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Beyond Use Date (BUD) Determination of Ambroxol Hydrochloride Syrup by High-Performance Liquid Chromatography – UV/VIS Detector

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ABSTRACT

Ambroxol HCl is a mucolytic agent often used to treat respiratory disorders associated with excess mucus secretion. This study aims to determine the beyond-use date (BUD) of ambroxol HCl syrup on the market based on analysis of the decrease in drug content using High-Performance Liquid Chromatography (HPLC) – UV/Vis detector. The HPLC conditions were reversed-phase with a C18 column, mobile phase containing acetonitrile - phosphate buffer 0.05 M pH 4.5 (60:40) at a flow rate of 1.0 ml/min using UV detection at 248 nm. To determine BUD, five syrup preparations (brands) obtained from the Jakarta area were analyzed in triplicate. The retention time for ambroxol HCl was 4.62 minutes. In the validation, ambroxol HCl showed good linearity with r = 0.99985 in the 6 to 36 µg/ml. LOD and LOQ for ambroxol HCl were 0.74 µg/ml and 2.25 µg/ml, respectively. It is also fulfilled the accuracy and precision parameters with a % recovery from 99.04% to 100.94% and CV<2%. This method meets the requirements according to the ICH Q2(R1) guidelines and can be used for the assay of ambroxol HCl syrup. The ambroxol HCl level on all samples was still higher than 90% (until the 36th day). Normality test data result indicated that data must be divided into two groups that are sample A and B, and sample C, D, and E. In conclusion, the extrapolation result showed that the BUD ambroxol HCl syrup was 83 days for sample A and B, and 49 days for sample C, D, and E.

Keywords: *ambroxol hydrochloride; Beyond Use Date (BUD); HPLC; UV/Vis Detector*

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INTRODUCTION

According to the Indonesian Minister of Health Regulation, drugs are substances or combinations of materials, including biological products, that are used to influence or investigate physiological systems or pathological conditions to establish a diagnosis, prevention, treatment, recovery, health improvement, and contraception for humans (Indonesian Health Law, 2009). The quality of a drug preparation is closely related to the stability of the drug (Herawati & Christina, 2012). Stability is defined as the capacity of a product to maintain its original properties and characteristics from the production, storage period, and usage. Drug stability consists of chemical, physical, microbiological, therapeutic, and toxicological stability (USP, 2017). Chemical, physical, or microbial changes can affect a drug product's quality, efficacy, and safety. Therefore, drug stability must be assured to maintain the efficacy and safety of the product (Velagaleti, 2010).

A drug product has a specific storage period to ensure that the product is still in a stable condition (Herawati & Christina, 2012). The period is known as the expiration date listed on the product packaging. The drug expiration date is the time limit for the drug usage after it is produced by a pharmaceutical factory before the primary packaging is opened for consumption or formulated (Ohler et al., 2019). Generally, people assume the expiration time of drugs before and after the primary packaging opens is the same, which is not accurate (Cokro et al., 2021). After the drug's primary packaging is opened, the time limit for using the medicine is no longer determined by the expiration date but by the beyond-used date (BUD). Beyond-used date (BUD) is the time limit for a sterile or non-sterile drug preparation starting after the drug is formulated or after the primary packaging has been opened or damaged. The BUD determination is conducted to discover the time limit for using drugs before the risk of physical and chemical degradation, contamination, and microbial proliferation in drugs emerge, which can harm the patients (United States Pharmacopeia, 2019).

BUD aspects are essential to observe in multiple doses of syrup preparations. One syrups that are often used in Indonesia is ambroxol hydrochloride syrup. Ambroxol hydrochloride, [trans-4-(2-Amino-3,5-dibromobenzylamino)-cyclohexanol hydrochloride], is a mucolytic agent that has been used since 1978 for the treatment of acute and chronic respiratory infections (Kantar et al., 2020). Ambroxol hydrochloride is unstable to oxidation and alkaline environment, affecting the decomposition process and reducing its safety (Jelić et al., 2021). Ambroxol syrup is widely circulated in Indonesia and is often used by the public, especially for pediatric patients. Its high frequency of use makes the determination of BUD in syrup preparations essential. Moreover, the active substance will generally be more susceptible to chemical degradation when it is in a liquid dosage than in a solid dosage form. (Gupta et al., 2018; Oncel et al., 2018).

