HELIUM: Journal of Science and Applied Chemistry
https://journal.unpak.ac.id/index.php/he_jsac

Volume 01, Number 02, December 2021, Page 57 - 62
e-ISSN: 2776-4508; p-ISSN: 2776-1711
Publisher : Universitas Pakuan

STUDY OF REACTION KINETICS AND IDENTIFICATION OF CLOTRIMAZOLE DEGRADATION COMPOUNDS IN ANTI-FUNGAL CREAM PREPARATIONS

Cellia Christiana Batubara [a)], Ani Iryani[a)], Linda Jati Kusumawardani[*)]

[a)] *Universitas Pakuan, Bogor, Indonesia*

[*)] *Corresponding Author: linda.wardani@unpak.ac.id*

Article history: *received 08 September 2021; revised 22 October 2021; accepted 28 November 2021; published 31 December 2021*

Abstract.

Fungal infections of the skin can be treated with antifungal compounds, one of which is clotrimazole. Under certain conditions, clotrimazole can be degraded to imidazole and (o-chlorophenyl) diphenyl methanol which can cause skin irritation. This is because the storage conditions of the drug product during or after use if it is not suitable can interfere with the stability of the drug product. Therefore, this study was conducted to obtain information regarding changes in clotrimazole levels, reaction order, reaction constant value, half-life, activation energy and the possibility of compounds formed after the clotrimazole degradation process to determine the good storage conditions of the product. Clotrimazole levels were tested in the open and closed state of the packaging every day at different storage temperatures for three months. Changes in clotrimazole levels were measured using HPLC every week. In addition, the identification of compounds resulting from degradation was also tested using HPLC every month and continued using LC-MS/MS in the last week. The results of the analysis of clotrimazole levels, there was a decrease in levels but still in accordance with the specifications required by the Indonesian Pharmacopoeia. The greatest decrease in levels occurred in samples stored at a temperature of 40±2ºC which reached 9133%. The decrease in clotrimazole levels followed a first-order reaction with the shortest half-life of 90 weeks and an activation energy of 18.9 kJ/mol. Identification of the degraded compounds using HPLC and LC MS/MS found imidazole compounds and (o-chlorophenyl) diphenyl methanol in samples stored at 25±2ºC and 40±2ºC. These compounds can cause skin irritation, but these compounds may not have a serious impact because they have a fairly large body threshold value of 220 mg/kg. This shows that clotrimazole cream stored at various temperatures, both at 5±2ºC, 25±2ºC and 40±2ºC can still be used for three months after the packaging is opened according to the in-use shelf life.

Keywords: Clotrimazole; Stability test; HPLC; LC MS/MS.

1. INTRODUCTION

Indonesia is a country with a tropical climate that has high temperature and humidity. In addition to the tropical climate, people's skin conditions are easily moist and sweaty and skin hygiene is not maintained is a factor in the emergence of skin infections due to fungi **[1].** One of the active compounds that can be used as antifungal drugs is clotrimazole. Clotrimazole has the principle that it can inhibit the performance of the enzyme lanosterol 14 α-demethylase. So that the amount of ergosterol in the fungus will decrease and can damage the structure and function of the fungal membrane so that it can inhibit fungal growth **[2].** Clotrimazole has the IUPAC name 1-(2-chloro-phenyl)diphenylmethyl-1H-imidazole and the molecular formula is $C_{22}H_{17}ClN_2$ with a molecular weight of 344.8 grams/mol **[3].**

In BPOM RI regulation number 24 of 2017 concerning drug registration procedures, the pharmaceutical industry not only attaches the results of stability tests during the shelf life but also attaches the results of stability tests during the period of drug use with the aim of protecting the public from the circulation of dangerous drugs **[4].** Inappropriate storage conditions for medicinal products can interfere with the stability of a drug product. In a study conducted by Tawakkol in 2019, under conditions that were not suitable, clotrimazole could be degraded. The structure of clotrimazole is degraded to imidazole and (o-chlorophenyl)diphenylmethanol **[5]**. These compounds can cause irritation to the skin and can cause skin burns according to the explanation on the material safety data sheet (LDKB). The purpose of this study was to obtain information about changes in clotrimazole levels, reaction order, reaction constant value, half-life, activation energy and the possibility of compounds formed after the degradation process. The storage conditions of the antifungal cream preparations can interfere with the stability of the product and under conditions that are not suitable for clotrimazole contained in the antifungal cream preparations can be degraded.

