *Lactobacillus leichmannii* as a test organism is very laborious [9]. Radioisotope method has been applied to the determination of vitamin B_{12} in food samples using intrinsic factor as recognition molecule but the cost of the assay is questionable [10,11]. High performance liquid chromatography (HPLC) has been employed by a number of workers to assay vitamin B_{12} in multivitamin and mineral tablets where as these matrices are less complex than

* Corresponding author. Tel.: +91 821 2515792; fax: +91 821 2517233.
E-mail addresses: msthakur@cftri.res.in, msthakur@yahoo.com (M.S. Thakur).

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foodstuffs and therefore, extraction and resolution of the vitamin is quite difficult in food matrix [12,13]. Other specific detection methods based on the dosage of cobalt either by atomic absorption spectrometry [14] or by inductively coupled argon plasma emission spectrometric detection [15] were proposed. However, the limit of detection (LOD) of these methods (150 mg g^{-1}) only allows the quantification of high amounts of vitamin B_{12} in food samples. Spectrophotometry [16,17], fluorimetric assay [18], capillary electrophoresis [19], and CL [20] are employed for analysis of vitamin B_{12}. However these methods are found to be less dependable in analyzing crude sample considering the time, cost and sensitivity. Such a situation demands more specific methods like biospecific assays which make use of biorecognition molecules like antibodies, for the detection of analyte [21]. Development of an immunological based methods, is therefore a promising field; taken care, the possibilities of exploiting the principle of antigen–antibody specific interaction. But the issues of raising antibody, IgG and monoclonal antibody against such analyte is always questionable due to its costly extraction procedure which is resolved with the development of efficient protocols for raising and extracting immunoglobulins from hen egg yolk, namely IgY [22]. In our previous report, we used hens as immunization hosts to produce vitamin B_{12} IgY antibody against a derived form of vitamin B_{12} by immunizing a hen with vitamin B_{12} conjugated to a carrier protein, Keyhole Limpet Hemocyanin. The sensitivity and specificity of developed antibodies were checked using ELISA [23]. Previous studies in our laboratory indicated that, IgY antibody in combination with CL was highly promising for detection of analytes with high sensitivity [24].

In present work, we advance the approach of using dipstick based format of immuno-CL method for the detection of vitamin B_{12}. CL-based analytical methods are rapid, specific, cost-effective, and requires no excitation source like in fluorescence, phosphorescence, monochromator (often not even a filter), or radioactive or hazardous chemicals. Hence, this method has become an attractive analytical tool for sensitive clinical diagnosis and environmental applications [25–27]. To achieve this objective, different parameters were optimized such as immobilization of vitamin B_{12} antibodies IgY on NC strip, optimization of vitamin B_{12}–ALP (alkaline phosphatase) conjugate concentration, optimization of substrate CDP star concentration, optimization of volume of substrate CDP star concentration along with possible mechanism of chemiluminescence reaction. The CL signal produced by biochemical reactions was indirectly proportional to the concentration of vitamin B_{12}. In addition, the CL analytical procedure was compared and validated with conventional colorimetric assays.

2. Materials and methods

2.1. Reagents

All the reagents were analytical grade and used without further purification. Doubly distilled water (DDW) was used throughout this work. Vitamin B_{12}, Alkaline phosphatase, Tween-20, *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) cabodiimide (EDC), XAD-2 Amberlite, Disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricycle [3.3.1.13,7]decan}-4-yl) phenyl phosphate (CDP star) chemilumeniscent substrate were procured from M s^{-1} Sigma–Aldrich Chemicals, USA. Energy drinks were procured from local market, Mysore, India. Vitamin B_{12} antibody generation and vitamin B_{12}–alkaline phosphatase conjugate preparation were done at Central Food Technological Research Institute (CFTRI), India.

2.2. Apparatus

Luminometer-Luminoskan TL Plus with photomultiplier tube was procured from Helsinki, Finland. Incubator shaker-INFORS HT, Ecotron was procured from Bottmengen, Switzerland. Vortex cyclo mixer was procured from Genie Bangalore, India. Flash evaporator was procured from Rotavac Senso Heidolph, Sweden. Nitrocellulose membrane was procured from Advanced Microdevices, Ambala Cantt Haryana, India.