In this study, the BUD of ambroxol HCl syrup was determined based on the amount of active substance in the preparation during storage time after the primary packaging was opened. The assay was carried out using High-Performance Liquid Chromatography (HPLC) with a UV-VIS detector.

METHODS

Optimization of Wavelength Analysis

The UV spectrum of ambroxol was obtained by measuring the standard solution of ambroxol HCl 10 µg/ml with UV-VIS spectrophotometry in the 200-400 nm range. Maximum absorption wavelength was used for HPLC analysis.

Optimization of Chromatography Condition

A standard solution of ambroxol HCl 30 µg/ml was injected into the HPLC system using the isocratic mode at the selected wavelength from the optimization results. The HPLC mobile phase system was acetonitrile: phosphate buffer pH 4.5 with the composition as the following: 40:60; 50:50; and 60:40 v/v with the flow rate was 1.0 ml/min. The composition referred to the report from Gadhvi et al. in 2013. The optimum conditions were determined based on chromatogram profile parameters such as retention time (tR), the number of theoretical plates (N), HETP, tailing factor, resolution, and peak area.

The process was continued to flow rate optimization. A standard solution of ambroxol HCl 30 µg/ml was injected into the HPLC system using selected wavelength and mobile phase composition. At a flow rate of 0.8 ml/min; 1.0 ml/min; 1.2 ml/min. The optimum conditions were determined based on chromatogram profile parameters such as retention time (tR), the number of theoretical plates (N), HETP, tailing factor, resolution, and peak area (Bose et al., 2014).

System Suitability Test

A standard solution of ambroxol HCl 30 µg/ml was injected into the HPLC system based on the optimization of the analytical conditions. In the system suitability test, the injection was carried out six times. Several parameters, include a coefficient of variance (retention time and peak area), resolution, tailing factor, HETP, and N. The test results meet the requirements of the HETP value being close to zero, the number of theoretical plates (N)>2,000, the associated factor (Tf) 2, and the coefficient of variation (CV) 2% (Harmita, 2015; ICH Q2(R1), 2022).

Validation of Method Analysis

Selectivity test

The selectivity test was carried out by comparing the chromatogram profile of the ambroxol standard solution (30 µg/ml) and blanks. The sampel (20 µl) was injected into the HPLC system using the selected method. The analysis was carried out on blanks, standard ambroxol HCl (30 µg/ml), simulation matrix (consisting of syrupus simplex 65%, methylparaben 0.2%, and aquadest), and samples of ambroxol HCl syrup with a 30 µg/ml concentration. The results were eligible if there was no interference around the retention time of the analyte (Harmita, 2015; ICH Q2(R1), 2022).

Linearity test

A linearity test was performed on six concentrations of standard ambroxol HCl, namely 6, 12, 18, 24, 30, and 36 µg/ml. As much as 20 µl standard solutions were injected into the HPLC system with selected method analysis. Based on the result, a calibration curve is made based. The linear regression line equation and the value were calculated. The linearity test meets the acceptance requirements if the correlation coefficient value (R) is $\geq$ 0.9990 and $V_{x0} \leq$ 2% (Harmita, 2015; ICH Q2(R1), 2022).

Accuracy and Precision test

This study chose the simulation (spiked-placebo recovery) as the accuracy test method. The test was carried out by making a simulation formula of ambroxol HCl syrup with three concentration levels, namely 80% (24 µg/ml), 100% (30 µg/ml), and 120% (36 µg/ml). The test was carried out three times (the 100% level was carried out six times). 20 µl sample solution was injected into the HPLC system. The accuracy test results are eligible if the recovery value is 98-102%. The precision test result fulfills the requirement if CV< 2% (Harmita, 2015; ICH Q2(R1), 2022).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limits of detection and quantitation were obtained based on statistical calculations through linear regression equations of the ambroxol HCl standard calibration curve. The LOD and LOQ were calculated by the following formula (ICH Q2 (R1), 2005).

$$LOD = \frac{3.3\left(S_{\frac{y}{x}}\right)}{S} \quad LOQ = \frac{10\left(S_{\frac{y}{x}}\right)}{S}$$

$S_{y/x}$ is the standard deviation of the residual, and S is the slope of the calibration curve between the response area to the analyte concentration in the linear regression equation.

