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Research Article

Stability Model Prediction Using the Response Surface Approach to Assess the Outcomes of Vitamin B_{12} Injection Post-Market Sampling

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ABSTRACT

B_{12} injections are one of the most widely used vitamins in Indonesia. The nature of vitamin B_{12}, which is easily degraded by environmental factors, has been widely reported. This research aimed to determine the effect of stability optimization model predictions with post-market sampling of vitamin B_{12} injection preparations by considering the variables of temperature, humidity, and distribution time. Factorial design was used to determine the stability optimization model. Validation of the analysis method using ultraviolet (UV) spectrophotometry showed valid results for testing. Stable product outcomes were seen during the research period based on the results of post-market sampling, optimization of stability conditions by evaluating pH parameters, and content determination. Post-market sampling data showed a pH decrease of 3.46% in the Palembang distribution area and an increase in pH of 4.54% and 5.33% in the Cirebon and Denpasar distribution areas. In the Palembang distribution area, the content of cyanocobalamin decreased by 0.90%, while in the Cirebon distribution area, it decreased by 0.94%. A paired t test for statistical analysis was used to determine the significance of the model. The results obtained show that there was no significant difference between the prediction model and the results of post-market sampling (p-values of 0.065 and 0.491) or H_0 received.

INTRODUCTION

Vitamin B_{12} is a water-soluble vitamin that functions for DNA synthesis, maintenance of the nervous system, erythropoiesis, protein metabolism, and brain development (Stabler, 2013). The average need for B_{12} a day is 2.4 – 3µg. Even though it is needed in small amounts, B_{12} is very important for the body. Several types of pharmaceutical preparations are available including tablets, caplets, syrup, and injections. One of the important criteria for achieving the desired therapeutic influence is maintaining the stability of the pharmaceutical preparation consumed (Tavakoli *et al.*, 2019). Inappropriate storage conditions can affect the stability of the preparation, thereby reducing the therapeutic influence. One of the problems in the pharmaceutical industry is the difficulty of controlling the environmental conditions of post-circulation products, especially non-cold chain products. Non cold chain products are products that do not require a cold chain in the production and storage process (Wang *et al.,* 2018).

The degradation of vitamin B_{12} can be influenced by environmental factors, including heat, light, and pH (Lee *et al.,* 2022). Oxygen can destroy vitamin B_{12} through oxidation reactions (Temova *et al.*, 2023). Heating at high temperatures, humidity, low pH, and light can damage the structure of vitamin B_{12} (Douglas de Brito *et al.*, 2016; Watanabe and Bito, 2018; Bajaj and Singhal, 2019; Hemery *et al.* 2020). According to other research, the contents of vitamin B12 preparations were reduced by up to 38% after 60 minutes of storage at 100 °C, 3% after 8 hours at 37 °C, and 80% after 9 weeks at

40 °C (Lie *et al.*, 2019; Monajjemzadeh *et al.* 2014). Therefore, monitoring the duration of exposure to humidity and temperature needs to be considered.

Response Surface Methodology (RSM) is a statistical method used to evaluate influences and interactions between components. The RSM helps determine an empirical model of the relationship between the independent variables and the response variable as well as the values of the independent variables which cause the value of the response variable to be optimal. RSM combines experimental design and regression analysis to optimize the value of the respons. RSM can reduce the time and resources required to optimize a process (Myers *et al.*, 2013).

There has never been any research done on the prediction model for vitamin B_{12} injection stability. It is recognized that B_{12} instability can develop under a variety of circumstances, as the description above indicates. Therefore, the purpose of this study was to investigate the relationship between the stability conditions of vitamin B12 injections given by post-marketing using the RSM method and the predictions made by the stability model.

METHODS

Materials

Vitamin B_{12} (Hebei Huarong Pharmaceutical, China), purified water (PW) and water for injection (GMP Ltd), and Vitamin B_{12} injections from Indonesian health market.

Instrumentation

Shimadzu Japan ultraviolet (UV)-1700 spectrophotometer, 2 ml volume pipette, 50 ml volumetric flask, 1000 ml measuring flask, drop pipette, Mettler Toledo pH meter, 30°C climatic chamber (Biolab CC-3000), 40°C pharma engineering climatic chamber, and Mettler Toledo XP26 balance with readability of 0.001 mg. The software used in this research includes Minitab 16 statistical software. The software is operated with an HP V190 computer processor, an Intel® Core ™ i3-2120 CPU @ 3.30GHz, system type 64-bit, and 4.00GB of RAM.

