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Method Validation of Chloramphenicol Analysis in the Shrimp Based on Diazotization Reaction

Abdul Wafi[1*], Ganden Supriyanto[2,3], Tjitjik S. Tjahjandarie[2]

[1]Department of Pharmacy, Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang, 65143, Indonesia

[2]Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya, 60115, Indonesia

[3]Biosensor Laboratory, Institute of Tropical Disease, Airlangga University, Surabaya, 60115, In-donesia

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*Corresponding author:wafi@farmasi.uin-malang.ac.id

Abstract

A simple, rapid and precise spectrophotometric method has been developed and validated for the determination of Chloramphenicol (CAP) in the shrimp based on diazotization reaction at room temperature. The CAP was reduced by zinc powder and the diazotization reaction was carried out in the presence of NaNO2, bismuth nitrate pentahydrate as catalyst. The 2-napthol used as coupling agent to form a red-violet solution and the absorbance of azo dye solution was measured by UV-Vis spectrophotometer at 554 nm. The method validation parameters including linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) have been investigated. The correlation coefficient (R2) was 0.996 for concentration range 0.70 – 4.65 µg/mL. The LOD and LOQ were 0.36 µg/mL and 1.19 µg/mL. Accuracy and precision of the method were performed by spiking of CAP in the shrimp sample at concentration 1.16; 2.33; 3.49 µg/mL. Analysis result showed that the accuracy and precision of the method were 92.77-97.37 % and 0.21-2.39 % respectively.

Keywords: Chloramphenicol, diazotization, method validation, shrimp, spectrophotometry

Validasi Metode Analisis Kloramfenikol pada Udang Berbasis Reaksi Diazotasi

Abstrak

Sebuah metode yang sederhana, cepat dan presisi telah dikembangkan untuk penentuan kloramfenikol (CAP) pada udang berdasarkan reaksi diazotasi pada suhu kamar secara spektrofotometri. CAP direduksi dengan menggunakan serbuk seng (Zn) dan reaksi diazotasi dilakukan dengan mereaksikan NaNO2, bismut (III) nitrat pentahidrat (Bi(NO3)3.5H2O) sebagai katalis, dan β-naftol sebagai agen pengkopling untuk membentuk senyawa azo yang berwarna merah-ungu dan absorbansi diukur dengan spektrofotometer UV-Vis pada panjang gelombang 554 nm. Parameter validasi yang ditentukan antara lain linearitas, akurasi, presisi, batas deteksi (LOD) dan batas kuantifikasi (LOQ). Koefisien korelasi (R2) yang diperoleh dalam penelitian ini sebesar 0,996 untuk rentang konsentrasi 0,70-4,65 µg/mL, LOD dan LOQ masing-masing sebesar 0,36 µg/mL dan 1,19 µg/mL. CAP yang ditambahkan (spiking) ke sampel udang Litopenaeus Vanamie dan Litopenaeus Monodon sebesar 1,16; 2,33; 3,49 µg/mL. Hasil analisis menunjukkan akurasi dan presisi masing-masing berkisar 92,77-97,37 % dan 0,21-2,39 %.

Kata Kunci: Kloramfenikol, reaksi diazotasi, spektrofotometri, udang, validasi metode

1. Introduction

Chloramphenicol (CAP) is a bacteriostatic anti-microbial compound originally derived from the bacterium Streptomyces venezuelae. It is now synthesized chemically and has an antibacterial effect by interfering with protein synthesis in micro-organisms. CAP has been commonly used in the treatment of bacterial disease in the aquaculture production such as shrimp and ornamental fish. The use of CAP antibiotic can promote growth and improve the production of aquatic products. However the excessive use of CAP will lead to exist of antibiotic residues in aquatic products. These residues may cause a risk for human consumption. It is well-known that CAP antibiotic may lead to bone marrow suppression, leukemia, and aplastic anemia in human beings. Hence, the residue of CAP in aqua culture product is strictly controlled.[1]