In this study, a stability test for antifungal drugs containing the active compound of clotrimazole will be conducted for three months according to the in-use shelf life according to the National Health Service **[6].** The results of the stability test are then evaluated for conformity based on the requirements set by the Indonesian Pharmacopoeia Edition V **[7].** The HPLC instrument was used to analyze clotrimazole levels and identify compounds resulting from degradation and will be continued with the identification of compounds using LC-MS/MS because LC-MS/MS can produce consistent, accurate isotope patterns and can provide

Volume 01, Number 02, December 2021, Page 57 - 62
e-ISSN: 2776-4508; p-ISSN: 2776-1711
Publisher : Universitas Pakuan

fast spectral acquisition so that it can be used for confirmation of compounds and identify compounds that are not yet known **[8].**

2. RESEARCH METHODS

The test sample used was antifungal cream brand X in a package that had been opened and closed and as a comparison or control an antifungal cream brand X was used which was still sealed. Sample packaging is opened and then closed again every day. Samples will be tested for 3 months. Samples and comparisons will be analyzed weekly for clotrimazole levels, identified compounds formed as a result of degradation using HPLC every month and followed by identification using LC MS/MS in the last week. Sample treatment and comparison can be seen in Table 1.

Table 1. Treatment of antifungal cream samples

Sample	Treatment		Sample Code
Anti-Fungus Cream	Temperature	5°C ± 2°C	A
		25°C ± 2°C	B
		Not controlled	C
		40°C ± 2°C	D
	Packaging	Open	1
		Closed	2

Where the sample treatment at various storage conditions consists of:
A1 : At a temperature of 5°C ± 2°C with an open package
A2 : At a temperature of 5°C ± 2°C with closed packaging
B1 : At a temperature of 25°C ± 2°C with an open package
B2 : At a temperature of 25°C ± 2°C with closed packaging
C1 : At uncontrolled temperature with open package
C2 : At uncontrolled temperature with closed packaging
D1 : At a temperature of 40°C ± 2°C with an open package
D2 : At a temperature of 40°C ± 2°C with closed packaging

The tools used include analytical balance, measuring flask, sharpening erlenmeyer, measuring cup, volumetric pipette, ultrasonic, magnetic stirrer, hotplate, centrifuge, membrane filter with a pore diameter of 0.2 µm, vial, syringe, HPLC, LC-MS /MS. The ingredients used include antifungal cream, methanol, acetonnitrile, potassium dihydrogen phosphate, potassium hydrogen phosphate, ammonium formate and formic acid.

2.1. Analysis of Clotimazole Levels

Samples were weighed as much as ± 2,5 grams, put into a centrifuge tube, added 25 mL of methanol, heated in a water bath at 50°C for 5 minutes, shaken vigorously for 5 minutes, cooled in an ice bath for 15 minutes, centrifuged, the clear solution was transferred into 50 mL volumetric flask, added 20 mL of methanol into a centrifuge tube, the extraction process was repeated, the clear solution from the second extraction was transferred to a 50 mL volumetric flask containing the first extract, diluted with methanol to the mark, homogenized, filtered with 0.2 PVDF membrane filter was inserted into the vial, then read using HPLC.