2.3. Experimental set up and principle of detection of vitamin B_{12} using dipstick based immuno-CL method

The principle is competitive binding between the immobilized vitamin B_{12} antibodies with free vitamin B_{12} and vitamin B_{12}–ALP conjugated on NC membrane. The CL signal generated during reaction was inversely proportional to the presence of vitamin B_{12} in the samples. Thereby, the concentration of vitamin B_{12} could be determined by measuring the CL intensity in the absence and presence of vitamin B_{12} as shown in Scheme 1a. The CL signals were plotted at 5 s intervals for a period of 10 min. The CL depends upon the light signals generated by the biochemical reactions between vitamin B_{12}–ALP and CDP-star as shown in Scheme 1b. Enzymatic dephosphorylation of dioxetane by alkaline phosphatase leads to the formation of the meta-stable dioxetane phenolate anion, which decomposes and emits light and is detected by the luminometer. The CL units (CLU) term used for the unit value of photons produced from the CL reaction between vitamin B_{12}–ALP and CDP-star over fixed time duration. Using this phenomenon, vitamin B_{12} concentration in analytical samples was determined.

2.4. Preparation of hapten–protein conjugates containing vitamin B_{12} epitopes and generation of vitamin B_{12} IgY antibodies

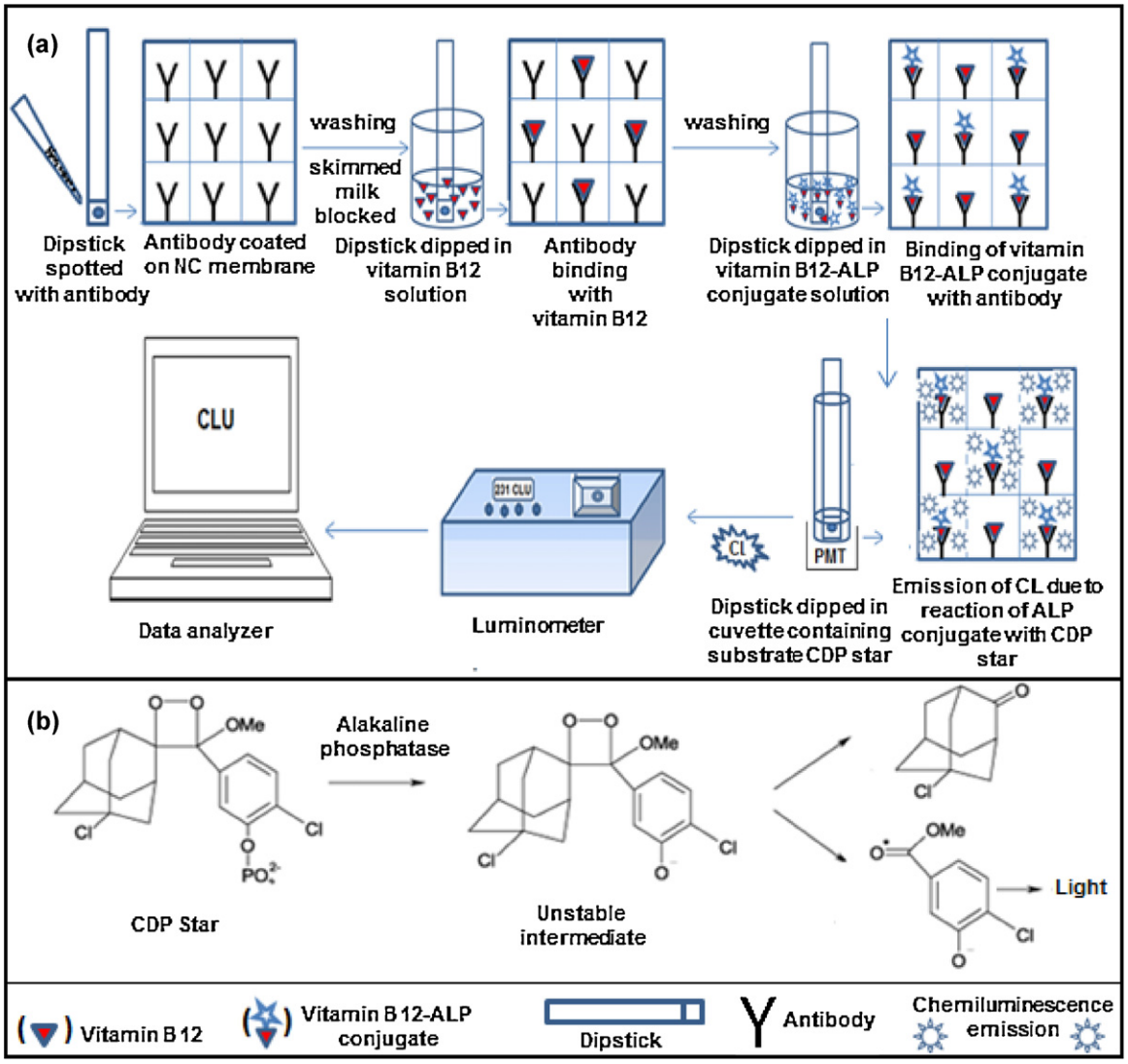
Preparation of hapten–protein conjugate containing vitamin B_{12} epitopes and production of the IgY polyclonal antibodies against vitamin B_{12} is described in our recent publication [23].