Preparation of standard solution

A total of 30 mg of secondary standard vitamin B_{12} was weighed using a Mettler Toledo XP26 China scale with a readability of 0.001 mg then diluted with PW to 1000 ml.

Test solution

A total of 1.5 ml of vitamin B_{12} injection sample was put into a 50 ml measuring flask, then diluted with PW until the mark and shaken until homogeneous. The solution that had been made was observed on a UV Spectrophotometer with λ 361 nm. The concentration levels at which test solutions can be made vary based on the testing needs.

Content determination and pH testing

Vitamin B_{12} produced in China by Hebei Huarong Pharmaceutical. GMP Ltd. provided PW and injection water. Injection of vitamin B12 purchased from the Indonesian medical market. A 361 nm Shimadzu UV-1700 Spectrophotometer from Japan was used to assess the injection dose content. A Mettler Toledo pH Meter was utilized to determine the preparation's pH level.

Method validation

The method of analyzing vitamin B_{12} injection using a UV spectrophotometer has been validated in terms of linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ). Measuring the absorbance of the standard solution, test solution, solvent (pure water), and placebo (water for injection) is how the specificity test is conducted. Linearity was established using test solutions at concentrations of 80%, 90%, 100%, 110%, and 120%. In compliance with ICH guidelines, a linear regression value is deemed satisfactory if the correlation coefficient (r^2) is near 0.999 (ICH, 2005). Test solutions (6 replications) and standard solutions were subjected to precision testing. Next, at λ 361 nm, absorption measurements were performed using a UV spectrophotometer. A standard solution and a test solution are created in order to conduct accuracy testing. Three replications, at concentrations of 80%, 100%, and 120%, were used to create the test solution. LOQ = (10 × σ)/S and LOD = (3.3 × σ)/S were the formulae used to compute LOD and LOQ from linearity. The selected method has a high sensitivity to the components tested, as indicated by small LOD and LOQ values.

Experimental design

Vitamin B_{12} produced in China by Hebei Huarong Pharmaceutical. GMP Ltd. provided PW and injection water. Vitamin B_{12} injection purchased from Indonesian health market. Full factorial design was used to find out the vitamin B_{12} degradation design. The fixed variable in this study was vitamin B_{12} injection, while the independent variables consisted of temperature, humidity and distribution time. The experiment

was done by placing the test solution in the climatic chamber 30°C Biolab CC-3000 and climatic chamber 40°C Pharma Tehnik according to the temperature, pH and time of experimental design.

Data analysis

Response surface methodology (RSM) uses factorial design with other statistical parameters processed using Minitab 16 statistical software. Factors are considered to have a significant influence if the *p*-value is <0.05.

Post market sampling

Vitamin B_{12} produced in China by Hebei Huarong Pharmaceutical. GMP Ltd. provided PW and injection water. Injection of vitamin B_{12} purchased from the Indonesian medical market. The investigation was done by paying attention to the distribution distance to find out the stability of the vitamin B_{12} injection product up to the distributor. Post market sampling was done in the distribution areas of western Indonesia (Palembang), central Indonesia (Cirebon) and eastern Indonesia (Denpasar) by considering anomalies in temperature, humidity and distribution time.

RESULTS AND DISCUSSION

Validation of the analytical methods

The working principle of UV spectrophotometry is light absorption using a wavelength between 200 and 400 nm with a colorless sample solution. The injection vitamin B_{12} level determination method was validated using the UV spectrophotometry method. Table 1 presents the validation data, with the concentration selected based on the sample concentration under analysis. A high degree of linearity is indicated by the r^2 value of 0.9994. Because the obtained accuracy, precision, and specificity values fall within the acceptability standards, the analytical method is precise and accurate. The quantitative limit (LOQ) in this investigation was 1.3858 µg/ml. In the meantime, 0.4573 µg/ml was the lowest analyte level in the sample that could be found and cause a reaction (LOD).

Optimized of the stability prediction model

Determination of the test design was done using Minitab 16 with a factorial design model. Obtained 8 runs according to Table 2. The prepared injections of vitamin B_{12} showed the observed reactions in terms of pH and content. According to the design results, vitamin B_{12} samples were evaluated in a climatic chamber at different temperatures, humidity levels, and storage times. The test was done by dissolving 1.5 ml of vitamin B_{12} sample into 50 ml of PW. Then scanning was done using a UV spectrophotometer with λ 361 nm. The pH test of the preparation was done using a Mettler Toledo pH meter.

