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Validation of Analytical Method for The Dissolution Test of Vitamin D3 5000 IU Chewable Tablets With HPLC

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ABSTRACT

Background: Vitamin D3 is a fat-soluble vitamin. The low solubility of vitamin D3 in water has attracted the researchers to develop a modified form of vitamin D3 in which is more soluble in water. High solubility will increase the bioavailability of vitamin D3 in the body and improving product performance both in vitro and in vivo.

Aim: This study aims to validate the dissolution analysis method of vitamin D3 chewable tablets using HPLC and the stability of samples vitamin D3 5000 IU up to 6 weeks of shelf life with storage conditions at 30 °C ± 2 °C and 40 °C ± 2 °C and relative humidity 75% ± 5%.

Method: This study refers to USP 43 NF 38 with modifications of mobile phase.

Result: The analytical method used shows linear results with a coefficient of correlation (r) equal to 0.9997. The value of LoD and LoQ are 0.0076 ppm and 0.02302 ppm, respectively. The Q values of intermediate precision test are 86.43% and 85.22%. The recovery percentage is 96.82% - 100.1338%. The specificity test shows the analyte can be clearly distinguished from other components. Stability test of solution resulting in percentage of dissolution 100.14% and %RSD equal to 0.13% for 0 hours and 99.28% and %RSD equal to 0.33% for 24 hours. In the accelerated stability test, the results at 0 week were 85.22%, the results at 6 weeks at 30 °C and 40°C are 86.72% and 82.81%, respectively.

Conclusion: Validation of analytical method has met the acceptance criteria from ICH and USP.

Keywords: Validation, analytical method, vitamin D3, dissolution test, accelerated stability test

INTRODUCTION

The Covid-19 pandemic caused by SARS-CoV-2 has received attention from all over the world. SARS-CoV-2 is a β-*coronavirus* which infects the lung interstitium by attaching to *Angiotensin Converting Enzyme* 2 (ACE2) receptors[1]. Several risk factors, such as age, increased body mass index, *diabetes mellitus*, and cardiovascular disease may contribute to worsen Covid-19 infection. However, the increased risk can also be caused by vitamin D deficiency in the body[2]. Sabico et al. (2021), conducted clinical trial in Riyadh, Saudi Arabia, with 36 confirmed Covid-19 positive patients given Ultra-D® 5000 IU (125 µg cholecalciferol) and 33 confirmed Covid-19 positive patients given Ultra-D® 1000 IU (25 µg cholecalciferol). After both group taken orally daily for 2 weeks, group of patients with vitamin D3 5000 IU show shorter time recovery and show mild to moderate symptoms[3].

Vitamin D3 can be synthesized in the skin by 7-*dehydrocholecalciferol* precursors through Ultraviolet B (UVB)[4] at a wavelength of 290-315 nm[5]. Vitamin D3 is attracting the researcher because it has several health benefits, such as lowering the risk of cancers and chronic diseases or supporting immune system and the bones

health[6]. However, vitamin D is fat-soluble vitamin[7]. The low solubility of vitamin D3 in water has attracted the researchers to modify vitamin D3 so that more soluble in water, thus increasing bioavailability of vitamin D3 in the body and improving product performance in vitro and in vivo[8]. The first step must be taken to ensure and control the quality of the active ingredients of new products[6]. Analytical method used in ensuring and controlling quality the active ingredient must be validated [9].