Formula for calculating clotrimazole levels (Equation 1)

$$(\%) = \frac{A_{sample}\ x\ C_{standard}(mg/ml)\ x\ \text{Potency std}}{A_{standard}\ x\ C_{sample}(mg/ml) x\ \text{Etiquette}}\ x\ 100$$

Information:
A sample = The peak area of the sample from the sample chromatogram
A standard = The standard peak area of the sample chromatogram.
Potency std = Standard purity (%)
C sample = Sample concentration (mg/ml)
C standard = Standard concentration (mg/ml)
Etiquette = Claims for the content of the active substance in the product

2.2. Identification of Degraded Compounds with HPLC

The sample was weighed as much as ±1000 mg, put into a sharpening erlenmeyer, added 50 mL absolute methanol, heated at 60°C for 5 minutes. Shaken for 3 minutes, the solution was filtered into a 100 mL volumetric flask, squeezed with absolute methanol, homogenized, the solution was pipetted 5 mL, put into a 10 mL flask, squeezed with absolute methanol, homogenized, filtered with 0.2 m PVDF filter membrane, put into vial, then read using HPLC.

2.3. Identification of Degraded Compounds with LC MS/MS

The sample was put into a centrifuge tube, added 25 mL of methanol, heated in a water bath at 50 °C for 5 minutes, shaken vigorously for 5 minutes, cooled in an ice bath for 15 minutes, centrifuged, filtered with 0.2 m PVDF membrane filter, put into vial, then read using LC-MS/MS.

3. RESULTS AND DISCUSSION

3.1. Analysis of Clotimazole Levels

The results obtained showed that there was a decrease in each sample in various storage conditions but still met the specifications required by the Indonesian Pharmacopoeia Edition V, namely 90.0%-110.0%. Various storage conditions but still met the specifications required by the Indonesian Pharmacopoeia Edition V, namely 90.0%-110.0%. Based on the graph of the comparison of clotrimazole levels against storage time, the largest decrease in levels occurred in sample D1, namely the antifungal cream sample which was placed at a temperature of 40°C ± 2°C with a concentration of 91.33% in the thirteenth week so that the antifungal cream that had been used should be stored at a maximum room temperature of 38°C so that the product remains suitable for use, but according to BMKG data in 2020, the highest temperature in Indonesia is only 37.2°C so that the temperature in Indonesia is still below the recommended storage temperature .

Volume 01, Number 02, December 2021, Page 57 - 62
e-ISSN: 2776-4508; p-ISSN: 2776-1711
Publisher : Universitas Pakuan