The observations of pH and content determination during the optimization of the stability prediction model conditions are displayed in Table 2. The findings of the observations indicated that vitamin B_{12} remained stable enough. The United States Pharmacopeial 41 states that injection preparations containing vitamin B_{12} must have a pH of 4.5 to 7.0 and a minimum level between 95% and 115% (USP-NF, 2018).

Responses to the factor

Multiple linear regression was used to analyze data from factorial designs and find out whether there are significant influences and interactions of each factor. In RSM factorial designs were often used as part of more complex experimental designs that involve creating a mathematical model for the desired response (Myers *et al.*, 2013). Based on the statistics above, using temperature (X_1), humidity (X_2), and time (X_3) as independent variables with content determination (Y_1) and pH (Y_2) as the responses obtained:

$$Y_1 = -18.3 + 0.0444\,X_1 + 0.222\,X_2 + 0.116\,X_3 \quad \textbf{(1)}$$

and

$$Y_2 = -0.05 + 0.00167\,X_1 - 0.000\,X_2 + 0.00192\,X_3 \quad \textbf{(2)}$$

A 3D graphs of the variables: temperature, humidity, and time are displayed in Figures 1A and 1B, along with the contour plot of the content determination. There is less of a difference in levels before and after treatment as the temperature and time are lower. According to Table 3's *p*-value of 0.124, the humidity variable, on the other hand, has the least effect on lowering content determination. Since the Table 4 ANOVA *p*-value was quite large at 1.000, it was concluded that the humidity level in Figure 2A has no significant effect on the pH factor. A 3D graph of temperature and time variables is displayed in Figure 2B, along with the contour plot of the pH response.

Table 1. Validation data of analytical methods vitamin B_{12}

No	Parameter	Acceptance criteria	Results
1	Precision	RSD ≤ 2%	RSD 0.2%
2	Accuracy	Average recovery for each concentration 98 – 102% RSD ≤ 2% for each concentration	101.0%; 101.5%; 100.5% 0.1%; 0.1%; 0.2%
3	Linearity	$r^2 \geq 0.98$	0.9994
4	Specificity	-The standard solution and test solution must have an absorbance at maximum λ 361 nm ± 2 nm -The solvent and placebo did not provide absorbance at maximum λ 361 nm ± 2 nm	According to specifications According to specifications
5	LOD	-	0.4573 µg/ml
6	LOQ	-	1.3858 µg/ml

Table 2. Results optimization of the stability prediction model vitamin B_{12}

StdOrder	RunOrder	CenterPt	Blocks	Temperature	Humidity	Time	**Δ** Content of active (%)[a]	**Δ** pH
1	1	1	1	28	76	1	0.136	0.020
2	2	1	1	43	76	1	0.140	0.010
3	3	1	1	28	77	1	0.664	0.000
4	4	1	1	43	77	1	0.666	0.020
5	5	1	1	28	76	14	1.242	0.000
6	6	1	1	43	76	14	2.659	0.070
7	7	1	1	28	77	14	1.249	0.030
8	8	1	1	43	77	14	2.488	0.050

[a] vitamin B_{12} content of active target 1000 µg/ml with 10% overage.

The pH difference between before and after treatment is less at lower temperatures and durations.

Post market sampling

Post market sampling products were then subjected to a quantitative examination by comparing them with the initial examination of the product before it was marketed (Wallach *et al.*, 2019). The results obtained in Table 5. All test parameters post market sampling showed results according to requirements. However, there was a decrease in pH (3.46%) for post-market sampling in the Palembang distribution area and an increase in pH (4.54% and 5.33%) in the Cirebon and Denpasar distribution areas. Changes in pH during storage indicate instability in the drug preparation. pH can influence the level of decomposition of the preparation. The addition of acids or bases to drug preparations caused decomposition to be accelerated, causing the drug to become unstable. The pH changes that occur can be influenced by humidity. Analysis of surface humidity in the first - third months of July 2023 in the Palembang area was higher than in the Cirebon and Denpasar areas (BMKG, 2023). Chemically, the absorbed moisture was a medium for chemical decomposition (hydrolysis). Mild acid hydrolysis of cyanocobalamin can induce nucleotide removal, and additional fragmentation occurs as the severity of acidic conditions increases. Exposure to alkaline conditions causes amide hydrolysis,

Table 3. Analysis of variance (ANOVA) based on active vitamin B_{12} content determination

