ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Comparison of Ampicillin Determination in Pharmaceutical Preparations with Iodometry Titration, HPLC, And Diffusion Methods.

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ABSTRACT

This study aims to determine Ampicillin concentration in generic and non-generic pharmaceutical preparations of a capsule, dry syrup, and injection with iodometry titration, HPLC, and diffusion methods. Three pharmaceutical samples containing Ampicillin trihydrate 500 mg in the form of the capsule, ampicillin trihydrate 125 mg/5ml dry syrup and sodium ampicillin 500 mg/vial for injection were determined. Two types of samples were generic and Nongeneric samples were used. Ampicillin determination followed Norman method. For iodometry titration, samples were added with sodium hydroxide 1.0N, let stand 15 minutes, then added HCl 1.2N and Iodine 0.01N. Let stand and dilute with sodium thiosulfate 0.01N with amylum indicator. For HPLC, Waters HPLC was used with C18 column, 10 ml injection volume, 225 nm wavelength. Eluent consisted of water: acetonitrile: potassium phosphate mono base: acetic acid (909:80:10:1). For microbiological method diffusion way by using tryptic soy broth and tryptic soy agar media was applied. The test bacteria used were Micrococcus luteus ATCC 9341. The procedure was performed aseptically for each standard comparative solution with 5 dose levels and test solution with one dose level for each sample type. Incubation period at 37^0C for 18-24 hours. Measured diameter resistor formed in the form of a clear zone around the hole by using a caliper. Against the results of the ampicillin assay and analysis in generic and nongeneric preparations for the three methods used to apply statistical analysis using random design, Anova analysis. Iodometric determination on generic, nongeneric capsule, generic and nongeneric syrup and generic and nongeneric injection obtained % ampicillin level 98.62%, 99.36%, 97.88%, 99.41%, 98.92% and 97.77%. Applicability of HPLC ampicillin results obtained 95.21%, 92.88%, 98.43%, 98.72%, 95.58% and 98.17%, whereas for the results of re-acquisition of ampicillin with a diffuse method, 97.75%, 95.67%, 95.00%, 93.98%, 93.03%, and 95.67%. Using data analysis for the accuracy of each method on each type of preparation with a significant level of $\alpha = 0.05$ and $\alpha = 0.01$, it could be concluded for the three methods used for generic and nongeneric samples did not give a significant difference of result with 95% and 99% confidence. The results of this study found that for the examination of ampicillin levels in the form of capsules, syrups and injections for both generic and nongeneric, the most appropriate method that could be used was the method of iodometry with the least variation of coefficient value for each type of dosage examined. It was also found that generic and non-generic dosage forms statistically did not have a significant effect on the value resulting from the examination of ampicillin levels.

Keywords: Iodometry, HPLC, Microbial diffusion method, ampicillin, pharmaceutical preparations.

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ISSN: 0975-8585

INTRODUCTION

Ampicillin is included in the List of Essential Medicines of the World Health Organization, the most effective and safe medicines needed in the health system. Discovered in 1958 and commercially used in 1961 to the present [1-3]. In America as well as in Indonesia, this drug exists as a generic and nongeneric drug. Resistance to ampicillin use has also been reported [4-6]. Ampicillin has a wide spectrum and is used for therapy for both positive and negative gram bacteria. These bacterial bacteria cause many common diseases in the community, such as influenza, ear infections, respiratory infections, urinary tract infections, ear infections, sinusitis, gonorrhea, typhoid, paratyphoid, and septicemia so that ampicillin use is widely used [6]. Ampicillin only serves to overcome bacterial infections, this drug cannot fight infections caused by viruses. Consumption of improper or excessive antibiotics can adversely affect the body and cause resistance [7-8].