The validation of analytical method is an analytical method that is determined through laboratory studies and has met the requirements for analytical purposes[10]. The dissolution test aims to represent the process of absorption of the product into the systemic circulation[11]. This study refers to USP (The United States Pharmacopeia) NF 38 (The National Formulary) with modifications to adjust the availability of tools and materials. The study uses dissolution system, 500 mL dissolution medium of 0.1% solution octoxynol-9, medium temperature 37 ℃ ± 0.5 ℃, apparatus type-2 (paddle), run time 30 minutes, speed 75 rpm, and chromatography system, UV detector 265 nm, column C18, column temperature 40 ℃, flow rate 1.0 mL/min, injection volume 100 µL, mobile phase Acetonitrile-Water (95:5). In research conducted by Kumar et al. (2015), validation of the vitamin D3 analytical method using the mobile phase Methanol-Water (95:5), column C18, and detector UV 265 nm resulting %Relative Standard Deviation (%RSD) peak area of 0.68%, retention time of 7 minutes, and correlation time (r^2) of 0.996. Selection of acetonitrile as the mobile phase is due to lower viscocity of acetonitrile (0.37 cP) than methanol (0.54 cP) [13]. Low viscosity may cause running time of chromatography is shorter, because mass transfer occurs more quickly[14]. This study aims to know the dissolution analysis method of vitamin D3 chewable tablets with HPLC and the stability of samples vitamin D3 5000 IU at 6th weeks of shelf life with storage conditions of 30 °C ± 2 ℃ and 40 °C ± 2 °C at Rh 75% ± 5%.

METHODS

Preparation of Dissolution Media

A 0.1% solution of octoxynol-9 was prepared with 4 mL of octoxynol-9 added to a measuring beaker glass 1 L which already contains 1 L of water. Octoxynol-9 solution was homogenized and poured into the dissolution medium container. Then the octoxynol-9 solution diluted with water up to 4 L.

Preparation of Standard Stock Solution

A standard stock solution concentration of vitamin D3 with concentration 250 ppm was made by putting 250 mg of vitamin D3 working standard into a 50 mL volumetric flask. Then 15 mL of isopropyl alcohol was added and sonicated for 20 minutes. Then the standard vitamin D3 solution was diluted with isopropyl alcohol until mark. One mL of the standard stock solution was pipetted into a 20 mL volumetric flask, then diluted with dissolution medium to the mark and homogenized to obtain a standard concentration of 250 ppm vitamin D3 solution.

Preparation of Sample Solution

Six samples of vitamin D3 5000 IU chewable tablets were put simultaneosly into each of the six dissolution vessels, containing 500 mL of dissolution medium which was previously maintained at 37 ℃ ± 0.5 ℃. The dissolution tester was turned on at 75 rpm for 30 minutes, while the vessel was covered with a black cloth.

System Suitability Test

System suitability test was carried out by injecting six replicate of 100% standard solution (containing 0,25 ppm vitamin D3) and analyzing the chromatogram for percentage of Relative Standard Deviation (%RSD) of peak area, tailing factor (T), and plate count (N). The acceptance criterion is %RSD ≤ 2,010; T < 2,0; and N > 200015.

Analytical Method Validation

Linearity

For linearity test, standard solutions were prepared with a concentration of 0.125 ppm; 0.1875 ppm; 0.25 ppm; 0.3125 ppm; and 0.375 ppm. Linearity test was carried out by injecting three replicates for each concentration. Calibration curve was plotted between concentration and peak area and correlation coefficient values (r) was obtained. The acceptance criteria for linearity is correlation coefficient (r) value ≥ 0,9810.

Limit of Detection (LoD) and Limit of Quantitation (LoQ)

Limit of Detection (LoD) and Limit of Quantitation (LoQ) were determined by the Standard Deviation (SD) and slope (S) of calibration curve. LoD and LoQ were determined by a formula:

LoD = 3,3 × SD/S

LoQ = 10 × SD/S

In which SD = Standar deviation, and S = Slope[16].

Precision

Precision was determined by measuring dissolution of six replicates of vitamin D3 samples. The acceptance criteria for vitamin D3 intermediate precision is that Q values is ≥ 75%, and the difference in the mean value for dissolution results between two different days does not exceed an absolute 10% at Q ≤ 85%[10].

Accuracy

For accuracy test, standard + placebo solutions were prepared with a concentration of 80% standar + placebo solution (containing 40 mg vitamin D3 standard and 80 mg placebo), 100% standar + placebo solution (containing 50 mg vitamin D3 standard and 100 mg placebo), and 120% standar + placebo solution (containing 60 mg vitamin D3 standard and 120 mg plaebo). Accuracy test was carried out by injection three replicate for each concentration.