2.5. Competitive direct ELISA for vitamin B_{12} determination

A microtiter plate was coated with 100 ng $well^{-1}$ of the purified antibody with 50 mmol L^{-1} of Na_2CO_3–$NaHCO_3$ solution (pH 9.6) and incubated overnight at 4 °C. After the coating solution was removed, blocking solution (200 μL $well^{-1}$ of 1% gelatin in phosphate buffer saline (PBS), containing 0.1% NaN_3) was added, and the wells were washed with phosphate buffer saline tween (PBST) after 2 h incubation at 37 °C. The plate was then filled with 100 μL $well^{-1}$ of different concentration of derivatized vitamin B_{12} sample, along with 100 μL $well^{-1}$ of serially diluted vitamin B_{12}–ALP conjugates, and incubated for 1 h at 37 °C. The wells were then washed thrice using 200 μL PBST, and color development was done using *p*-nitrophenyl phosphate (1 mg mL^{-1}; 100 μL $well^{-1}$) in 1% diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by adding 3 M NaOH (40 μL $well^{-1}$), and the absorbance was read at 405 nm in an ELISA microplate reader. All analysis was done in triplicates.

2.6. Immobilization of vitamin B_{12} antibodies IgY on NC strip

Different concentrations of antibodies were immobilized, from 1, 10, 100, 1000 ng μL^{-1} by direct spotting on to the surface of NC membrane and dried at room temperature (RT) for 60 min. Blocking was done with 2% skimmed milk in PBS for 30 min in shaking condition. Strips were washed several times with PBS



Scheme 1. (a) Schematic representation of immuno-CL based dipstick technique for detection of vitamin B_{12}. (b) Enzymatic dephosphorylation of dioxetane by alkaline phosphatase.
Adopted from https://e-labdoc.roche.com/LFR_PublicDocs/ras/11759051001_en_08.pdf.

then dipped in optimized concentration of vitamin B_{12}–ALP conjugate. After 60 min of incubation the strips were again washed with PBS (pH 7.4) and dried. The CL of the strip was checked using optimized concentration/volume of CDP star in a luminometer. The photons generated as a result of the reaction was recorded for a period of 10 min using the luminometer and converted into a readable format as CLU using hexa terminal software.

2.7. Optimization of vitamin B_{12}–ALP conjugate concentration

NC membrane strips were coated with optimized concentrations of antibodies and dried at RT for 60 min. Blocking was done with 2% skimmed milk in PBS for 30 min in shaking condition. Strips were washed several times with PBS then dipped in different concentration of vitamin B_{12}–ALP conjugate from 6.25, 12.5, 25, 50, 100 $\mu g\,mL^{-1}$. After 60 min of incubation the strips were again washed with PBS (pH 7.4) and dried. The CL of the strip was checked using optimized concentration/volume of CDP star in a luminometer. The photons generated as a result of the reaction was recorded for a period of 10 min using the luminometer and converted into a readable format as CLU using hexa terminal software.

2.8. Optimization of substrate CDP star concentration

NC membrane containing strips were coated with optimized concentrations of antibodies and dried at RT for 60 min. Blocking was done with 2% skimmed milk in PBS for 30 min in shaking condition. Strips were washed several times with PBS then, dipped in optimized concentration of vitamin B_{12}–ALP conjugate. After 60 min of incubation the strips were again washed with PBS (pH 7.4) and dried. The CL of the strip was checked using different concentration of CDP star from 3.125, 6.25, 12.5 and 25 μM in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format as CLU using hexa terminal software.