Ampicillin is produced by many factories in Indonesia in the forms of caplets, capsules, dry syrups and injectable forms with different trade names [9-10]. With this brand of drugs being circulated in the community, a good method is needed to examine the medicines whether the ampicillin content is consistent with the ones listed on the label. Previous researchers have examined ampicillin by HPLC method [11-13], Diffusion method [14-15], iodometry titration [16-17] , and Colormetry [18-20] individually. Unlike these determinations, this article reports the comparison of three methods, iodometry titration, HPLC, and Diffusion methods and statistically verified by Anova.

METHODS

The methods used were based on Norman et.al method [21].

Iodometry titration: This iodometric titration refers to Pharmacopoeia Indonesia III edition [22]. The standard comparative solution was prepared with 50 mg ampicillin (Pharos) into 50 ml water. For the test solution, for capsule weighed 20 capsules one by one to find the average weight of the capsule. Weighed 20 capsules each for generic and non-genetic capsules. The twenty capsule content was homogenized by crushing and was equivalent to 50 mg ampicillin, dissolved in 100 ml of water. For both generic and nongeneric syrup test solutions, the dried syrup was suspended with water to obtain a concentration of 125 mg/5 ml, shaken. The specific gravity of syrup was determined by using picnometer. Then weigh carefully the stock of suspense equivalent to 50 mg ampicillin. For the injection injection test solution, 10 ml of water was added to the vial quantitatively, then 1.0 ml planted into a 100 ml measuring flask and added water to the boundary marker. The titration process was carried out by adding 2.0 ml of 1.0N sodium hydroxide solution to 5 ml of the solution to be titrated, leaving it for 15 minutes. Then 2.0 ml HCl 1.2N and 10.0 ml of Iodine solution was added. Volumetric flask closed and left again for 15 minutes. After that, it was titrated with the 0.01N Na2S2O3 solution. Towards the end point was added 1 drop of the starch solution. Titration continued until the blue color was disappeared. Titrations were performed for each of the standard solutions and generic and nongeneric test solutions.

Assessment of ampicillin content by HPLC: According to Pharmacopoeia Indonesia IV [23] determination of antibiotic content can be by using HPLC. In this HPLC study Waters, 490E at 225 nm wavelength was used. The column was C18 15 cm long contains octadecylsilane. The eluent was the mixture of water: acetonitrile: potassium phosphate mono base 1M: 1 M acetic acid (909: 80: 10: 1). Injection volume was 10 µL. Standardized eluent preparation using Millipore 0.5 µm sieve and degassing with Bransonic ultrasonic at 18,000 Hz frequency for 15 min. The standard solution was made by weighing 50 mg Ampicillin (Pharos) dissolved and sonicated. This raw solution was made fresh. Test solutions for generic and nongeneric capsules were prepared by weighing capsule preparations equivalent to 50 mg ampicillin and dissolved in 100 ml water and sonicated. For preparations originating from syrup and injection were treated similarly to the preparation of the sample in an iodometric titration. These solutions were used as soon as they were made.

Determination of ampicillin content by a microbiological method of diffusion method: Made ampicillin standard solution with 0.1 mg/ml level. This solution was diluted to 5 levels of 1.25, 2.5, 5.10, and 20 µg/ml. Generic and nongeneric test solutions for capsules, dried syrup and injections were prepared with 5µg/ml. The media used were tryptic soy broth and tryptic soy agar from Oxoid brand. Tryptic soy broth composition per liter was 17 g Pancreatic digest of casein, 3 g Papaic digest of soybean meal, 5 g NaCl, 2.5 g Dibasic potassium phosphate and 2.5 g Dextrose. Weighed 3 g Tryptic soy broth incorporated container and added 100 ml water,

shaken until dissolved. Tryptic soy to be made by weighing 15 g Tryptic soy broth inserted into the container and added 5 g agar. Then dissolved in 500 ml cold water and heated until the solution becomes clear. Media was sterilized with autoclave 1210C for 15 minutes. Test bacteria used Micrococcus luteus ATCC 9341. The procedure worked as follows: The test bacteria were suspended in 5 ml Tryptic soy broth in a test tube and incubated at 37 °C for 18-24 hours. Included 0.2 ml of bacterial suspense into a sterile petri dish, then 20 ml of Tryptic soy agar was added. Shake and wait until cool and hardened. The petri dish was divided into 5 equal zones, then made a hole on the agar with a perforator on each part. The incorporated solution would be tested as much as 50 µL in each hole. Then incubated for 18-24 hours at 370C. The inhibitory diameter was measured using a caliper.