Robustness test

Robustness tests were conducted on the variations of mobile phase and flow rate. Strength tests were carried out on variations in the analytical method, namely on variations in the composition of the mobile phase and variations in flow rate. For each variation of the analytical method, the test was repeated three times (triplicates). As much as 20 µl ambroxol HCl standard solution 30 µg/ml was injected into the HPLC system. The acceptance criteria for robustness test is coefficient of variation ≤ 2% (Harmita, 2015; ICH Q2(R1), 2022).

Ruggedness test

The ruggedness test was conducted using different instruments. The test objective is to evaluate the reproducibility of the method. In the test, a 20 µl standard solution of 30 µg/ml ambroxol HCl was injected into the HPLC system three times (triplicates). The result was evaluated. The test results meet the requirements of the variation coefficient value of ≤ 2% (Al-Hakkani, 2019; Harmita, 2015).

Assay

The assay was performed on five ambroxol HCl syrup brands commercially available on the market. In each brand, the assays were carried out using three samples. The assay procedure is a modification from the paper reported by Gadhvi et al., 2013. Before assay, the specific gravity of each dosage brand was determined. The ambroxol HCl syrup sample was weighed equivalent to 3 mg of ambroxol HCl and put into a 10 ml volumetric flask. Then, the mobile phase was added to half the volumetric flask and sonicated for 5 minutes. Next, the solution was added with the mobile phase to the limit mark to obtain 300 µg/ml sample concentration. The sample solution was diluted tenfold by mobile phase to obtain a concentration sample of 30 µg/ml. After that, the sample solution was filtered using a 0.45 µm nylon syringe filter. The sample solution was injected into the HPLC system, and sample content was calculated.

Table 1. Sampling schedule and analysis of ambroxol HCl syrup product

Week	Day						
0	0	1	2	3	4	5	6
1	7	8	9	10	11	12	13
2	14	15	16	17	18	19	20
3	21	22	23	24	25	26	27
4	28	29	30	31	32	33	34
5	35	36	37	38	39	40	41

Determination of Beyond Use Date (BUD)

The determination of the beyond-use date (BUD) of the ambroxol HCl syrup was conducted by quantitative analysis of the ambroxol HCl syrup content since the primary packaging was opened (day 0) for 5 weeks of storage. According to the ICH Q1F guidelines, the sample was stored in zone IVB at 30°C ± 2°C and in a dry place. The sample was opened occasionally according to the dose regimen three times a day and stored in a medicine box so that storage conditions comply with storage guidelines. The test was performed by analyzing the ambroxol content from the sample. The test interval is shown in Table 1.

Beyond use date was determined based on changes in ambroxol HCl content in the syrup sampel. According to the USP, the general content requirement of an ambroxol syrup is not less than 90.0% and not more than 110.0% of the amount stated on the label. The BUD of the sample was determined when the sample concentration no longer met the general level requirement, which is less than 90.0% of the amount stated on the label.

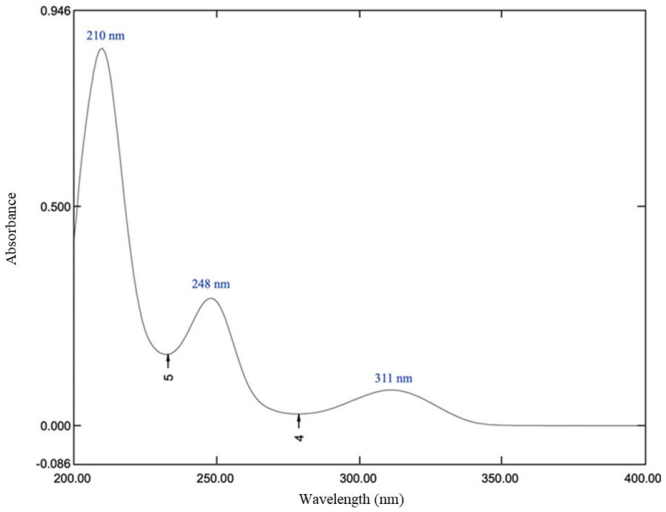

Figure 1. UV Spectrum of ambroxol HCl (10 µg/mL) in methanol

Data analysis

The chromatograms were processed using the Shimadzu LC solution program. The data analysis was processed using the Microsoft® Excel for Mac application version 16.61.1, and statistical testing was processed using the IBM SPSS application version 28.0.1.1.

RESULTS AND DISCUSSION

The results of the measurement of the UV spectrum of ambroxol HCl can be seen in Figure1. From this spectrum, it was determined that the optimum wavelength for the ambroxol HCl analysis is 248 nm.