Specificity

Specificity was carried out by injecting six replicates of 100% standard solution, three replicate of 100% standar + placebo solution, and three replicates of placebo solution.

Solution Stability

In order to demonstrate the stability of test solution, the solution stability was determined by injecting three replicates of 100% standard + placebo solution and comparing the peak areas after storage for 24 hours. Acceptance criteria for stability solution test is the recovery 95% - 105%[10].

Stability Testing

Stability testing of vitamin D3 chewable tablets was carried out at storage condition of 30 ℃ ± 0,2 ℃ and 40 °C ± 2 °C and Rh 75% ± 5% for 6 weeks of storage[17]. Acceptance criteria for stability testing is Q values ≥ 75%[10]

RESULTS AND DISCUSSION

Performing system suitability test is important to verify the instrument or the chromatography system18. The result of system suitability test shown in Table 1. The result of %RSD peak area is 0.3136%; %RSD retention time is 0.0649%; T values is 1.02; and the N values is 11554.3333. These results suggest that the system suitability test has met the acceptance criteria.

Table 1. System Suitability Test

No.	Peak Area	Retention Time (tR)	Tailing factor (T)	Plate Count (N)
1	70483	9.708	1.01	11601
2	70255	9.711	1.02	11532
3	70136	9.710	1.03	11655
4	70187	9.721	1,01	11569
5	70494	9.723	1.00	11427
6	70708	9.718	1.02	11542
Total	**422263**	**-**	**-**	**-**
Mean	**70377.17**	**9.7152**	**1.02**	**11554.33**
SD	**220.70**	**0.0063**	**-**	**-**
%RSD	**0.31**	**0.0649**	**-**	**-**

The result of linearity test of the five concentrations is shown in the Table 2 which further plotted in a curve in which x-axis is the concentration and the y-axis the peak area (Figure 1). From the equation of the calibration curve results, the coefficient of correlation is 0.9997. LoD and LoQ values of this study were determined by the SD and slope (S) of calibration curve, which further calculated using formula. The obtained LoD and LoQ values are 0.0076 ppm and 0.02302 ppm, respectively.

Table 2. Linearity Test Result

Concentration (ppm)	Peak Area
0.125	544.3333
0.1875	841
0.25	1137
0.3125	1399.33
0.375	1681

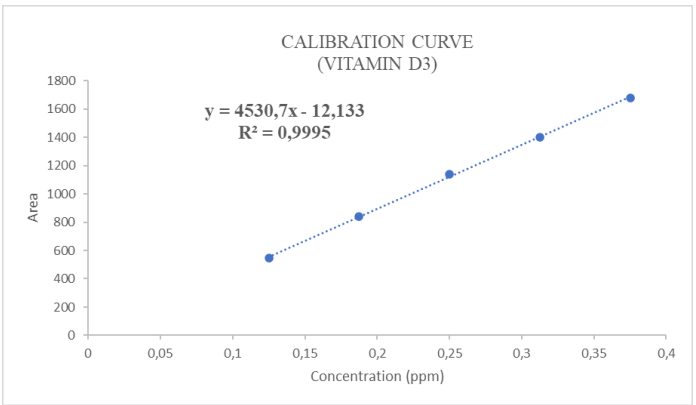

Figure 1. Calibration Curve Linearity Test Analysis of Vitamin D3

The result of intermediate precision of the six concentrations is shown in the Table 3. Based on Table 3, the Q values of intermediate precision I and intermediate precision II are 86.46% and 85.22%, respectively. The difference in the Q values for intermediate precision I and intermediate precision II is 1.24%.