RESULTS AND DISCUSSION

Ampicillin levels in pharmaceutical preparations by iodometric titration:

From the titration results of Na2S2O3 0.1N volume obtained, then calculated levels of ampicillin contained in capsules, dry syrup and injection as follows:

The equivalence in µg per ml of Na2S2O3 0.1N used by the standard solution was calculated by the formula:

$$\frac{(5CP)}{B-I}$$

Where: C = standard reference in mg/ml of standard solution
P = Potential in ug/mg reference standard
B = Volume (ml) of 0.01N thiosulfate solution used for blank determination
I = Volume (ml) of 0.01N thiosulfate solution used for the test samples.

The ampicillin content tested in the preparation is calculated by the formula:

$$[T/5000]\,[F/[B-I]$$

Where: T = ampicillin levels in mg/capsule as shown on the label.
F = levels of ampicillin mg/ml in test solution

The result of ampicillin determination by titration of iodometry is shown in Table 1.

Table 1: The results of ampicillin levels in a pharmaceutical preparation by iodometric titration

	PHARMACEUTICAL PREPARATIONS					
AMPICILLIN	CAPSULE	CAPSULE	DRY SYRUP	DRY SYRUP	INJECTION	INJECTION
CONCENTRATION	GENERIC	NONGENERIC	GENERIC	NONGENERIC	GENERIC	NONGENERIC
(MG)	(PER CAPSULE)	(PER CAPSULE)	(PER 5 ML)	(PER 5 ML	(PER VIAL)	(PER VIAL)
1	499.6	493.41	124.96	124.96	478.2	478.2
2	495.88	493.42	121.5	121.43	492.65	492.5
3	485.23	510.32	118.92	124.98	499.65	478.2
4	492.41	493.42	124.91	124.98	499.65	495.8
5	492.46	493.42	121.45	124.98	502.9	499.55
MEAN	493.12	496.80	122.35	124.27	494.61	488.85
CONCENTRATION (%)	98.62	99.36	97.88	99.41	98.92	97.77

% concentration of ampicillin was obtained by dividing the mean by the value written on the packaging label. In this study, all the pharmaceutical preparations examined met the requirements listed in Indonesian Pharmacopeia IV, which contained ampicillin with not less than 90.0% and not more than 120.0% C16H19N3O4S of the amount indicated on the label. Putra[24] reported the results of the determination of ampicillin levels in tablets with generic names = 99.69% ≤μ≤104, 91.03% ≤μ≤97, 95.70% ≤μ≤102.44% and for non-generic preparations 93.38% ≤μ≤99.44%, 91.41% ≤μ≤97.95%, 97.19% ≤μ≤101.67%, and 95.60% ≤μ≤ 98.58%. Blazheyevskiy *et.al* [25] claimed that their unified procedure was able to quantify penicillin by the iodometric method using potassium hydrogen peroxomonosulfate (KHSO5) with RSD ≤ 2. 35% (δ = -0.1 ... + 1.0%).

Ampicillin levels in pharmaceutical preparations with HPLC: By calculating the peak area obtained in the test solution analysis against the peak area of the reference standard solution, ampicillin levels were obtained as shown in Table 2.