Method Analysis Optimization

In the optimization method analysis, a mobile phase combination was used to achieve the optimum polarity for the analyte. Acetonitrile and methanol are commonly used organic solvents in the analysis of HPLC. Acetonitrile was chosen because it has a lower viscosity than methanol. Lower viscosity in the mobile phase can result in a sharper chromatogram peak (Harmita, 2015). Phosphate buffer was used as one of the compositions of the mobile phase. Buffer utilization aims to maintain the pH at a target value to produce a stable retention time. Other pH values, such as 3.5 and 5.5, have been tried; however, they gave poor peak characteristics compared to 4.5.

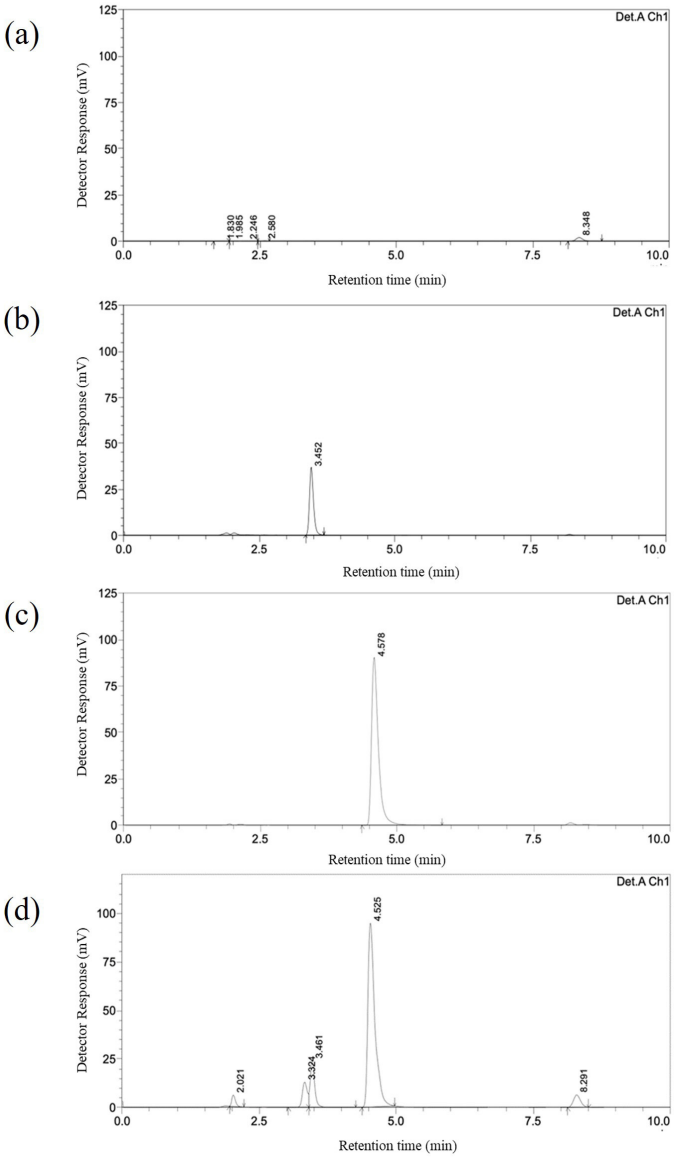

Figure 2. Chromatogram of the selectivity test. (a) blank, (b) matrix simulation, (c) ambroxol HCl standard (30 µg/ml), and (d) sample (30 µg/ml). Analytical conditions: Waters® Spherisorb ODS2 C18 (250 × 4.6 mm, 5 µm); Mobile phase acetonitrile – phosphate buffer 0.05 M pH 4.5 (60:40 v/v); flow rate 1.0 mL/menit; UV detection at 248 nm; injection volume 20 µL; analysis time 10 min.

Table 2. Accuration and precision test results

Ambroxol HCl Standard added (µg/ml)	Area (mV/s)	Ambroxol HCl measured (µg/ml)	% Recovery	Mean	CV (%)
24 (80 %)	636456	23.8861	99.5257	99.18	0.72
	642488	24.1110	100.4625		
	645552	24.2252	100.9383		
30 (100 %)	793807	29.7511	99.1704	100.31	0.22
	790996	29.6463	98.8211		
	795540	29.8157	99.3857		
	793668	29.7459	99.1531		
	793632	29.7446	99.1486		
	795894	29.8289	99.4297		
36 (120 %)	955368	35.7730	99.3694	99.41	0.38
	959587	35.9302	99.8062		
	952224	35.6558	99.0439		

Furthermore, the pH condition had been optimized according to the report by Gadhvi et al. in 2013. The evaluated criteria were retention time, tailing factor, and theoretical plate (N). Acetonitrile:phosphate buffer (60:40) provides optimum chromatographic profile.