Table 3. Intermediate Precision Test Results

No.	Intermediate Precision I		Intermediate Precision II	
	Peak Area	Dissolution (%)	Peak Area	Dissolution (%)
1	945	87.54	910	84.12
2	947	87.73	907	83.85
3	943	87.36	973	89.95
4	948	87.82	975	90.13
5	898	83.19	886	81.91
6	919	85.13	880	81.35
Mean		**86.46**		**85.22**

Accuracy test were determined by measuring three concentrations and three replications for each concentration. Accuracy test is evaluated by percent recovery (Table 4). Based on Table 7, the % recovery result for concentrations of 80%, 100%, and 120% are 96.82%, 100.13%, and 97.66%. respectively.

Table 4. Accuracy Test Results

No.	Concentration (%)	Actual Weight (mg)	Peak Area	Recovery Weight (mg)	% Recovery	% Recovery Average	%RSD
		40.0	54695	38.86	97.15		
1	80	40.0	54641	38.82	97.05	96.81	0.51
		40.0	54189	38.50	96.25		
		50.0	70472	50.07	100.13		
2	100	50.0	70562	50.13	100.26	100.13	0.13
		50.0	70380	50.00	100.00		
		60.0	82733	58.78	97.96		
3	120	60.0	82379	58.53	97.54	97.66	0.27
		60.0	82319	58.48	97.47		
	Mean		**69152.22**			**49.13**	**1.55**

Specificity testing is carried out to ensure that measurement of analytes are not affected by placebo [15]. Chromatogram of specificity test results is shown in Figure 2. Furthermore, data from chromatogram of standard, placebo, and standard + placebo specificity tests are summarized in Table 5.

Table 5. Specificity Test Results

Solution	Peak Area Mean	Retention Time Average (minute)	Concentration (%)	%RSD
Standard	70377.17	9.715	101.19	0.31
Placebo	-	-	-	-
Standard + Placebo	70612	9.511	100.33	0.1776

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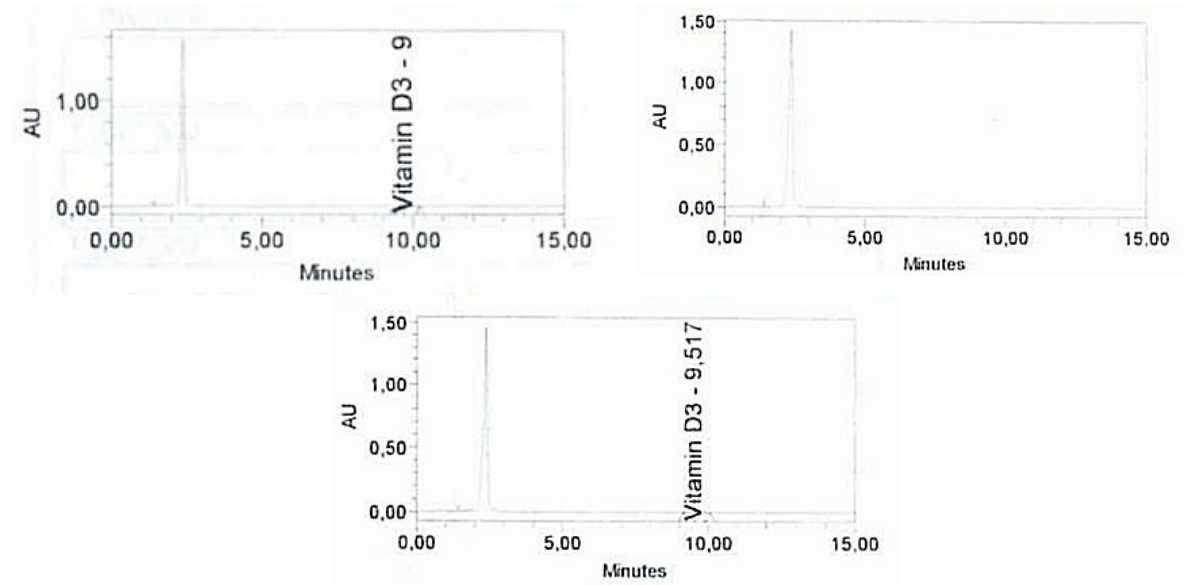

Figure 2. Vitamin C Serum Gel Preparation with Optimum Formula.

