Validation of TLC-contact bioautography and TLC-densitometry methods for simultaneous limit test of streptomycin sulfate and kanamycin sulfate residues in fresh fish meat

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ABSTRACT

A simple, sensitive, and accurate TLC method was combined and validated for a simultaneous limit test of streptomycin sulfate and kanamycin sulfate residues in fresh fish meat. The optimum mobile phase was 10% potassium dihydrogen phosphate. TLC-contact bioautography used Staphylococcus aureus ATCC 29737 as a test organism. For TLC-densitometry, analyte spots were detected using 1% w/v ninhydrin in ethanol and maximum absorbance at 400 nm. TLC-contact bioautography and TLC-densitometry methods were validated according to USP guidelines for the limit test with respect to selectivity and limit of detection (LOD) added with the accuracy of a recovery test of streptomycin sulfate and kanamycin sulfate in fresh fish samples was developed and validated. The result of LOD from the TLC-contact bioautography method for streptomycin sulfate and kanamycin sulfate were obtained 0.7028 µg and 0.8032 µg, respectively. Meanwhile, LOD of the TLC-densitometry method for streptomycin sulfate and kanamycin sulfate were obtained 0.0631 µg and 0.0685 µg, respectively. In this study, TLC-contact bioautography method showed better results than TLC-densitometry. TLC-contact bioautography appears a good choice for the simultaneous limit test of streptomycin sulfate and kanamycin sulfate residues in fresh fish samples.

INTRODUCTION

Antibiotics, such as streptomycin sulfate and kanamycin sulfate, have an important role in treating or preventing infections. As an aminoglycoside antibiotic, streptomycin sulfate and kanamycin sulfate bind to the ribosome 30S protein subunit to inhibit protein synthesis, and then an RNA reading error occurs which causes the binding of the wrong amino acids to the peptide to form a non-functional protein (Jiang et al., 2019). However, abuse of streptomycin and kanamycin can leave residues in animal tissues, causing allergic reactions in humans. As a consequence, an important impact of antibiotic residues is antibiotic resistance (Bacanli and Nursen, 2019).

Several methods have been carried out to detect streptomycin and kanamycin residues in various animal tissues, such as LC-MS (Tao et al., 2012), ELISA (Chen et al., 2008) and HPLC (Chen et al., 2009). However, these methods still have several weaknesses; for example, most of their applications take several hours for processing the derivatization of antibiotic compounds and impractical for simul-
taneous analysis (Jiang et al., 2019).

Therefore, it is important to develop simple, sensitive, accurate, and valid methods for the simultaneous limit test of streptomycin sulfate and kanamycin sulfate residues.

Thin Layer Chromatography (TLC) has several advantages, such as simple, short analysis time, and practical for simultaneous analysis. TLC can be combined with bioautography and densitometry. TLC-densitometry is a sensitive and specific analysis method (Reich and Maire-Widmer, 2013). On the other hand, TLC-bioautography is a sensitive and accurate method for screening the activity of antibiotics (Patil et al., 2013).

Various bioautography techniques, such as direct, immersion, and contact, have been widely used for antibiotics screening. Direct and immersion bioautographies are specifically applied for bacteria that can grow directly on TLC plates. Contact bioautography can be applied for bacteria that can grow directly or indirectly on the TLC plate (Marston, 2011).

The aim of this study was obtained a valid, sensitive, and accurate method for the simultaneous limit test of streptomycin sulfate and kanamycin sulfate residues in fresh fish meat samples.

MATERIALS AND METHODS

Chemicals
All chemicals were analytical chemical grade. Some chemical materials used were streptomycin sulfate (PT. Meiji), kanamycin sulfate (PT. Meiji), Staphylococcus aureus ATCC 29737, potassium dihydrogen phosphate (Merck), nutrient broth (Merck), nutrient agar (Merck), ninhydrin (Merck), trichloroacetic acid (Merck), sodium hydroxide (Merck), sodium chloride (Merck), ethanol (Merck), and distilled water (Otsuka).

Instrumentations
Glasswares (Pyrex), chamber (Camag), TLC silica gel 60 F$_{254}$ plates (Merck), Petri dishes (Pyrex), vortex (Thermo), incubator (Memmert), micropipette (Socorex), polypropylene centrifuge tube (Onemed), autoclave (Huxley HV-340 Speedy), densitometry (Shimadzu TLC Scanner), and spectrophotometry (Lovibond Spectro PC 22).

Preparation of culture media
Add 9 g of nutrient agar and 4 g of nutrient broth to 500 ml of distilled water. Heat to homogeneous, dispense aseptically and sterilize by autoclave at 121°C for 15 min.

Preparation of inoculum
Staphylococcus aureus ATCC 29737 colony from the stock culture was taken as much as one ose, then etched on a sloping surface and incubated at 32-35°C for 24 h. After growth, a bacterial suspension is made by adding 10 ml of sterile sodium chloride solution to the culture to tilt, and then the suspension is shaken with vortex until the entire colony is released from the agar. The optical density of bacterial suspensions was measured with a spectrophotometer at a wavelength of 580 nm, and then volume adjustment was made to obtain 25% transmittance.