The optimum flow rate was 1.0 ml/min since it generated a retention time of less than 10 minutes, The tailing factor was slightly larger than in 1.2 ml/min; however, it gave a lower pressure column.

System Suitability Test

The objective of this test is to ensure that the selected method is suitable for the analysis (Bose et al., 2014). It is essential because the validated analytical methods do not guarantee their applicability to different instruments or environments. The method should be evaluated by whether it gives a precision value on retention time or peak area of the analyte. The chosen analytical method was acetonitrile–phosphate buffer 0.05 M pH 4.5 (60:40 v/v), flow rate 1.0 ml/min, at a wavelength of 248 nm. The result showed that consecutively, the standard deviation (from retention time data and area) was 0.52, and 1.18, which adhere to the requirement of less than 2%.

Analysis Method Validation

Selectivity

The selectivity test objective is to assess the ability of the analytical method to separate and differentiate between the analyte and the other components that may be present in the sample, such as matrix, impurities, or degradation products. This parameter can be assessed by comparing the analyte, matrix, and solvent chromatogram. (ICH Q2 (R1), 2005; Ministry of Health RI, 2020).

According to the chromatogram described in Figure 2, there is no interference peak in the range of ambroxol HCl retention time (4.58 – 4.67 min). Moreover, the resolution value between the ambroxol HCl and other compounds is more than 1.5. It shows that the analytical method meets the criteria of the selectivity test.

Linearity Test, Limit of Detection (LOD), and Limit of Quantification (LOQ)

The linearity test objective assesses the relation between detector response and analyte concentration. (ICH Q2 (R1), 2005; Ministry of Health RI, 2020). The result of the linearity test was as follows: the linear regression equation of y = 26829x - 4386.1 with the correlation coefficient value (r) of 0.9998 and the coefficient of variance regression function (V_{x0}) of 1.07%. The results comply with the requirement since the criteria for the linearity are $r \geq 0.999$ and $V_{x0} \leq 2$ %. It showed that the calibration curve is linear in the range concentration of 6 – 36 µg/ml.

Determining the limit of detection (LOD) and limit of quantification (LOQ) was based on calculations using linear regression equations obtained from the calibration curve. The detection limit (LOD) and the method's quantification were 0.74 µg/ml and 2.25 µg/ml, respectively.

Accuracy and precision test

The accuracy and precision of the method were determined by spike-placebo recovery. The ambroxol HCl standard was added to the matrix. The test was conducted using three different concentration levels as follows: 80% (24 g/ml); 100% (30 g/ml); and 120% (36 g/ml). The result is shown in Table 2.

Table 3. Robustness test result

Variance Factor		Area (mV/s)	CV (%)
Flow rate	0.8 ml/min	1237089	
		1256464	0.89
		1237473	
	1.2 ml/min	823219	
		827047	0.26
		826829	
Acetonitrile	Acetonitrile – phosphate buffer 0.05 M pH 4.5 (50:50)	785050	
		789083	
		782960	0.40
	Acetonitrile – phosphate buffer 0.05 M pH 4.5 (40:60)	771766	
		797417	1.88
		797365	

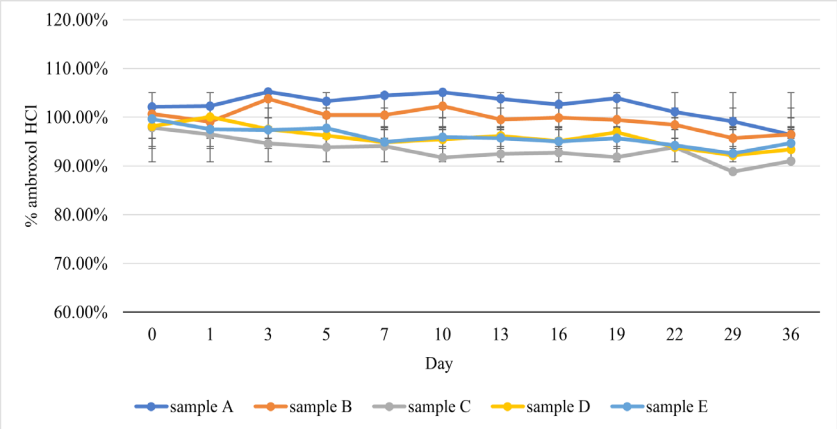

Figure 3. The curve of % ambroxol HCl content (average data from each sample, n=3) on each analysis time.