In stability test, % recovery and % RSD of solution in 0 hour test are 100.13% and 0.13%, respectively. Meanwhile, after storage 24 hours, %recovery and % RSD are 99.28% and 0.33%. The results of solution stability is shown in Table 6.

Table 6. Solution Stability Test Results

No.	0 Hours			24 Hours		
	Peak Area	**%Recovery**	**%RSD**	**Peak Area**	**%Recovery**	**%RSD**
1	70472	100.14		70214	99.02	
2	70562	100.26	0.13	70660	99.65	0.33
3	70380	100.00		70316	99.16	
Mean		**100.13**			**99.28**	

The Q values for vitamin D3 with a shelf life of 0 week is 825,2182%; with a shelf life of 6 weeks at 30 ℃ and 40 ℃ *are* 86.72% and 82.81%. The results of accelerated stability test is shown by Table 7.

Table 7. Accelerated Stability Test Results

No.	0 Weeks		6 Weeks (30 ℃)		6 Weeks (40 ℃)	
	Peak Area	**Dissolution (%)**	**Peak Area**	**Dissolution (%)**	**Peak Area**	**Dissolution (%)**
1	910	84.12	975	89.69	906	83.35
2	907	83.85	1005	92.45	906	83.35
3	973	89.95	905	83.25	911	83.81
4	975	90.13	909	83.62	901	82.89
5	886	81.91	927	85.27	880	80.95
6	880	81.35	935	86.01	897	82.52
Mean		**85.22**		**86.72**		**82.81**

DISCUSSION

Analytical method validation aims to ensure that an analytical method will work in accordance with the desired conditions and objectives in terms of experimental testing and scientific evidence. However, the quality of the results obtained in an analysis process may deviate which may be caused by several factors, such as decreased efficiency or malfunctions of the instruments used. Therefore, it is necessary to carry out a system suitability test both before and simultaneously with the analysis proces[16], to verify that the instrument or chromatographic system that will be applied for the further analysis[18]. Injection of six replications aims to evaluate the suitability of the injector and mobile phase pump of the instrument used[16]. Parameters of system suitability test suggest that the test has met the acceptance criteria and good precision, so the chromatography system can be applied for futher analysis.

Linearity test is the ability to obtain test results that are directly proportional to the concentration of the analyte[19]. Linearity test is evaluated by calibration curve and correlation cofficient (r). The correlation coefficient (r) is a measure of the relationship between variables (concentration and peak area)[15]. The calibration curve is presented in Figure 1. From the equation of the calibration curve results, the coefficient of determination is 0.9995 and the correlation coefficient (r) is 0.9997. This result indicates a linearity.

The limit of detection is the lower limit of the analyte contained in the sample that still can be detected. Meanwhile, the limit of quantitation is the lowest limit of analytes that can be expressed in terms of precision and accuracy[18]. Based on the calibration curve, LoD values of 0.0076 ppm and LoQ values of 0.02302 ppm.

Precision test is a measure that shows the degree of uniformity, obtained from the distribution of average individual results through repeated procedures and sampling[20]. Precision is determined by injection of six replicates of vitamin D3 dissolution samples and carried out in two different days. The results of intermediate precision is shown in Table 3. The Q values of intermediate precision I of 86.46% and the Q values of intermediate precision II of 85.22%. The difference in the Q values for intermediate precision I and intermediate precision II is 1.24%. This suggest that the precision test has met the acceptance criteria with the Q values are ≥ 75% and the difference in the Q values for intermediate precision I and II is ≤ 10%[10].

Accuracy is the closeness between the expected value and the results obtained from testing[19]. Accuracy test is determined by injecting three replicates for each concentrations. In this test, the sample consists of a mixture of placebo and standard. Accuracy test is expressed by recovering the theoritical amount of analyte added[16]. Based on the result (Table 4), the accuracy test has met the acceptance criteria.