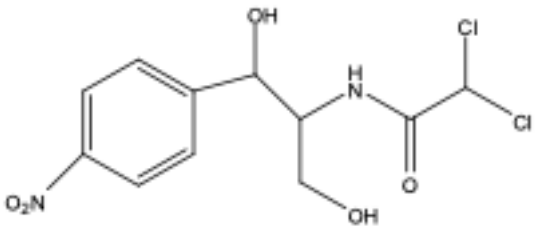

Figure 1. Chemical structure of CAP

Indonesia shrimp production had reached to 785,900 tons in 2015 and most of them were exported to several countries such as Japan, Eu-ropean Commission and the United States. Those countries have strictly implemented zero tolerance of CAP residue in aquatic products. Furthermore, Indonesia also has banned the use of CAP as mentioned in Permenkes No. 722/Menkes/Per/IX/88. However, the illegal use of CAP still exists due to its low price and con-sistent antibiotic effectiveness. Therefore, it is still needed a routine controlling and monitoring of CAP residue in aquaculture products.[2] The chemical structure of CAP is summarized in Figure 1.

Several analytical methods have been employed for analysis and determination of CAP in aquatic products, including Gas Chromatography–Mass Spectrometry (GC-MS),[3] Liquid Chromatog-raphy–Mass Spectrometry (LC-MS),[4,5] En-zyme-Linked Immunosorbent Assay (ELISA),[6,7] High Performance Liquid Chromatography (HPLC).[8] However, these methods involved special and high cost equipment. Hence, it is still necessary to develop different or simple approaches.

Spectrophotometric method is a very popular method or equipment owing to its easy-to-operate, specificity and low cost. In this work, an analytical protocol for determination of CAP in the shrimp based on diazotization reac-tion was established. The diazotization reaction occurred at room temperature in present of $Bi(NO_3)_3.5H_2O$ as catalyst. 2-napthol used as coupling agent to form azo dye solution and the absorbance of azo dye solution was measured by UV-Vis spectrophotometer. The validation parameters such as linearity, precision, accuracy, LOD and LOQ of this method have been stud-ied.

2. Method

2.1. Material

CAP reference standard was purchased from Sigma Aldrich, Singapore. Bismuth nitrate pen-tahydrate (Bi(NO3)3.5H2O) was purchased from Merck, Germany. Ethanol, sodium nitrite, ethyl acetate, concentrated hydrochloride acid, 2-napthol, zinc powder (Zn) were pure analytical grade. Litopenaeus vanamie and Litopenaeus monodon shrimp were obtained from local market in Surabaya, Indonesia.

2.2. Preparation of reagent

CAP 970µg/mL : CAP powder (0.097 g) was weighed quantitatively and dissolved with ethanol. The solution was transferred to a 100 mL volumetric flask and made up with same diluent to mark.

NaNO2 8x10-2 M : NaNO2 powder (0.5520 g) was weighed quantitatively and dissolved in distilled water. The solution was trans-ferred to a 100 mL volumetric flask and made up same diluent to mark.

2-napthol 4x10-3 M : 2-napthol powder (0.0576 g) was weighed quantitatively and dissolved in 50 mL ethanol. The solution was

transferred to a 100 mL volumetric flask and made up with distilled water to mark.

2.3. Reduction of CAP

A 2.50 mL of CAP solution 970µg/mL was added with 1 mL distilled water, 1 mL of con-centrated hydrochloride acid and 0.15 g zinc powder, subsequently allowed for 15 minutes for reduction process. The solution was filtered and transferred quantitatively to a 25 mL volu-metric flask and made up with distilled water to mark to obtain the reduced CAP 97 µg/mL.

2.4. Diazotization reaction and formation of azo dye

A 0.15 g of Bi(NO3)3.5H2O was added with 2,0 mL NaNO2 8x10-2 M. The solution was then added with 3 mL of reduced CAP solution and 3 mL 2-napthol 4x10-3 M, allowed at room tem-perature for 8-9 minutes to form azo dye solu-tion.

2.5. Spectrophotometric calibration curve

The standard solutions for calibration curve were prepared by transferring 1.5; 3.0; 5.0; 8.0 and 10.0 mL of reduced CAP 97 µg/mL in five different 25 mL volumetric flask and diluted with distilled water. The obtained standard solu-tions were 5.82; 11.63; 19.39; 31.02; 38.78µg/mL.

Furthermore, the diazotization reaction was car-ried out for the five standard solutions. Subse-quently, the obtained azo dye solution of each concentration was transferred to a 25 mL volu-metric flask and diluted with distilled water to obtain CAP 0.70; 1.40; 2.33; 3.72 and 4.65µg/mL. The absorbance of azo dye solu-tions were measured with UV-Vis spectropho-tometer Shimadzu-1800 at 554 nm.