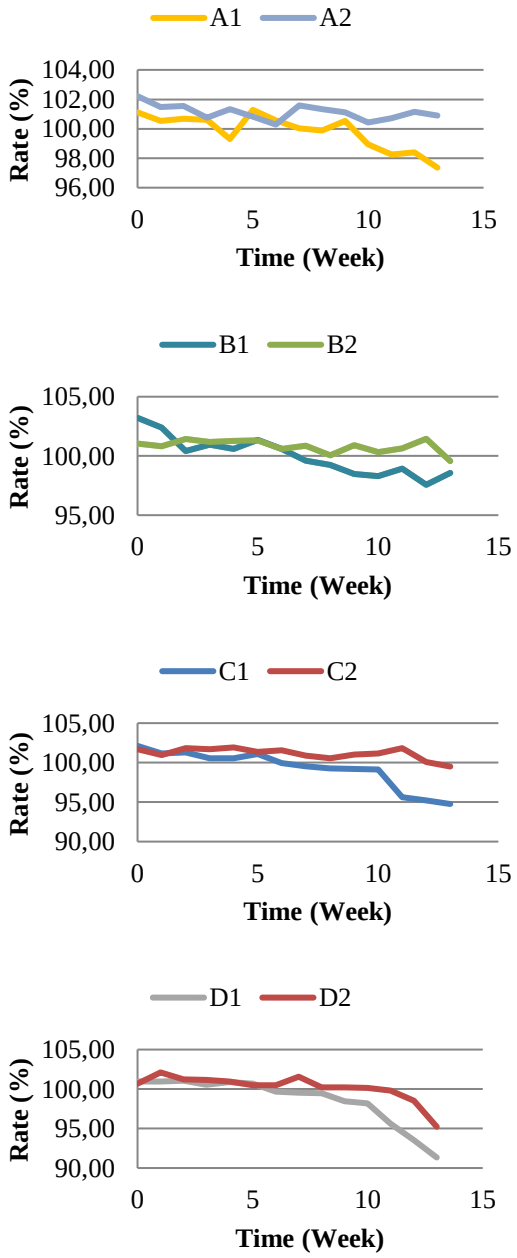


Figure 1. Graph of Clotrimazole Levels in Various Storage Conditions

In the four graphs shown in Figure 1, it is known that the antifungal cream samples with packages that have been opened and closed have a greater decrease in clotrimazole levels than the control samples whose packages are still sealed.

Table 2. T-Test on Various Storage Conditions

Sample	t_{table}	t_{count}
A	2.776	7.361
B		3.478
C		13.177
D		13.795

In Table 2 with a percent error of 5% or α of 0.05 it was found that the decrease in clotrimazole levels in all samples with the condition of the packaging being opened and closed at various storage temperature conditions was significantly different when compared to each control because t count obtained is greater than the t table. This indicates that the variable condition of the packaging that has been opened and closed and temperature affect the decrease in clotrimazole levels in the antifungal cream.

Determination of the order of the reaction using the graphical method, namely by applying the straight-line function y=ax + b to the equation of the order that has been derived. The order of the reaction can be determined by looking at the R^2 value that is closest to 1. The degradation reaction of clotrimazole in antifungal cream takes place with a first-order reaction due to the R^2 value. The first order reaction indicates that the concentration of clotrimazole affects the rate of the degradation reaction. Figure 2 is a first-order graph by plotting ln Ct (level at a certain time) with t (time). In the graph, The highest R^2 value is 0.872.

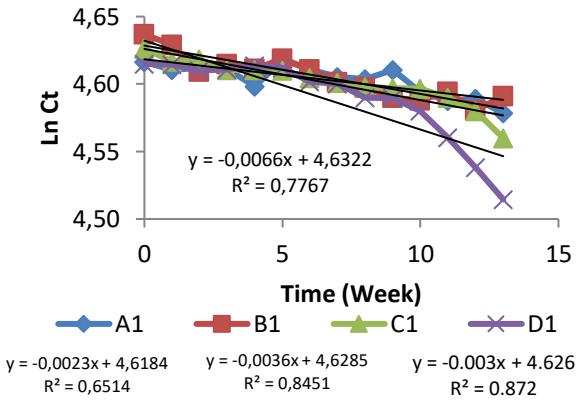


Figure 2. First Order Graph

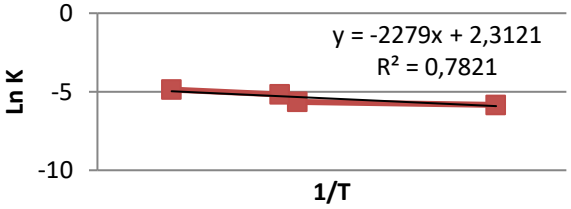


Figure 3. Graph of ln K with 1/T

After obtaining the order of the reaction, the value of k and the half-life of each sample can be determined. The value of k and the half-life can be calculated using the following formula.

Volume 01, Number 02, December 2021, Page 57 - 62
e-ISSN: 2776-4508; p-ISSN: 2776-1711
Publisher : Universitas Pakuan

$$k = \frac{\ln Co - \ln Ct}{t} \quad t_{1/2} = \frac{0.693}{k}$$

Information:
k = reaction constant (week-1)
C_0 = Initial concentration (%)
C_t = Concentration at a certain time (%)
t = Time (week)
$t_{1/2}$ = half-life (weeks)

Table 3. Value of Reaction Rate Constants and Half-Life

Sample	Reaction rate constant (week^{-1})	Half-life (weeks)
A1	0.0029	238
A2	0.0010	697
B1	0.0035	196
B2	0.0011	615
C1	0.0058	120
C2	0.0017	414
D1	0.0077	90
D2	0.0043	161

Based on the results in Table 3, the sample with the largest reaction rate constant and the shortest half-life was the sample at 40ºC with a k value of 0,0077 week^{-1} and a half-life of 90 weeks. The greater the value of the reaction constant indicates that the reaction rate that occurs in the sample is getting faster, so within 90 weeks the sample will reach half the initial level.