As described in Table 2, the accuracy method meets the criteria since the recovery percentage ranges from 99.04 – 100.2 % (the acceptance value is 98-102 %). The precision also complied with the requirements with the coefficient of variation between 0.22 – 0.72 % (the requirement is less than 2 %). The result showed that the method complies with the criteria.

Robustness and ruggedness

Robustness is performed to evaluate the reliability of method analysis concerning deliberate variations in method parameters (ICH Q2 (R1), 2005). In this test, the mobile phase composition and flow rate were varied. The test was repeated three times in each variation, and the coefficient of variation (% CV) was evaluated. The robustness result is shown in Table 3.

As shown in Table 3, all variance condition has CV less than 2 % (the acceptance criteria), indicating that the method unaffected by slight variation. Thus, the method complies with the robustness test criteria.

The ruggedness test is performed to evaluate the analytical method's reproducibility using different laboratories, instruments, analysts, or time (Harmita,

Table 4. Ruggedness test result

Test	Area (mV/s)
1	751214
2	753989
3	746372
SD	3854.96
CV (%)	0.51

2015). the selected analytical method was tested using other instruments (the instrument was the same type and model as the previous one). Table 4 shows the ruggedness test result. The result shows that the CV value is 0.51 % less than 2 %; thus, the method complies with the ruggedness test requirement.

Determination of Beyond Use Date of ambroxol HCl syrup

The beyond beyond-use date (BUD) of ambroxol HCl syrup was determined based on the ambroxol HCl concentration change of the sample. It was conducted by analyzing the ambroxol HCl content from the sample for five weeks. It started from day 0 (sample opening time) to day 36. The test was performed on 5 brands (sample A, B, C, D, and E) with 3 bottles from each brand. The sample was stored in a medicine box at room temperature. The sample bottle was opened according to the dosage regiment for pediatrics, three times a day for seven consecutive days from day 0 to reflect consumer usage. The result of the ambroxol HCl assay is shown in Figure 3.

In general, Figure 3 shows the decrease of ambroxol HCl levels in all samples. The ambroxol HCl level on all samples was still higher than 90%. According to the USP, the general requirement for the syrup preparation is to contain 90.0 – 110.0% active ingredients of the amount stated on the label. If the ambroxol HCl syrup sample no longer meets these requirements, the syrup has reached beyond the use date (BUD). It means that the BUD of all samples is longer than 36 days. Therefore, the BUD of the ambroxol HCl syrup samples was obtained by extrapolation of the linear regression equation from the curve of % ambroxol level vs. time (ICH Q1E, 2003).

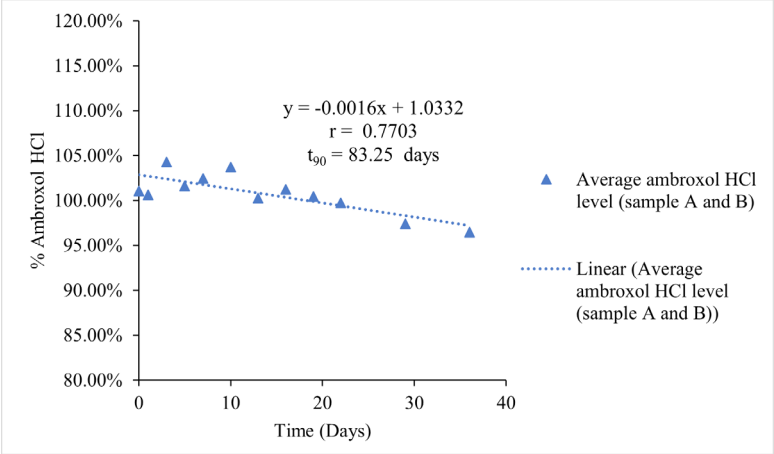

Figure 4. The curve of the % ambroxol HCl level (average data obtained from samples A and B) versus analysis time. The linear regression line obtained from the curve was used for BUD determination.