2.9. Optimization of volume of substrate CDP star concentration

NC membrane containing strips were coated with optimized concentrations of antibodies and dried at RT for 60 min. Blocking was done with 2% skimmed milk in PBS for 30 min in shaking

condition. Strips were washed several times with PBS then, dipped in optimized concentration of vitamin B_{12}–ALP conjugate. After 60 min of incubation the strips were again washed with PBS (pH 7.4) and dried. The CL of the strip was checked using optimized concentration of CDP star varying volume from 100, 200, 400, 800 μL in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format as CLU using hexa terminal software.

2.10. Detection of vitamin B_{12} by dipstick based immuno-CL assay

NC membrane containing strips was coated with optimized concentrations of antibodies and dried at RT for 60 min. Blocking was done with 2% skimmed milk in PBS for 30 min in shaking condition. Strips were washed several times with PBS and finally dipped in different concentration of derivatized vitamin B_{12} from 1, 10, 100 and 500 ng mL^{-1} followed by 60 min of incubation in optimized concentration of vitamin B_{12}–ALP conjugate and dried. The CL of the strip was checked using optimized concentrations/volume of CDP star. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format using as CLU hexa terminal software.

2.11. Extraction of vitamin B_{12} from energy drinks and its correlation study

200 mL of energy drinks having vitamin B_{12} labeled at concentration of 2 μg 100 mL^{-1} was extracted using cationic resin XAD-2 Amberlite [8]. The sample was loaded onto Amberlite XAD-2, prepared as a methanolic suspension of the resin packed to a bed height of 15–16 cm. The column was equilibrated with water. The sample was eluted with 80% (v/v) methanol and concentrated using rotavapor (Buchi). The concentrate was derivatized and diluted for further analysis of vitamin B_{12} by ELISA followed by analysis using dipstick based immuno-CL. Energy drinks was further spiked with 10 ng of concentration of derivatized vitamin B_{12} for its accuracy and recovery before and after addition.

3. Results and discussion

3.1. Effect of antibody concentration for immobilization

Different concentrations of vitamin B_{12} antibody were immobilized by direct spotting on NC membrane to see the optimum binding of antibody with vitamin B_{12}-conjugate. Out of which 100 ng μL^{-1} was found to be optimum because beyond 100 ng μL^{-1} CL signals obtained were almost constant which shows the saturation binding of antibody and antigen reaction therefore, 1000 ng μL^{-1} was ruled out. Comparatively lower CL signals were obtained with lower antibody concentration due to less binding of antibody with vitamin B_{12}-conjugate as shown in Fig. 1.

3.2. Effect of vitamin B_{12}–ALP conjugate concentration

Optimized immobilized concentrations of vitamin B_{12} antibody were dipped in different concentration of vitamin B_{12}–ALP conjugate to check for optimum binding of vitamin B_{12}–ALP conjugate to vitamin B_{12} antibody. Out of which, 50 μg mL^{-1} was found to be optimum because beyond 50 μg mL^{-1} CL signals obtained were almost constant which shows the saturation binding of antibody and antigen reaction therefore, 100 μg mL^{-1} was ruled out. Comparatively lower CL signals were obtained with lower vitamin B_{12}–ALP conjugate concentration due to less binding of vitamin B_{12}–conjugate to vitamin B_{12} antibody as shown in Fig. 2.

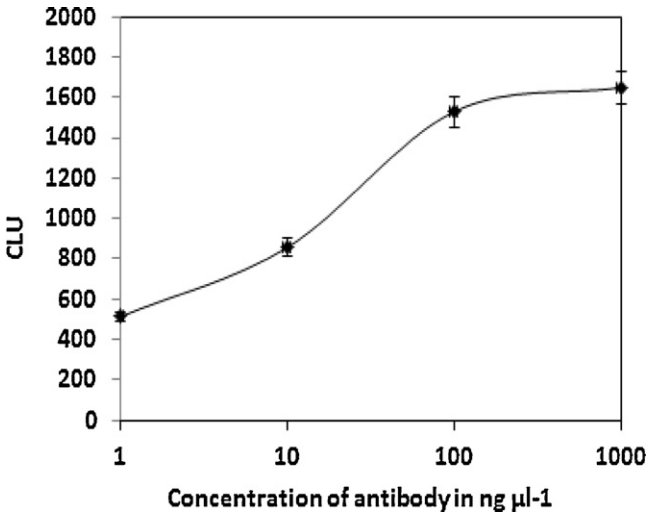


Fig. 1. Effect of antibody for immobilization. The reaction mixtures contained different concentrations of antibody from 1, 10, 100, 1000 ng μL^{-1} by direct spotting on to the surface of NC membrane followed by blocking with 2% skimmed milk. Strips were dipped in 50 μg mL^{-1} concentration of vitamin B_{12}–ALP conjugate to check for optimum CL generation using 12.5 μM (400 μL) concentration CDP star in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format using hexa terminal software as CLU. Data were derived from triplicate assays ($n = 3$) with error bars of 5%.