Preparation of sample
Catfish and Nile tilapia samples were washed and their scales, heads, tails, fins, skin, and bones were set aside. Fish fillets were taken from head to tail and from top to back of the stomach on both sides to get the fish meat. Blend for 5 min until smooth (AOAC, 2019). Weighed accurately 10 g of fresh fish meat and put into a centrifuge tube. Catfish and Nile tilapia samples were added with 5 ml of 20% trichloroacetic acid, ultrasonication was performed for 15 min, and centrifuged at 9000 r/min for 5 min.

The supernatant was taken and put into beaker glass. The extraction process was repeated again by adding 5 ml of 20% trichloroacetic acid, ultrasonication was carried out for 15 min, and centrifuged at 9000 r/min for 5 min. The supernatant was taken and put together with the previous supernatant into a beaker glass. The pH was neutralized to 7 with 4 M sodium hydroxide, then put into a 20 ml volumetric flask and the volume was sufficient with distilled water addition.

Optimization of mobile phase concentration
Optimization of mobile phase concentration was carried out using 7.5%, 10%, and 12.5% potassium dihydrogen phosphate solution as mobile phase. The streptomycin sulfate and kanamycin sulfate standard solution of 300 μg/ml were spotted as much as 10 μl on the TLC plate and then developed in the mobile phase. The elution plate was dried.

Validation Method
Selectivity
The selectivity of the method was established by analyzing the sample solution containing streptomycin sulfate and kanamycin sulfate simultaneous on the silica gel 60 F$_{254}$ TLC plate. Elution was carried out with 10% potassium dihydrogen phosphate solution. The selectivity of streptomycin sulfate and kanamycin sulfate was assessed by resolution (Rs) value.
**LOD**

Streptomycin sulfate and kanamycin sulfate standard solutions with a concentration range, such as 6-100 μg/ml were spotted as much as 10 μl on the TLC plate, then eluted using a 10% potassium dihydrogen phosphate solution. When the eluent has reached the developer limit, the plate is lifted and dried. The Minimum Inhibitory Concentration (MIC) was the limit of detection of TLC-contact bioautography.

**Linearity**

Linearity was made by the dilution of the standard reference solutions of streptomycin sulfate and kanamycin sulfate to the required concentration. For this aim, a series of solutions concentration 100.4-301.2 μg/mL of streptomycin sulfate solutions and 80.3-301.2 μg/mL of kanamycin sulfate solutions were spotted as much as 10 μl on the TLC plate. The plates were developed using a 10% potassium dihydrogen phosphate solution. The calibration curve was plotted between peak area versus concentration for TLC-densitometry and inhibitory zone versus concentration for TLC-contact bioautography. The coefficient correlation, slope, and intercept of obtained calibration plots were reported.

**Accuracy**

The accuracy of the method was evaluated by measurement of recovery. Percent recovery was performed by the standard addition method. For this aim, known amounts of standard solution streptomycin sulfate and kanamycin sulfate were added to the sample. This analysis was performed three times. The percentage of recovery for both drug components was calculated.

**TLC-contact bioautography**

The sample solutions (10 μl) were spotted manually on the TLC plates, then developed with a selected eluent of 10% potassium dihydrogen phosphate solution. After the eluent front was reached, the TLC plate was dried aseptic to remove the eluent from the silica gel 60 F254 TLC plate. The TLC plate was placed on the inoculated agar surface with 5 μl of *Staphylococcus aureus* ATCC 29737 inoculum and storage in a refrigerator for 1 h to allow diffusion process. Furthermore, the TLC plate was removed and the petri dish was incubated at 32-35°C for 24 h. The growth-inhibitory zone diameter was measured.

**TLC-densitometry**

The sample solutions (10 μl) were spotted manually on the TLC plates. Ten percent of potassium dihydrogen phosphate was used as the mobile phase. After dried, the TLC plate was sprayed with 1% w/v ninhydrin solution in ethanol and heated at 150°C for 10 min. The spot obtained was scanned using a densitometer at a wavelength of 400 nm.

**RESULTS AND DISCUSSION**

Fish have high protein content. Streptomycin sulfate and kanamycin sulfate are binds strong to proteins because of the formation of insoluble anions (Tao et al., 2012). Extraction of streptomycin sulfate and kanamycin sulfate from fish samples requires a low pH to release of these antibiotics from proteins. Usually, this pH is achieved by using a strong acid solution, such as 20% trichloroacetic acid in a mixture of water (Tao et al., 2012; Díez et al., 2015; Li et al., 2016).

**Optimization of mobile phase concentration**

The results of the analysis using some of potassium dihydrogen phosphate solution as mobile phase with various concentrations appear in densitogram (Figure 1) and bioautogram (Figure 2) which show the separation between streptomycin sulfate and kanamycin sulfate in all the mobile phase concentrations used, namely 7.5%, 10%, and 12.5%. Based on the Rf and Rs values of the streptomycin sulfate and kanamycin sulfate standard analysis, meet the requirements is potassium dihydrogen phosphate solution with a concentration of 10%.