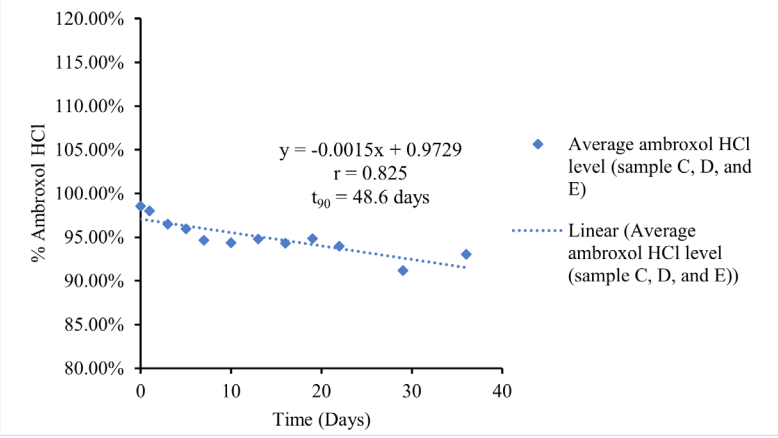

Figure 5. The curve of the % ambroxol HCl level (average data obtained from sample C, D, and E) versus analysis time. The linear regression line obtained from the curve was used for BUD determination.

The objective was to obtain BUD of ambroxol HCl syrup in general (applicable for most ambroxol preparation in Indonesia). Hence, the obtained data were evaluated to determine whether they could be combined or analyzed separately. Before the BUD determination extrapolation, a normality test was performed on the data from the entire sample. The normality test evaluates whether the data distribution is typically distributed normally or not. In this study, the normality test was Kolmogorov-Smirnov since the obtained data were more than 50. The result shows that all samples have $p > 0.05$, which means that the test data distribution is normal. Afterward, the homogeneity data was tested to assess the variance differences in each sample. This test is generally used as a prerequisite for ANOVA testing. The result showed that the significant value $p > 0.05$, which means that the obtained data was homogeneous.

After that, the sample data was tested by ANOVA one way. The result showed no significant difference between data samples A and B ($p > 0.05$). However, it significantly differs from samples C, D, and E ($p < 0.05$). Due to the significant difference between the data groups, the BUD was calculated using two data groups: the first group, data from samples A dan B, and the second from samples C, D, and E. The average from each data group was calculated and used for the next step.

The curve between the average data vs. time was generated. The linear regression equation for each curve was obtained. The linear regression equation for the first group (A and B) and the second group (C, D, and E) was $y = -0.0016x + 1.0332$ and $y = -0.0015x + 0.9729$ consecutively. The curve from both groups is shown in Figure 4 dan Figure 5.

To obtain the BUD value, the ordinate of the linear regression equation was substituted with 90 %, and the x value obtained is the BUD. Figure 3 dan Figure 4 shows that the BUD for samples A and B was 83 days. Meanwhile, samples C, D, and E are 49 days.

Based on the results of the substitution of 90% in the linear regression equation, the BUD for samples A and B was obtained for 83 days. Meanwhile, samples C, D, and E are 49 days. The different stability of each brand can cause difference because of varying formulations from each manufacturer. The different types of excipients, the excipient quality, and the production process could affect the stability of active ingredients. Hence, that differences in BUD values from several data groups are things that are likely to occur. In general, it is concluded that the ambroxol HCl syrup BUD is 49 days, in which the smaller values was chosen.

In addition to observing the ambroxol HCl level changes, organoleptic testing was also carried out to evaluate the syrup instability (EMEA, 2001). The result shows no organoleptic change (in color or aroma) in each ambroxol HCl syrup from the start to the end of the experiment. This indicates that there is no indication of syrup instability in terms of organoleptic.

CONCLUSION

The optimum HPLC-UV condition for analyzing ambroxol HCl syrup is Waters® Spherisorb ODS2 C18 column (250 x 4.6 mm, 5 m), wavelength UV detection 248 nm; mobile phase acetonitrile – phosphate buffer 0.05 M pH 4.5 (60:40); flow rate of 1.0 ml/min. The method has met the requirements of the validation parameters based on the ICH guidelines, namely selectivity, linearity, LOD, LOQ, accuracy, precision, robustness, and ruggedness. Beyond use date (BUD) of ambroxol HCl syrup used in this was 49 days. An increasing number of the sample should give more description about BUD of ambroxol HCl syrup. The information can be used as a reference to enlighten the public about beyond-use date.

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