3.3. Effect of substrate CDP star concentration

Optimized concentrations of vitamin B_{12} antibody/vitamin B_{12}–ALP conjugate was dipped in different concentration of ALP specific CDP star substrate to check for optimum CL signals. Out of which, 12.5 μM concentration was found to be optimum because beyond 12.5 μM concentration substrate availability to enzyme is more results in higher undesirable background CL signals therefore 25 μM concentration was ruled out. Comparatively lower CL signals were obtained with lower CDP Star concentration due to

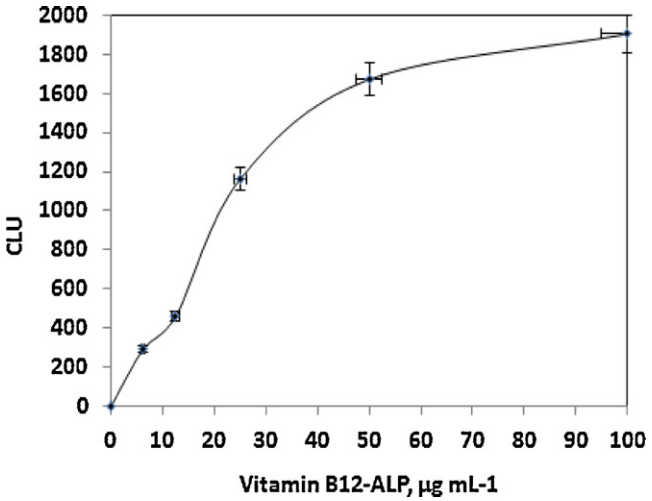


Fig. 2. Effect of vitamin B_{12}–ALP conjugate concentration. The reaction mixtures contained antibody of 100 ng μL^{-1} by direct spotting on to the surface of NC membrane followed by blocking with 2% skimmed milk. Strips were dipped in different concentration of vitamin B_{12}–ALP conjugate from 6.25, 12.5, 25, 50, 100 μg mL^{-1} to check for optimum CL generation using 12.5 μM (400 μL) concentration CDP star in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format using hexa terminal software as CLU. Data were derived from triplicate assays ($n = 3$) with error bars of 5%.

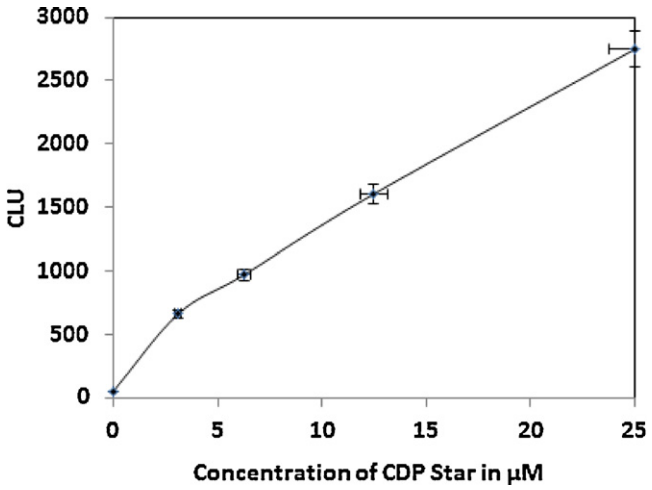


Fig. 3. Effect of substrate CDP star concentration. The reaction mixtures contained antibody of 100 ng µL^{-1} by direct spotting on to the surface of NC membrane followed by blocking with 2% skimmed milk. Strips were dipped in 50 µg mL^{-1} concentration of vitamin B_{12}–ALP conjugate to check for optimum CL generation using optimized volume of different concentration of CDP star from 3.125, 6.25, 12.5 and 25 µM in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format using hexa terminal software as CLU. Data were derived from triplicate assays ($n = 3$) with error bars of 5%.