2.6. Application in shrimp (standard addition)

After removing shells, Litopenaeus vanamie and Litopenaeus monodon shrimp were pulped. A 1.0 g homogenous shrimp was weighed and trans-ferred into three different glass beaker. Each glass beaker was added by 5, 10, 15 mL of re-duced CAP solution 97µg/mL. Subsequently, 5.0 ml of ethyl acetate, 1.00 mL of distilled wa-ter, 1.00 mL of concentrated hydrochloride acid and 0.15 of zinc powder were added. The solu-tion was filtered and transferred to a 50 mL volumetric flask and diluted with distilled water to obtain filtrate reduced CAP 9.7, 19.4 and 29.1µg/mL. Furthermore, 3.00 mL of each fil-trate was pipetted and the diazotization reaction was carried out. The azo dye solution was transferred to 25 mL volumetric flask and dilut-ed with distilled water to obtain spiked CAP concentration of 1.16; 2.33 and 3.49µg/mL, re-spectively. The absorbance of azo dye solution was measured with UV-Vis spectrophotometer Shimadzu-1800 at 554 nm at 554 nm.

3. Results

3.1. Formation of azo dye

The principle reaction of this method is diazoti-zation and following by coupling reaction to form red-violet azo dye solution as shown in Figure 2.

Figure 2. Photograph of Azo dye solution

3.2. Method validation

A. Linearity

The linearity was tested with the standard CAP concentration range of 0.70 – 4.65µg/mL as shown at Bable 1.

The calibration curve, linear regression equa-tions, and correlation coefficient (R) are shown in Fig. 3. The result showed good linearity with correlation coefficient (R2)

Table 1. Linearity result of CAP

CAP standard solution No.	Concentration (µg/mL)	Absorbance
1	0.70	0.220
2	1.40	0.291
3	2.33	0.342
4	3.72	0.454
5	4.65	0.523

0.996. The sensitivity of this method was 0.0749.

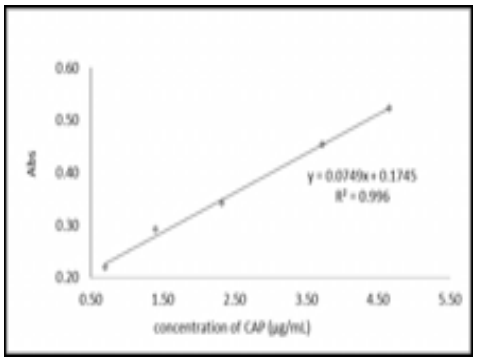

Figure 3. Calibration curve of CAP

B. Limit of detection and quantification

The limits of detection (LOD) and the limit of quantitation (LOQ) of the proposed method were 0.36µg/mL and 1.19µg/mL, respectively as shown at Table 2.

C. Accuracy and precision

The accuracy and precision of the proposed method were determined at three different con-centration of standard CAP including 1.16; 2.33; 3.49µg/mL. The results of accuracy and preci-sion were 92.77-97.37 % and 0.21-2.39 as shown at Table 3.

3.3. Application in shrimp

The standard CAP was spiked in the shrimp sample at concentrations 1.16; 2.33; 3.49µg/mL, respectively. The result showed that the recover-ies of CAP spiked in Litopenaeus vanamie and Litopenaeus monodon were 92.64-97.37 % and 93.83-104.25 % respectively. The relative standard deviations (RSD) of CAP spiked in Litopenaeus vanamie and Litopenaeus monodon were 0.39-1.96 % and 0.68-1.68 % respectively as shown at Table 4.