Table 2: Ampicillin levels in pharmaceutical preparations by HPLC method

	PHARMACEUTICAL PREPARATIONS					
AMPICILLIN	CAPSULE	CAPSULE	DRY SYRUP	DRY SYRUP	INJECTION	INJECTION
CONCENTRATION	GENERIC	NONGENERIC	GENERIC	NONGENERIC	GENERIC	NONGENERIC
(MG)	(PER CAPSULE)	(PER CAPSULE)	(PER 5 ML)	(PER 5 ML	(PER VIAL)	(PER VIAL)
1	509.7	479.9	124.5	129.88	449.2	466.2
2	475	440.1	124.9	116.2	503.8	496.3
3	488.9	454	127.2	128.5	479.8	491.8
4	468	468.2	119.7	121.3	467.3	489.99
5	438.75	479.9	118.9	121.1	489.4	509.9
MEAN	476.07	464.42	123.04	123.40	477.9	490.83
CONCENTRATION (%)	95.21	92.88	98.43	98.72	95.58	98.17

Table 2 showed that the six types of pharmaceutical preparations studied fulfill the requirements stipulated in Indonesia Pharmacopoeia IV containing ampicillin in the range of not less than 90.0% and not more than 120.0% of the ampicillin number indicated on the label. Percent concentrations of ampicillin were obtained by dividing the average value of the weight indicated on the packaging label.

HPLC and eluent conditions used in this study where the mixture of water: acetonitrile: 1M mono-based potassium phosphate: 1M acetic acid (909: 80: 10: 1), and 225 nm wavelengths which were different from those used by other researchers. Injac *et.al* [26] used detection at a 220 nm wavelength. The optimal operating conditions were: injection volume 10 µL, flow rate 3 ml min-1, mobile phase a distilled water with 0.1% trifluoroacetic acid (TFA), mobile phase B 100% acetonitrile with 0.1% TFA, elution gradient 0-1 min 100 % A, 1-4 min 100-89% A, 4-5min 89% A, 5-10 min 89-88% A, 10-11 min 88-100% A, 11-13 min 100% A. Douša and Hosmanová [27] stated a rapid analytical procedure for routine identification and quantification of amoxicillin in premixes by high performance liquid chromatography was developed and tested. The ground premix samples were extracted for 10 min using 100 ml extraction mixture of water: methanol (800: 200, v/v). The extract was analyzed by reversed-phase on Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, id, 5 µm particle size) with water-methanol-phosphoric acid-triethylamine (842: 150: 4: 4) containing 10 mM hexane-1-sulfonic acid sodium salt (pH 3.5) as a mobile phase. UV detection was carried out at 230 nm. While de Abreu and Ortiz[28] using combined reversed-phase liquid chromatography and UV detection (λ = 229 nm). Amoxicillin and cefadroxil (internal standard) were extracted from the plasma by addition of cold methanol. The separation was achieved using Lichrosorb®10µm, C18 reversed phase column at room temperature. The mobile phase consisted of a 95% phosphate buffer (0.01 mol / L), pH = 4.8 and 5% acetonitrile mixture.

ISSN: 0975-8585

However, although different columns, tools, and conditions were applied by different researchers, as long as they used standard comparative solutions, the results obtained would be acceptable.

Ampicillin levels in the preparation by diffusion method: In fact, Rolinson and Russell in 1972 introduced a method of diffusion for the determination of antibiotics [29]. Balouiri *et.al* [30] of 2016 had reviewed methods for in vitro evaluating antimicrobial activity. It was stated that several bioassays such as disk-diffusion, well diffusion and broth or dilution were well known and commonly used, but others, such as flow cytofluorometric and bioluminescent were used for evaluation and standardization, even if they could provide rapid results of the antimicrobial agent's effects and a better understanding of their impact on the viability and cell damage inflicted to the tested microorganism. The principle of the diffusion examination was mentioned by Bonev *et.al* [31].

Our study used both diffusion and broth or dilution and was a modification of the Kirby-Bauer Disk Diffusion Susceptibility Test [32]. To calculate ampicillin levels in the examined preparation, a table of the inhibitory diameter of the standard ampicillin solution as shown in Table 3 was first prepared.