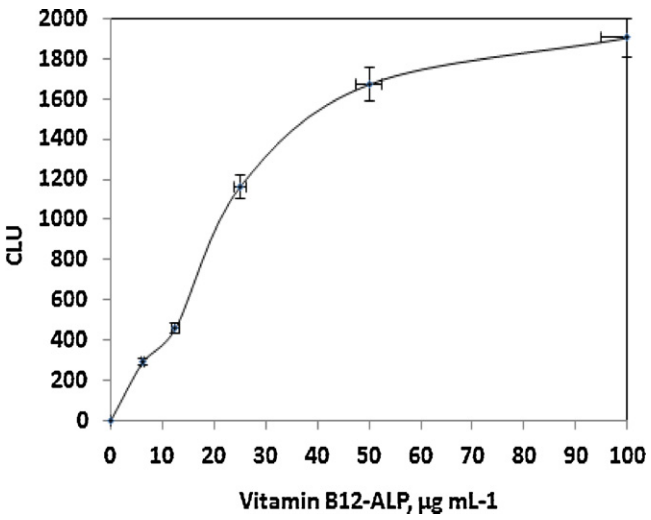


Fig. 4. Effect of substrate CDP star volume. The reaction mixtures contained antibody of 100 ng µL^{-1} by direct spotting on to the surface of NC membrane followed by blocking with 2% skimmed milk. Strips were dipped in 50 µg mL^{-1} concentration of vitamin B_{12}–ALP conjugate to check for optimum CL generation using different volume from 100, 200, 400, 800 µL of 12.5 µM of concentration of CDP star in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format using hexa terminal software as CLU. Data were derived from triplicate assays ($n = 3$) with error bars of 5%.

less substrate available for enzyme to generate CL signals as shown in Fig. 3.

3.4. Effect of substrate CDP star volume

Optimized concentrations of vitamin B_{12} antibody/vitamin B_{12}–ALP conjugate were dipped in different volume of optimized concentration of ALP specific CDP star substrate in cuvette to check for optimum CL signals and minimum volume require for dipstick to immerse in substrate solution. Out of which, 400 µL was found to be optimum and minimum because beyond 400 µL, volume is too high as substrate availability to enzyme is more results in higher undesirable background CL signals and wastage of substrate, therefore 800 µL was ruled out. Comparatively lower CL signals were obtained with lower volume of CDP Star due to incomplete immersion of dipstick in cuvette results in less substrate available for enzyme to generate CL signals as shown in Fig. 4.

3.5. Dipstick based immuno-CL assay

After the generation of antibody against vitamin B_{12} ELISA was performed for its sensitivity and specificity. The limit of detection is 10 ng mL^{-1} and linear up to 100 µg mL^{-1} with a regression coefficient of 0.989 [23], but it suffer from drawbacks such as time consuming and cost, in order to resolve the issues, dipstick based format was followed for its analysis. The principle is competitive binding between the immobilized vitamin B_{12} antibodies with free vitamin B_{12} and vitamin B_{12}–ALP conjugated on NC membrane containing strips was the key principle behind the detection of vitamin B_{12} by immuno-CL based immunoassay. Hence when the vitamin B_{12} concentration is less, more conjugate bind to the strip and more CL are observed. The signal generated during reaction was inversely proportional to the presence of vitamin B_{12} in the samples.

Studies on different dilutions of vitamin B_{12}–ALP conjugate were done to obtain optimal CLU readings, and a 50 µg mL^{-1} was found to be optimal for vitamin B_{12} detection. Further optimization of antibody concentrations showed that 100 ng µL^{-1} was suitable for the detection of varying concentrations of vitamin B_{12} using an optimized vitamin B_{12}–ALP conjugate. This method was able to detect vitamin B_{12} at 1 ng mL^{-1} with range of 1 to 500 ng mL^{-1}; below 1 ng mL^{-1}, the signals were not reproducible and no significant difference was observed in the CL response. In the dipstick technique, CLU response was generated by bound vitamin B_{12}–ALP conjugate with CL reaction, and the results obtained were inversely proportional to vitamin B_{12} concentration. For different concentrations of vitamin B_{12}, different CLU responses were obtained, as shown in the inset picture in Fig. 5. In Fig. 5, the CL response