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Model	6	6.601	6.601	1.100	287.330	0.045
Linear	3	5.533	5.533	1.844	241.710	0.033
Temperature	1	0.885	0.885	0.885	231.360	0.042
Humidity	1	0.986	0.098	0.098	25.760	0.124
Time	1	4.549	4.549	4.549	1188.00	0.018
Interaction	3	1.067	1.067	0.355	92.950	0.076
Temperature*Humidity	1	0.004	0.004	0.004	1.070	0.489
Temperature*Time	1	0.877	0.877	0.877	229.230	0.042
Humidity*Time	1	0.185	0.185	0.185	48.540	0.091
Residual error	1	0.003	0.003	0.003		
Total	7	6.605				

Table 4. Analysis of variance (ANOVA) from pH

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Model	6	0.003	0.003	0.000	0.710	0.720
Linear	3	0.002	0.002	0.000	1.040	0.601
Temperature	1	0.001	0.001	0.001	1.560	0.430
Humidity	1	0.000	0.000	0.000	0.000	1.000
Time	1	0.001	0.001	0.001	1.560	0.430
Interaction	3	0.000	0.000	0.000	0.370	0.799
Temperature*Humidity	1	0.000	0.001	0.000	0.060	0.844
Temperature*Time	1	0.000	0.000	0.000	1.000	0.500
Humidity*Time	1	0.000	0.000	0.000	0.060	0.844
Residual error	1	0.000	0.000	0.000		
Total	7	0.004				

producing biologically inactive vitamin B_{12} carboxylic acid derivatives.

A decrease in levels of 0.90% was seen in the Palembang distribution region and 0.94% in the Cirebon distribution area as a result of the content parameters being checked. In the meantime, there was no drop in levels in the Denpasar distribution region. One of the causes of changes in the levels of preparation is environmental temperature. The temperature of the surrounding environment can affect how quickly a pharmaceutical treatment reacts (Ceribeli *et al.*, 2023; Yamada *et al.*, 2008). In general, a reaction happens more quickly at higher temperatures, where the reaction rate constant, or k, will be higher (Chandra-Hioe *et al.,* 2020). Based on the analysis of surface temperatures, it can be observed that Bali has lower temperatures than the Palembang and Cirebon area (BMKG, 2023).

Paired t-test

To determine the average of two sets of data, the paired t-test was employed (Priyanto, 2013). This study compared the pH and content determination between multiple linear regression using response factor and post-market sample data using a paired t-test. The test findings were shown in Table 6. The stability optimization results equation was used to determine the content and pH of vitamin B_{12}. Post-market sampling using a paired t test revealed that the difference was not significant, with *p*-values of 0.065 and 0.491 (<0.05) or H_0 obtained.

Table 5. Difference pH, content determination pre market and post market sampling

Specification	pH (4.5 – 7.0)			Content determination (95.0 – 115.0)		
	Pre market	Post market	Δ pH	Pre market	Post market	Δ Content determination
Palembang	6.640	6.410	0.230	114.250	113.220	1.030
Cirebon	6.600	6.900	0.300	113.580	112.510	1.070
Denpasar	6.370	6.710	0.340	111.150	111.150	0.000

Table 6. Results paired t-test

Response	Content determination		pH	
	MLR[a] (Prediction test)	Post market sample (Real test)	MLR (Prediction test)	Post market sample (Real test)
Mean	2.211	0.700	0.016	-0.137
Variance	1.711	0.368	4E-06	0.101
Observations	3.000	3.000	3.000	3.000
Pearson Correlation	0.998		0.998	
Hypothesized Mean Difference	0.000		0.000	
df	2.000		2.000	
t Stat	3.718		0.837	
P(T<=t) one-tail	0.033		0.245	
t Critical one-tail	2.920		2.920	
P(T<=t) two-tail	0.065		0.491	
t Critical two-tail	4.303		4.303	

[a] MLR : Multiple linear regression.