Specificity is the ability to strictly assess the analyte and other additional components[10]. Specificity testing is carried out to ensure that the expected analytes are not affected by placebo, so that results of linearity, precision, and accuracy can be guaranteed [15]. Specificity test resulting in the presence of analyte peaks and empty peaks in the placebo test[21]. Based on Figured 2, the peak of vitamin D3 is not interfered by placebo and based on Table 5. concentration results between standard solution and standard + placebo solution are not too different. These results suggest that the specificity test method can clearly shown the presence of vitamin D3 peaks.

The stability of the test solution is carried out by storing the test solution at room temperature and reanalyzing it at the specified time variation[18]. Testing the stability of the test solution was carried out by injecting 100% standard together with placebo solution 3 times replication into the HPLC instrument. Furthermore, the solution will be stored at room temperature and tested again after 24 hours. Based on Table 6, there is a decrease in % recovery after 24 hours storage.

Stability test aims to determine the resistance of a product to certain limits during a certain shelf life, so that the product still has the same characteristics as when it was made[22]. Stability testing of vitamin D3 samples for chewable tablets was carried out at storage conditions of 30 ℃ ± 2 ℃ and 40 °C ± 2 °C and Rh 75% ± 5% for 6 weeks of storage. In stability studies that are only carried out under a narrow range of conditions, testing is generally carried out for 6 – 24 weeks at 40 °C ± 2 °C and Rh 75% ± 5%[17]. Based on Table 7, the stability test has met the acceptance criteria with the Q values is ≥ 75%.

CONCLUSION

The conclusion of this study is the validation of analytical method has met the acceptance criteria from ICH and USP, with a value of r=0.9997, LOD value is 0.0076 ppm, and LOQ value is 0.0230 ppm. In accuracy test, % recovery value are 96.81% for concentration of 80%; 100.13% for 100% concentration and 97.66% for 120% concentration. In precision test, the average of intermediate precision I and II are 86.46% and 85.22%, respectively. There is no other compound which can interfere analyte measurement in this test. In stability test, % recovery at 0 hours, 24 hours, and 6 weeks are 100.14%, 99.28%, 86.72% (for 30°), and 82.81% (for 30°).

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CONFLICT OF INTEREST

The authors does not have conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors read and approved the final manuscript.

AUTHOR DETAILS

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REFERENCES

1. Adami G, Giollo A, Fassio A, et al. Vitamin D and disease severity in coronavirus disease 19 (COVID-19). 2020;72(4):189-196.
2. Orchard L, Baldry M, Nasim-mohi M, et al. Vitamin-D levels and intensive care unit outcomes of a cohort of critically ill COVID-19 patients. 2021;59(6):1155-1163. doi:https://doi.org/10.1515/cclm-2020-1567
3. Sabico S, Enani MA, Sheshah E, et al. Effects of a 2-Week 5000 IU versus 1000 IU Vitamin D3 Supplementation on Recovery of Symptoms in Patients with Mild to Moderate Covid-19 : A Randomized Clinical Trial. *Nutrients*. 2021;13(7):270. doi:https://doi.org/10.3390/nu13072170
4. Oscanoa TJ, Amado J, Vidal X, Laird E, Ghashut RA, Romero-ortuno R. The relationship between the severity and mortality of SARS-CoV-2 infection and 25-hydroxyvitamin D concentration — a metaanalysis. Published online 2021:145-157. doi:10.5603/ARM.a2021.0037
5. Saponaro F, Saba A, Zucchi R. An update on vitamin d metabolism. *Int J Mol Sci*. 2020;21(18):1-19. doi:10.3390/ijms21186573
6. Andri B, Lebrun P, Dispas A, et al. Optimization and validation of a fast supercritical fluid chromatography method for the quantitative determination of vitamin D3 and its related impurities. *J Chromatogr A*. 2017;1491:171-181. doi:10.1016/j.chroma.2017.01.090
7. Handono K, Kalim H, Nurdiana, et al. *VITAMIN D DAN AUTOIMUNITAS*. Cetakan Pe. UB Press; 2018.
8. Braithwaite MC, Choonara YE, Kumar P, Tomar LK, Du Toit LC, Pillay V. A novel bile salts–lipase polymeric film-infused minitablet system for enhanced oral delivery of cholecalciferol. *Pharm Dev Technol*. 2016;21(7):832-846. doi:10.3109/10837450.2015.1069329
9. Sugihartini N, Fudholi A, Pramono S. VALIDATION METHOD OF QUANTITATIVE ANALYSIS OF EPIGALLOCATHECIN GALLAT BY HIGH PERFORMANCE. *Pharmaciana*. 2014;4(2):111-115.
10. U.S. Pharmacopeia. *The United States Pharmacopeia, USP 43/The National Formulary, NF 38*. Volume 1. U.S. Pharmacopeial Convention, Inc.; 2020.
11. Sofyani CM, Rusdiana T, Chaerunnisa AY. REVIEW: Validasi Metode Analisis Kromatografi Cair Kinerja Tinggi untuk Penetapan Kadar Uji Disolusi Terbanding Tablet Amoksisilin. *Farmaka*.