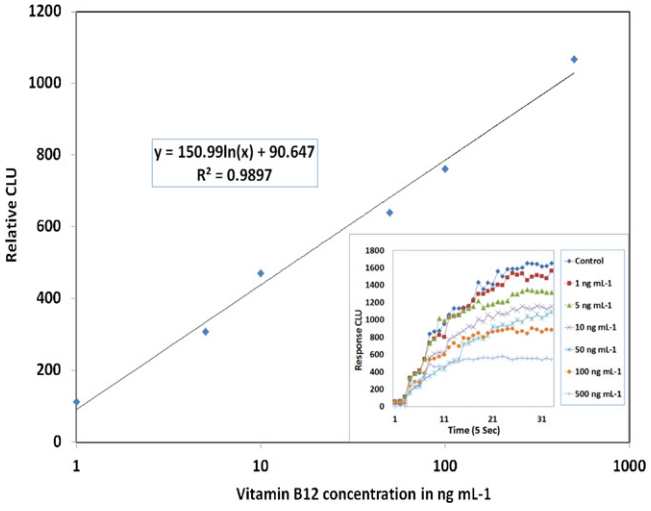


Fig. 5. The response graph and the logarithmic plot of concentration of vitamin B_{12} vs CLU in dipstick based immunochemiluminescence assay. The reaction mixtures contained 100 ng µL^{-1} antibodies by direct spotting on to the surface of NC membrane followed by blocking with 2% skimmed milk. Strips were dipped in different concentration of derivatized vitamin B_{12} from 1, 10, 100 and 500 ng mL^{-1} followed by dipping in 50 µg mL^{-1} concentration of vitamin B_{12}–ALP conjugate to check for optimum chemiluminescence generation using 12.5 µM (400 µL) concentration CDP star in a luminometer. The photons generated as a result of the reaction were recorded for a period of 10 min using the luminometer and converted into a readable format using hexa terminal software as CLU. Data were derived from triplicate assays ($n = 3$) with error bars of 5%.

Table 1
Precision of spiked derivatized vitamin B_{12} concentration estimates determined with a dipstick based immuno-CL. Samples were assayed in triplicate of 5 assays per day over 5 days ($n = 3$).

Sample (ng mL^{-1})	1	10	100
Triplicates/day	5	5	5
Days	5	5	5
N	25	25	25
Mean (ng mL^{-1})	0.82	9.34	99.36
Recovery (%)	90	93.4	99.36
%CV (intra assay)	0.1	0.03	0.003
%CV (inter assay)	0.1	0.2	0.05

for each vitamin B_{12} concentration was plotted, and the area covered under each concentration was calculated by taking the total reading from the initial to final CLU as an integrated value that is presented as a single line graph. In the inset picture, the *y*-axis is represented as CLU, and in the main figure, it represents integrated CLU against vitamin B_{12} concentration in ng mL^{-1}. Different CLU signals were monitored over a fixed time period (450 s) for each vitamin B_{12} concentration. From the response graph (Fig. 5, inset picture) obtained for different dilutions of vitamin B_{12}, a linear standard graph of vitamin B_{12} was constructed (Fig. 5), and it was found that a good regression value ($R^2 = 0.9897$) was obtained in the range of 1–500 ng mL^{-1}.

3.6. Comparison between colorimetric and CL detection

The optimized CL enzyme immunoassay was compared with a conventional colorimetric method. By comparing the dose response curves shown in Fig. 5, it can be observed that the CL method provided a lower detection limit with respect to the colorimetric assay (i.e. 10 ng mL^{-1} to 1 ng mL^{-1}, respectively). A further advantage obtained by using the CL detection is the rapidity of the assay, since the CL signal can be measured immediately after substrate addition, while the colorimetric assay requires a 20–30 min incubation step, as well as an enzyme activity blocking step, prior to signal detection. Indeed, thanks to the glow type emission kinetics of the enhanced CL substrate, the steady state light emission is reached 2–3 min after substrate addition and it is maintained for at least 15 min, thus allowing easy handling and standardization of the experimental conditions. In this particular experiments, ALP was chosen as enzyme due to its stable CL response when compare to Horse Radish Peroxidase (HRP) as its counterpart. The CL generated by HRP reactions with urea–hydrogenperoxide (Urea–H_2O_2) and luminol was not reproducible due to the unstable nature of Urea–H_2O_2. But in case of ALP, substrate CDP star is very stable and gives stable CL response as shown in Fig. 5. In addition to that, the ALP tagged dipstick is easy to dip in cuvette containing single substrate CDP star where as in case of HRP, two reactants are necessary, i.e. luminol and Urea–H_2O_2 which leads to non-reproducible CL response. Therefore ALP enzyme could be an ideal tool in dipstick based immuno-CL assay format.