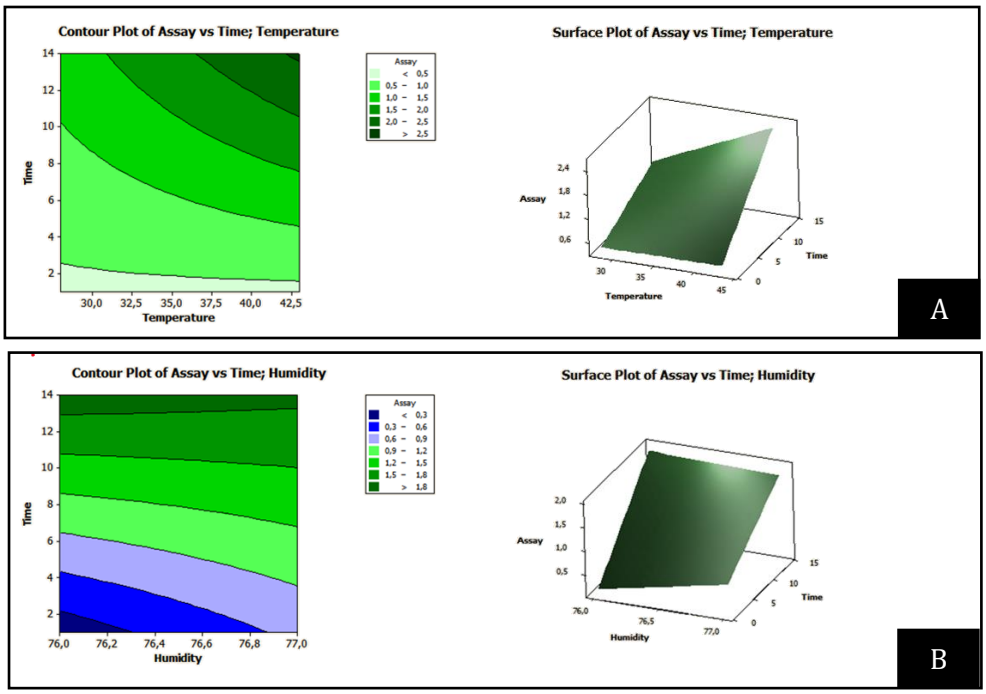

Figure 1. Contour plot and surface plot of content determination response on the time vs temperature (A), and time vs humidity (B)

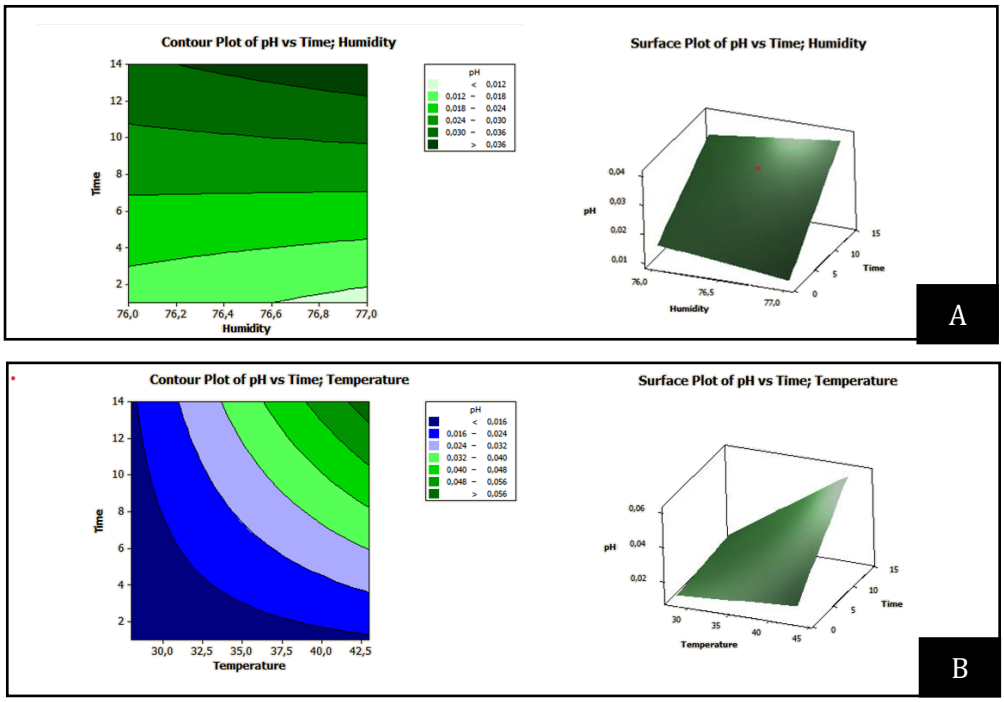

Figure 2. Contour plot and surface plot of pH response on the time vs humidity (A), and time vs temperature (B).

CONCLUSIONS

The stability of vitamin B_{12} injections is contingent upon temperature, humidity, and distribution time, as determined by content determination and pH factors. By reducing the temperature, humidity, and distribution time to the lowest values stated on the label, ideal test points and pH can be preserved. Results from post-market sampling and stability condition optimization using content determination and pH parameter testing demonstrated consistent product performance throughout the study period. When compared to the outcomes of post-market sampling tests, the stability optimization condition prediction model is intended to ascertain how temperature, humidity, and distribution time affect the preparation's content and pH. According to the data, there is no discernible discrepancy between the prediction model and the post-market sample results, or H_0 is accepted. So, the prediction model can be used to determine the stability of vitamin B_{12} injections.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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