By using the Arrhenius equation, the value of k obtained can then be used to determine the value of the activation energy (Ea) at temperature T with the following formula:

$$k = Ae^{\frac{-Ea}{RT}}$$

$$\ln k = -\frac{Ea}{R}\frac{1}{T} + \ln A$$

Information:
k = reaction constant (week^{-1})
A = Pre exponential (L/ mol s)
Ea = activation energy (J/mol)
R = Constant (8.314 J/mol K)
T = Temperature (K)

To determine the value of the activation energy, it is necessary to make a curve of the relationship between 1/T and the reaction rate constant Ln (*k*). On this curve, the slope value will be obtained which then when multiplied by the constant R, the activation energy value will be obtained. Figure 3 is a curve for determining the activation energy.

In calculating the activation energy value at an uncontrolled room temperature, a temperature of 300 K is used as T, this is based on the average room temperature measurement just before the clotrimazole level measurement takes place.

Table 4. Activation Energy Value

Sample	1	2
Activation Energy (kJ/mol)	18.9	26.3

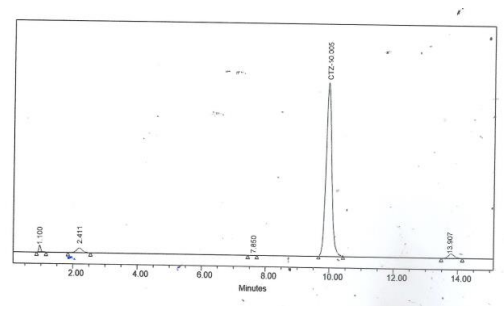
Based on the results in Table 4, the smaller the activation energy value obtained indicates that the faster a reaction in the sample can start so that the degradation reaction that occurs in the sample occurs faster than the control which causes a greater decrease in clotrimazole levels than the control.

3.2. Identification of Degraded Compounds with HPLC

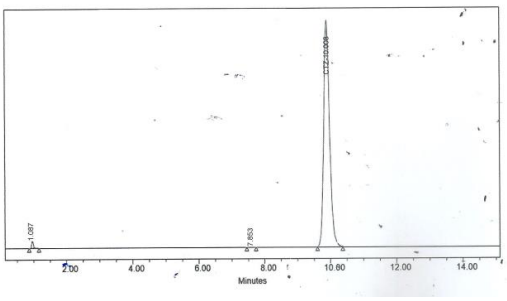
Identification of compounds resulting from clotrimazole degradation in antifungal cream preparations followed the method contained in the journal **[5].** From the retention time data for each sample, the relative retention time value can be calculated. The relative retention time is the ratio of the retention time of an analyte peak to the retention time of the main or comparison peak obtained in a chromatogram. Table 4 is the relative retention time of each compound resulting from clotrimazole degradation reported in the journal.

Table 5. Relative Retention Time of Compounds in Reference **[5]**

Compound	Relative Retention Time
Clotrimazole	1.000
Imidazole	0.241
(o-chlorophenyl) diphenyl methanol	1.338



(a)



(b)

Figure 4. Chromatogram of Identification of Degraded

Volume 01, Number 02, December 2021, Page 57 - 62
e-ISSN: 2776-4508; p-ISSN: 2776-1711
Publisher : Universitas Pakuan

Compounds (a) First Month and Second (b) Third Month Figure 4a is a chromatogram in the first and second month of the study. Three peaks of analyte were found on the chromatogram, but the three peaks were not peaks of imidazole and (o-chlorophenyl) diphenyl methanol but only peaks of clotrimazole and peaks of other compounds which may be additives in antifungal creams or impurities because these two compounds are compared with peaks. The main reason is that clotrimazole does not give the value of the relative retention time of imidazole is 0.241 and (o-chlorophenyl) diphenyl methanol is 1.338. Table 6 is the relative retention time of each compound obtained during the analysis of degradation compounds with HPLC in the first and second months.