Table 3: Inhibitory diameter of ampicillin standard solution

Inhibitory diameter (mm)	Doses (µg/50 µL)													
	0.063		-1.204	0.13		-0.903	0.25		-0.602	0.5		-0.301	0.1		0
1		23.2			24.3			25.2			26.4			28.1	
2		23.2			24.5			25.2			26.7			27.9	
3		23			25.1			26.4			26.5			28.4	
4		23.2			25			26.3			26.4			28.5	
5		23.1			24.6			23.1			25.4			24.6	
Mean		23.1			24.7			25.2			26.3			27.5	

From the data in Table 3, we made an equation between log dose and inhibitory diameter with the equation of the straight line as shown in Figure 1.

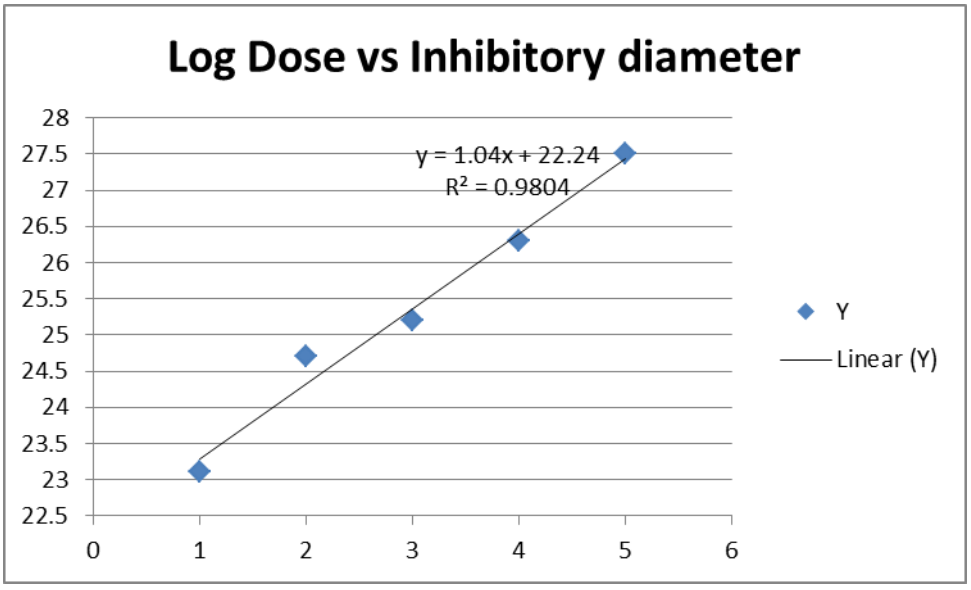

Figure 1: Ampicillin standard curve

ISSN: 0975-8585

It was found the equation of Y = 1.04X + 22.24, where Y = Inhibitory diameter of the test solution and X = log dose of the test solution (µg / 50 ml).

From the result of the inhibitory diameter of the test solution then plotted to the above equation so that the value as shown in Table 4.

Table 4: Ampicillin potential in pharmaceutical preparations by diffusion method

	PHARMACEUTICAL PREPARATIONS					
AMPICILLIN	CAPSULE	CAPSULE	DRY SYRUP	DRY SYRUP	INJECTION	INJECTION
POTENTIAL	GENERIC	NONGENERIC	GENERIC	NONGENERIC	GENERIC	NONGENERIC
(%)	(PER CAPSULE)	(PER CAPSULE)	(PER 5 ML)	(PER 5 ML	(PER VIAL)	(PER VIAL)
1	99.9	97.12	97.11	97.1	91.9	99.92
2	97.13	91.9	94.46	94.47	94.7	94.7
3	97.12	94.72	94.45	94.48	89.52	97.1
4	99.92	94.72	91.9	91.92	91.9	91.9
5	94.7	99.91	97.1	91.92	97.11	94.71
MEAN	97.75	95.67	95.00	93.98	93.03	95.67

All inspected dosage forms met the requirements of the Indonesian Pharmacopoeia, i.e ampicillin equivalent of not less than 90.0% and not more than 120.0% of the amount stated on the label. The diffusion method had also been tried by other researchers [31] against various types of antibiotics such as ampicillin, chloramphenicol, erythromycin, streptomycin, tetracyclin, and trimethoprim by giving a good inhibitory diameter and potent antibiotics.