3.7. Precision

In precision studies, samples were spiked with vitamin B_{12} and extracted using XAD-2 amberlite and derivatized at three different concentrations and each measured five times in triplicate on five different days are shown in Table 1. Recovery was observed to be 90–99.36% with coefficients of variation between 0.003 and 0.2% for both inter and intra assay. But in case of energy drinks, vitamin B_{12} was to be extracted and derivatized for precision studies.

Table 2
Recovery of externally added (ng mL^{-1}) derivatized vitamin B_{12} in energy drinks was analyzed using dipstick method. Data were derived from triplicate assays ($n = 3$).

Energy drinks	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%)	Recovery (%)
Sample 1	0	9.26	46.4	
	10	18.4	5.22	92
	50	58.6	1.52	97.6
	100	109.4	0.5	99.4
Sample 2	0	8.98	26.3	
	10	18.86	8.9	94
	50	58.8	2.52	98
	100	109.4	0.8	99.4

Table 3
Analysis of vitamin B_{12} in energy drinks using ELISA in comparison with dipstick based immuno-CL. The average of five measurements (±D). Amount of vitamin B_{12} labeled in energy drinks are 2 μg 100 mL^{-1}.

Energy drinks	Amount labeled (μg 100 mL^{-1})	Amount found	
		ELISA	Dipstick Immuno-CL
Sample 1	2	1.72 (±0.42)	1.74 (±0.41)
Sample 2	2	1.96 (±0.28)	1.90 (±0.41)

3.8. Accuracy

The accuracy was assessed by analyzing the energy drinks before and after the addition of derivatized vitamin B_{12}. On average, 99–101% of added derivatized vitamin B_{12} was recovered as shown in Table 2.

3.9. Comparison study

Two different brands of energy drinks labeled with vitamin B_{12} samples were compared using the ELISA and dipstick based immuno-CL. The results obtained by the ELISA and HPLC agreed well with the labeled values for vitamin B_{12} in energy drinks as shown in Table 3. A comparison of two different immunological methods, ELISA and dipstick based immuno-CL, demonstrates the usefulness of the vitamin B_{12} immunoassay as shown in Fig. 6. There was no difference between the two methods as determined. To compare the results of the two different immunological methods (ELISA vs dipstick based immuno-CL) at different concentration ranges, the ELISA concentration values were well correlated to fall within the detection range of the dipstick based immuno-CL method.

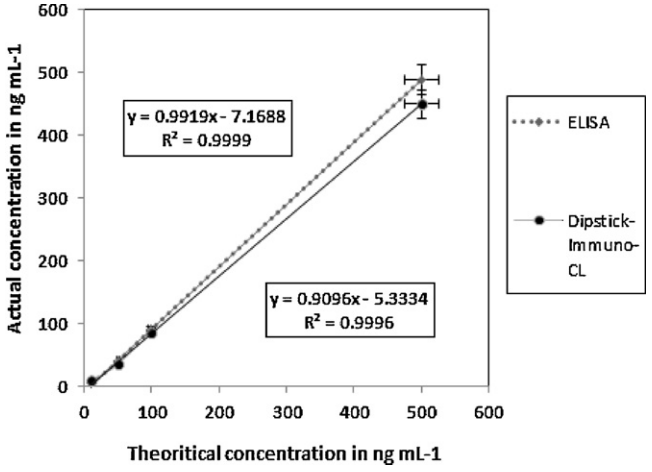


Fig. 6. Linear regression graph of correlation between ELISA and HPLC. Data were derived from triplicate assays ($n = 3$) with error bars of 5%.

4. Conclusion

From the studies it can be proved that dipstick based detection can be better alternative to conventional immunological method such as ELISA in terms of its cost, time duration, robustness and easy for handling. Dipstick technique coupled with CL for the sensitive detection will be an important aspect of the biosensor for cheaper as well as less time consuming and field applicable techniques used for the trace level detection of vitamin B_{12} in food and pharmaceutical industries. The methods are reliable and cost-effective, and have several advantages over standard gas chromatography, HPLC, ELISA, and enzymatic methods. As a futuristic approach, this dipstick technique can be further applied for nanotechnological based analysis of vitamin B_{12} by using quantum dots and gold nanoparticles for visual detection of analytes in food samples.

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