Table 6. Relative Retention Time of Compounds in the First and Second Months

Compound	Relative Retention Time
Clotrimazole	1.000
Compound 1	0.109
Compound 2	0.785

Figure 4b is a chromatogram of samples C1, D1 and D2 in the third month. In the third month, five analyte peaks were found, namely in samples C1, D1 and D2. After comparing the relative retention time obtained in the journal and the relative retention time in this study, in samples C1, D1 and D2 in the third month there were imidazole compounds and (o-chlorophenyl) diphenyl methanol. Table 7 is the relative retention time of each compound obtained during the analysis of degradation compounds with HPLC for samples C1, D1 and D2 in the third month.

Table 7. Relative Retention Time of Compounds in the Third Month

Compound	Sample Relative Retention Time		
	C1	D1	D2
Clotrimazole	1,000	1,000	1,000
Compound 1	0,110	0,110	0,110
Compound 2	0,241	0,241	0,241
Compound 3	0,785	0,785	0,785
Compound 4	1,390	1,390	1,390

3.3. Identification of Degraded Compounds with LC MS/MS

The results of the identification of compounds resulting from degradation continued using LC MS/MS which were carried out in the last week of the study showed that there were compounds derived from clotrimazole degradation, namely imidazole and (O-chlorophenyl) diphenyl methanol in samples B1, C1, D1 and D2. The two compounds can be formed because of the possibility of a hydrolysis reaction in the sample which causes the amine bond to break in the clotrimazole compound. Meanwhile, in samples A1, A2, B2 and C2, no degradation products were found. The compounds found in the four samples were only sorbitol and clotrimazole. Table 8 is the result of identification of compounds using LC-MS/MS on samples in the last week.

Table 8. Results of Identification with LC-MS/MS

Sample	Compound	Molecular Formula
A1	Sorbitol	$C_6H_{14}O_6$
	Clotrimazole	$C_{22}H_{17}ClN_2$
A2	Sorbitol	$C_6H_{14}O_6$
	Clotrimazole	$C_{22}H_{17}ClN_2$
B1	Sorbitol	$C_6H_{14}O_6$
	Imidazole	$C_3N_2H_4$
	(O-chlorophenyl) diphenyl methanol	$C_{19}H_{15}ClO$
	Clotrimazole	$C_{22}H_{17}ClN_2$
B2	Sorbitol	$C_6H_{14}O_6$
	Clotrimazole	$C_{22}H_{17}ClN_2$
C1	Sorbitol	$C_6H_{14}O_6$
	Imidazole	$C_3N_2H_4$
	(O-chlorophenyl) diphenyl methanol	$C_{19}H_{15}ClO$
	Clotrimazole	$C_{22}H_{17}ClN_2$
C2	Sorbitol	$C_6H_{14}O_6$
	Clotrimazole	$C_{22}H_{17}ClN_2$
D1	Sorbitol	$C_6H_{14}O_6$
	Imidazole	$C_3N_2H_4$
	(O-chlorophenyl) diphenyl methanol	$C_{19}H_{15}ClO$
	Clotrimazole	$C_{22}H_{17}ClN_2$
D2	Sorbitol	$C_6H_{14}O_6$
	Imidazole	$C_3N_2H_4$
	(O-chlorophenyl) diphenyl methanol	$C_{19}H_{15}ClO$
	Clotrimazole	$C_{22}H_{17}ClN_2$