Statistical analysis

The accuracy of each method against each type of preparation: ANOVA was used to analyze the results and methods of this study. Anova (Analysis of Variance) was a statistical technique that assesses the potential differences in a scale-level dependent variable by a nominal-level variable having 2 or more categories [33]. To calculate the accuracy of each method for each type of preparation, calculated average price *(x)*, median (*M)*, range (*R*), mean deviation (*d*), mean deviation (*drelative*), standard deviation (s), and coefficient of variation (*v*) on the result of ampicillin content obtained by using tables 1,2 and 4. Examples of accuracy value calculation and accuracy of each method for each type of preparation can be seen in Table 5.

Table 5: Example of the accuracy value of each method for each type of preparation

	METHOD			
TYPE OF	STATISTIC.	IODOMETRIC	DIFFUSION	HPLC
PREPARATIONS	SYMBOL	TITRATION	METHOD	
	X	493.13	97.75	476.07
	M	492.41	97.11	472.1
	R	14.27	5.2	72.5
GANERIC	*D*	3.71	1.73	19.22
CAPSUL	*DRELATIVE*	0.75%	1.77%	4.04%
	S	5.29	2.2	26.82
	N	1.07%	2.25%	5.64%
	X	488.85	95.67	490.83
	M	492.40%	94.71	491.70%

	R	21.45	8	43.4
NON GENERIC	*D*	8.57%	2.27	11.66%
INJECTION	*DRELATIVE*	1.75%	2.375	2.38%
	S	10.1	3	16.07
	N	2.07%	3.13%	3.285

Etc.

Seen from Table 5, the comparison of the coefficient value of variation was an indication of the accuracy of a method. It was found from all experiments the coefficient variation of iodometric titration has the lowest value.

The influence of generic and nongeneric forms on the value of the assay in the preparation: Perfect Random Design was applied to this statistical analysis. It was found that the results of ampicillin cocentration on capsules, dry syrup and both generic and nongeneric injection did not give significant differences in results with 95% and 99% confidence. The following table shows the example of Anova's calculation of ampicillin levels for generic and nongeneric capsules for HPLC methods.

Table 6: ANOVA checklist of ampicillin levels in generic and nongeneric capsules by HPLC

VARIATION				F	F TABLE	
SOURCE	**DK**	**JK**	**KT**	**CALCULATE**	**0.05**	**0.01**
MEAN	1	2207026.42				
BETWEEN	1	290.52	290.52	0.57	5.32	11.26
TREATMENTS						
MISTAKE	8	4079.73	509.97			
SUM	10	2211396.68				

From table 6 with the real level of $\alpha = 0.05$ and $\alpha = 0.01$ then obtained Ftable0.05 (1.8) = 5.32 and Ftable0.01 (1.8) = 11.26 so it could be concluded that the results of ampicillin levels on two kinds of capsules either generic or non generic by using HPLC did not give any significant difference in results with 95% and 99% confidence. The results of this study was in line with a study by Norman *et.al* [21].

CONCLUSIONS

The results of this study found that for the examination of ampicillin concentrations in the form of capsules, syrups and injections for both generic and non-generic, the most appropriate method that could be used was the method of iodometry titration with the least variation of coefficient value for each type of dosage examined. It was also found that generic and non-generic dosage forms statistically did not have a significant effect on the value resulting from the examination of ampicillin levels.

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