4. Discussion

In this study, the development of method for CAP analysis based on the diazotization

Table 2. LOD and LOQ of CAP

CAP (µg/mL)	$\hat{y}i^a$	yi^b	LOD (µg/mL)	LOQ (µg/mL)
0.70	0.227	0.220		
1.40	0.279	0.291		
2.33	0.349	0.342	0.36	1.19
3.72	0.453	0.454		
4.65	0.523	0.523		

$\hat{y}i^a$ = abs (theoretical); yi^b =abs (measurement)

Table 3. Accuracy and precision of CAP

CAP (µg/mL)	Detected (µg/mL) (n=3)	Recovery (%)	RSD (%)
1.16	1.08	92.77	0.49
2.33	2.24	96.12	2.39
3.49	3.40	97.37	0.21

Table 4. The recoveries and RSD of CAP spiked in shrimp sample

Shrimp type	Spiked level (µg/mL)	Detected (µg/mL) (n=3)	Recovery (%)	RSD (%)
Litopenaeus vanamie	1.16	1.08	92.77	1.96
	2.33	2.24	96.12	1.19
	3.49	3.40	97.37	0.39
Litopenaeus monodon	1.16	1.21	104.25	1.68
	2.33	2.18	93.83	1.22
	3.49	3.38	96.98	0.68

reaction has been performed by reducing of nitro group to amine group on CAP structure using zinc powder. Reduced CAP acts as primary aro-matic amine for establishing the diazonium salt on diazotization reaction. Generally, diazonium salt has poor thermal stability and must be han-dled around 0-5°C. But in this study, it kept sta-ble at room temperature because it was synthe-sized in presence of $Bi(NO_3)_3.5H2O$ as catalyst.[9]

Generally, the reduced CAP, $NaNO_2$, and $Bi(NO_3)_3.5H_2O$ were mixed homogeneously for 1 minute to form a diazonium salt. Futher, the 2-napthol as a coupling agent was added to the diazonium salt and mixed homogeneously for 8-9 minutes to form a red-violet solution. The absorbance was measured by UV-Vis spectro-photometer at 554 nm. The proposed reaction and optimization of each analytical parameter had been reported.[10]

In order to evaluate the performance of this method, the validation parameters such as linearity, precision, accuracy, LOD and LOQ have been evaluated. The result showed a good correlation coefficient (R_2 = 0.996) and sensitivity (0.0749). Alshirifi and Alhameedi reported a spectrophotometric method of CAP analysis base on condensation reaction. The result showed an identical correlation coefficient (R_2 = 0.9983) and lower sensitivity (0.0577). However, the LOD and LOQ were lower compared with the present work with nearly 0.068 and 0.207 µg/mL, respectively.[11] Al-Abachi, et al., also proposed the determination of CAP in eye drop by using diazotization reaction in low tem-perature. The result exhibited a lower sensitivity compared with present method with nearly 0.0288. However, it had a better (lower) LOD of 0.1334µg/mL.[12] From this point of view, the present method exhibited a higher sensitivity and LOD.

The accuracy of this study was 92.77-97.37 %. The result showed a slightly lower accuracy compared with previous method with nearly 98.5-104.4% and 98.6-100 %. In addition, the precision of this study was 0.21-2.39%. Alshirifi and Alhameedi; Al-Abachi, et al., have reported the precision of their methods were 0.23-0.67 % and 0.67-0.91 %.[11,12] The com-paration of some parameters for CAP analysis in the proposed method and

Table 5. The comparison of CAP analysis in the proposed method and other literatures method

Analytical parameters	Proposed Method	Literature Method 8	Literature Method 13	Literature Method 13	Literature Method 14
Type of method	Diazotization	HPLC	Diazotization	Diazotization	Diazotization
Reagent	2-napthol	-	Imino dibenzyl	3-amino phenol	N-methyl aniline
λ_{max} (nm)	554	225	590	470	504
Color of dye	red-violet	-	violet	orange	orange
Temperature (°C)	room temperature	25	0-5	0-5	0-5
Recovery (%)	92.77-97.37	81.1	-	-	-
LOD (µg/mL)	0.36	0.024	-	-	100

other previous method is shown at Table 5.

The standard additions method on the shrimp sam-ples (Litopenaeus vanamie and Litopenaeus monodon) was investigated to confirm the direct procedure. As shown in Table 4. The method ex-hibits a good accuracy (% recovery) and precision (%RSD) with nearly 92.77-104.25% and 0.39-1.96%. It indicated no interferences appeared during preparation of samples. On other word, the method is reliable enough on the determination of CAP in the shrimp samples.

5. Conclusions

A spectrophotometric method validation of CAP analysis based on simple diazotization reaction has been successfully performed. From the present study it can be concluded that the proposed meth-od was simple, rapid, precise and accurate.

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