Sorbitol is used as an additive in cream preparations for the purpose of being a moisturizer. Imidazole exposure in the short term can cause skin burns, eye irritation, and respiratory tract irritation. While in the long term can cause reproductive system disorders. However, these compounds may not be dangerous because the imidazole threshold in the body is quite large, namely 220 mg/kg so that the presence of imidazole can be said to be dangerous for consumers if the levels are above 220 mg **[9].** The impact of exposure to (O-chlorophenyl) diphenyl methanol can cause skin irritation but until now no threshold data have been found for this compound **[10].**

Volume 01, Number 02, December 2021, Page 57 - 62
e-ISSN: 2776-4508; p-ISSN: 2776-1711
Publisher : Universitas Pakuan

4. CONCLUSION

Clotrimazole cream stored in various temperatures, both at low temperature, room temperature and high temperature can still be used for 3 months. There was a decrease in clotrimazole levels but still met the specifications required by the Indonesian Pharmacopoeia. The results of the t test stated that the decrease in clotrimazole levels in the condition of the packaging that had been opened and closed was significantly different from the comparison or control sample. The decrease in clotrimazole levels followed a first-order reaction with the fastest half-life of 90 weeks in samples placed at 40^{o}C ± 2^{o}C. The activation energy in the open sample was smaller, namely 18,8894 KJ/mol compared to the control. The results of the identification of the degraded compounds using HPLC and LC MS/MS found that the degraded compounds were imidazole and (o-chlorophenyl) diphenyl methanol in open packaged samples placed at room temperature and high temperature. Imidazole has a fairly high threshold value of 220 mg/kg, so the antifungal cream is still safe to use three months after the packaging is opened according to the in-use shelf life.

REFERENCES

[1] Soetojo, Shinta Dewi Rahmadhani, Linda Astari. 2016. Profile of New Patients with Candida Infection in Skin and Nails. Surbaya: Department/Functional Medical Staff of Dermatology and Venereology Faculty of Medicine Universitas Airlangga/ Regional General Hospital Dr. Soetomo Surabaya. pp. 34-41.

[2] Crowley PD, Gallagher HC. 2014. Clotrimazole as a pharmaceutical: past, present and future. J Appl Microbiol. 2014, 117(3): 611-617.

[3] Hoogerheide JG, Wyka BE. 1982. Clotrimazole. In: Florey K (ed.), Analytical Profiles of Drug Substances, Vol. 11. Academic Press, Salt Lake City. Thing. 225-255.

[4] POM RI. 2017. Criteria and Procedure for Drug Registration. Jakarta: Food and Drug Supervisory Agency of the Republic of Indonesia.

[5] Tawakkol, Shereen, et al. 2019. Monitoring of Clotrimazole Degradation Pathway in Presence of its Co-formulated Drug. Journal of chromatographic science. 57. Pg. 518-527.

[6] Lowe, RA 2001. Storage, Stability and In Use Shelf Life Guidelines for Non Sterile Medicines. London: National Health Service.

[7] Indonesian Ministry of Health. 2014.Indonesian Pharmacopoeia Edition V. Jakarta: Directorate General of Pharmaceutical and Medical Devices.

[8] Zhang Z., et al. 2015.Quadrupole time-of-flight mass spectrometry as a powerful tool for demystifying traditional Chinese medicine. TrAC Trends in Analytical Chemistry Vol. 109(2):910-922.

[9] Imidazole. SDS No. 814223. Merck:Germany.2016.https://www.merckmillipore.com/ID/id/product/msds/MDA_CHEM814223#:~:text=Imidazole%20MSDS%20%2D%20814223%20%2D%20Merck. (Accessed March 2021)

[10] (2-Chloro phenyl) diphenyl methanol.SDS No. C377905. Toronto Research Chemical: 2 Brisbane Road, Toronto, Canada. 2021.https://www.trc-canada.com/prodimg/MSDS/C377905MSDS.pdf. (Accessed August 2021)