2018;16(1):324-330.
12. Kumar S, Chawla D, Tripathi AK. An Improved and Sensitive Method for Vitamin D3 Estimation by RP-HPLC. *Pharm Anal Acta*. 2015;6(8):6-11. doi:10.4172/21532435.1000410
13. Lerrick RI. *FOTOLUMINESENSI ORGANIK : SINTESIS, DESAIN, DAN APLIKASINYA*. UGM PRESS; 2022.
14. Susanti M, Dachriyanus. *Kromatografi Cair Kinerja Tinggi*. Cet.1. Andalas University Press; 2014.
15. Ravisankar P, Pentyala A, Sai CB, Hemasri P, Babu PS. VALIDATION CHARACTERISTICS AND STATISTICS IN ANALYTICAL METHOD DEVELOPMENT. *High Technol Lett*. 2021;27(7).
16. Marson BM, Concentino V, Junkert AM, Fachi MM, Vilhena RO, Pontarolo R. VALIDATION OF ANALYTICAL METHODS IN PHARMACEUTICAL QUALITY SYSTEM: AN OVERVIEW FOCUSED ON HPLC METHODS. *Quim Nov*. 2020;43(8):1190-1203. doi:https://dx.doi.org/10.21577/0100-4042.20170589
17. Waterman KC, Carella AJ, Gumkowski MJ, et al. Improved protocol and data analysis for accelerated shelf-life estimation of solid dosage forms. *Pharm Res*. 2007;24(4):780-790. doi:10.1007/s11095-006-9201-4
18. Direktorat Jenderal Kefarmasian dan Alat Kesehatan. *FARMAKOPE INDONESIA*. VI. Kementerian Kesehatan RI; 2020.
19. Alquadeib BT. Development and validation of a new HPLC analytical method for the determination of diclofenac in tablets. *Saudi Pharm J*. 2019;27(1):66-70. doi:10.1016/j.jsps.2018.07.020
20. Riyanto. *Validasi & Verifikasi Metode Uji: Sesuai Dengan ISO/IEC 17025 Laboratorium Pengujian Dan Kalibrasi*. Edisi 1, C. Deepublish; 2015.
21. Sharma S, Goyal S, Chauhan K. A REVIEW ON ANALYTICAL METHOD DEVELOPMENT AND VALIDATION. 2018;10(6). doi:http://dx.doi.org/10.22159/ijap.2018v10i6.28279
22. Deviarny C, Lucida H, Safni. Uji Stabilitas Kimia Natrium Askorbil Fosfat Dalam Mikroemulsi Dan Analisisnya Dengan HPLC. *J Farm Andalas*. 2012;1((1) April).