**Validation**

The proposed TLC-contact bioautography and TLC-densitometry for the simultaneous limit test of streptomycin sulfate and kanamycin sulfate residues in fresh fish meat samples was validated according to USP guidelines with respect to selectivity and LOD added with the accuracy of a recovery test of streptomycin sulfate and kanamycin sulfate in fresh fish samples was developed and validated.

**Selectivity**

Selectivity were carried out in catfish and Nile tilapia samples, each of which was added with streptomycin sulfate and kanamycin sulfate standards. Based on the value of Rs obtained from the standard solution of streptomycin sulfate and kanamycin sulfate in the sample, matrices of catfish and nile tilapia meet the selectivity parameters with a value of Rs ≥ 1.0, which means that the two analytes separate well is shown in Table 1. If the value of Rs = 1.0, then the two peaks separate is good because only 2% between peak 1 and peak 2 overlaps.

**LOD**

The results of LOD of the TLC-contact bioautography and TLC-densitometry methods are presented
Figure 1: The results of densitogram of streptomycin sulfate (1), kanamycin sulfate (2), and a mixture of both (3) with potassium dihydrogen phosphate solution as mobile phase with concentration (A) 7.5%, (B) 10%, and (C) 12.5%.

Figure 2: Bioautogram results of streptomycin sulfate, kanamycin sulfate, and a mixture of both (1) with potassium dihydrogen phosphate solution as mobile phase with concentration (A) 7.5%, (B) 10%, and (C) 12.5%.
Figure 3: The LOD results of streptomycin sulfate and kanamycin sulfate

![LOD results graph](image)

Figure 4: Streptomycin sulfate calibration curves use the TLC-contact bioautography (a) and TLC-densitometry (b)

![Calibration curves for streptomycin sulfate](image)

Figure 5: Kanamycin sulfate calibration curves use the TLC-contact bioautography (a) and TLC-densitometry (b)

![Calibration curves for kanamycin sulfate](image)

Table 1: The Rs results of streptomycin sulfate and kanamycin sulfate addition sample solution

| Analyte        | Sample     | TLC-Contact Bioautography | TLC-Densitometry |
|----------------|------------|---------------------------|------------------|
| STR* + KAN**   | Catfish    | 1.81                      | 3.38             |
|                | Nile Tilapia | 1.82                      | 3.50             |

*STR = Streptomycin sulfate
**KAN = Kanamycin sulfate
Table 2: Recovery results of streptomycin sulfate and kanamycin sulfate in catfish and tilapia samples

| Analyte          | Sample          | Concentration (µg/mL) | % Recovery |
|------------------|-----------------|-----------------------|------------|
|                  |                 | TLC-Contact Bioautography | TLC-Densitometry |
| Streptomycin Sulfate | Catfish         | 301.20                | 100.02     |
|                  |                 | 401.60                | 99.92      |
|                  |                 | 502.00                | 99.22      |
|                  | Nile Tilapia    | 301.20                | 99.50      |
|                  |                 | 401.60                | 99.13      |
|                  |                 | 502.00                | 98.90      |
| Kanamycin Sulfate | Catfish         | 150.60                | 99.02      |
|                  |                 | 200.80                | 99.60      |
|                  |                 | 251.00                | 100.65     |
|                  | Nile Tilapia    | 150.60                | 99.18      |
|                  |                 | 200.80                | 95.23      |
|                  |                 | 251.00                | 99.25      |

in Figure 3. Based on the LOD test, streptomycin sulfate and kanamycin sulfates were 0.0631 µg and 0.0685 µg, respectively. In TLC-contact bioautography, the Minimum Inhibitory Concentration (MIC) value was expressed as a LOD. The LOD results of streptomycin sulfate and kanamycin sulfates were 0.7028 µg and 0.8032 µg, respectively.

**Linearity**

Determination of linearity is determined by comparing the concentration with inhibitory zones for TLC-contact bioautography and concentration with the area for TLC-densitometry. From the concentration and response data obtained, then a calibration curve is made is shown in Figure 4 and Figure 5 to get the regression equation.

**Accuracy**

The accuracy of the method was evaluated by measurement of recovery (Table 2). When known amounts of streptomycin sulfate and kanamycin sulfate were added to fish samples. According to the AOAC, the requirement for the mean recovery at a concentration of > 0.01% is 85-110%.

The recovery value of streptomycin sulfate using the TLC-densitometry method did not meet the accuracy acceptance criteria. This is due to the more dominant structure of streptomycin sulfate by secondary amine groups so that streptomycin sulfate has a low absorption ability due to the presence of 5 secondary amine groups (NH) which causes impaired electron conjugation.

**CONCLUSION**

According to the results of the present study, validation of the TLC-contact bioautography and TLC-densitometry methods for simultaneous limit test met validation requirements according to USP guidelines. The TLC-contact bioautography method is more sensitive and accurate than the TLC-densitometry method for the simultaneous limit test of streptomycin sulfate and kanamycin sulfate in fresh fish samples.

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**Conflict of Interest**

The authors declare that they have no conflict of interest for this study.

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