Validated TLC-Contact Bioautography Method for Identification of Kanamycin Sulfate in Injection Preparation

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Abstract

***Background**: TLC-contact bioautography is one of an effective method for identification antibiotics, by which many antibiotics could be identification and determination simultaneously. **Objective**: To evaluate kanamycin sulfate in injection preparations based on its inhibitory activity against Escherichia coli ATCC 8739 as test organism. **Methods**: Sample and standard solutions were spotted onto TLC silica gel 60 F254 plate and developed in 10% potassium dihydrogen phosphate solutionas as mobile phase. The TLC-contact bioautography method was validated according to USP guidelines by considering specificity, LOD, LOQ, linearity, accuracy and precision parameters. **Results**: The TLC-contact bioautography method was found to be high sensitivity with LOD of 0.75 µg and LOQ 2.31 µg. Linearity range of 100-350 µg/mL with r = 0.9993 and linear regression equation was y = 0.0019x + 0.0338. The recovery obtained from addition of blank samples by three different concentrations of kanamycin sulfate standard was 101.40% ¬+ 2.02%. The precision of the method was good with coefficient of variation 0.080%. The TLC-contact bioautography method was supported by determination of kanamycin sulfate potency ratio in the injection preparation and kanamycin sulfate standard using 3-3 design. Random block design obtained the potential for kanamycin sulfate in injection preparations compared to kanamycin sulfate standard was 100.6%. **Conclusion**: The TLC-contact bioautography for kanamycin sulfate in injection preparations could be applied to the quality control analysis of the investigated drugs.*

***Keywords**: kanamycin sulfate, TLC-contact bioautography, Escherichia coli*

Abstrak

Pendahuluan: KLT-bioautografi kontak merupakan salah satu metode yang efektif untuk mengidentifikasi antibiotik secara simultan. **Tujuan**: Untuk mengevaluasi kanamisin sulfat dalam sediaan injeksi berdasarkan aktivitas antibiotik menghambat pertumbuhan bakteri *Escherichia coli* ATCC 8739 sebagai organisme uji. **Metode**: Larutan sampel dan larutan standar ditotolkan pada pelat KLT silika gel 60 F254 dan dielusi dalam larutan kalium dihidrogen fosfat 10% sebagai fase gerak. Validasi metode KLT-bioautografi kontak mengacu pada USP yang meliputi parameter spesifisitas, LOD, LOQ, linearitas, akurasi, dan presisi. **Hasil**: Metode KLT-bioautografi kontak memiliki sensitivitas tinggi dengan LOD 0,75 µg dan LOQ 2,31 µg. Rentang linearitas 100-350 µg/mL dengan nilai r = 0,9993 dan persamaan regresi linier adalah y = 0,0019x + 0,0338. Uji perolehan kembali diperoleh dari penambahan sampel blanko dengan tiga konsentrasi standar kanamisin sulfat yang berbeda adalah 101,40% + 2,02%. Presisi metode baik dengan koefisien variasi 0,080%. Metode KLT-bioautografi kontak didukung oleh penentuan rasio potensi kanamisin sulfat dalam sediaan injeksi dan standar kanamisin menggunakan desain 3-3. Rancangan blok acak memperoleh rasio potensi kanamisin sulfat dalam sediaan injeksi dibandingkan dengan standar kanamisin sulfat adalah 100,6%. **Kesimpulan**: Metode KLT-bioautografi kontak untuk kanamisin sulfat dalam sediaan injeksi dapat diaplikasikan pada analisis kontrol kualitas dari obat yang dianalisis.

Kata kunci: *kanamisin sulfat, KLT-bioautografi kontak, Escherichia coli*

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INTRODUCTION

Kanamycin belongs to the aminoglycoside antibiotics, which works by growth preventing or killing pathogenic bacteria, such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella* spp., and *Proteus* spp., as well as other bacteria. Kanamycin injection is usually used for serious bacterial infections for which other medicines may not work. However, it may also cause some serious side effects, including damage to hearing, sense of balance, and kidneys.

This medicine inhibits bacteria protein synthesis by tightly binding to the 30S ribosomal RNA, which causes misreading of the genetic code. The ues of this medicine is for short-term only (usually 7 to 10 days) and is to be administered only under supervision of the doctor. Therapeutic drug monitoring is needed, bacuase of its narrow therapeutic range. Therefore, a fast and accurate method is the important way for ensuring adequate therapy (Papich, 2016)

Several methods have been applied for determine kanamycin in different matrices such as gas chromatography (Stead, 2000), enzyme-linked immunosorbent assay (Chen *et al*., 2008), spectrophotometry (Omar *et al*., 2013), liquid chromatography-mass spectrometry (Santos & Ramos, 2016), high performance liquid chromatography (Zhang *et al*., 2019) and among others. Although these methods are sufficiently accurate, most of their applications is low sensitive. Therefore, simple and fast, but specific and very sensitive method is needed for identification the kanamycin sulfate.

Thin-layer chromatography combined with densitometry has been reported by Hubicka *et al*. (2009) for identification and quantitative determination of kanamycin. The method has high sensitivity with LOD < 1.5 µg and the precision of the determination was very good (Hubicka *et al*., 2009)

Thin-layer chromatography combined with a biological detection method, it is known as TLC-bioautography. This method is an effective method for identification of antibiotics and belongs to microbiological screening methods commonly used for detection of antibiotics activity. This method very sensitive and specific which performed with a minimum sample. Bioautography measures antibacterial properties of analyzed substances, i.e. inhibits bacterial growth (Choma & Grzelak, 2011). TLC-direct bioautography and TLC-immersion bioautography are specifically used for microorganisms that can grow directly on the TLC plate. While TLC-contact bioautography can be used for microorganisms that can grow directly or indirectly on the TLC plate (Marston, 2011).

This method has been successfully applied to kanamycin sulfate analysis in injection preparations. This method was supported by determination of potential ratio between kanamycin sulfate in injection preparations and kanamycin sulfate standard, by which the growth of *Escherichia coli* ATCC 8739 as a test organism was inhibited.

MATERIALS AND METHODS

Chemicals

Kanamycin sulfate p.g. (PT. Meiji), *Escherichia coli* ATCC 8739, kanamycin injection (PT. Meiji) obtained from pharmacy, potassium dihydrogen phosphate (Merck), nutrient broth (Merck), nutrient agar (Merck), sodium chloride (Merck) and distilled water (Otsuka).

Instrumentation

Chamber 10 x 10 x 6 cm^3 (Camag), TLC silica gel 60 F_{254} plate (Merck), incubator (Memmert), autoclave (Huxley HV-340 Speedy), vortex (Thermo), micropipet (Socorex) and spectrophotometer (Lovibond Spectro PC 22).

Preparation of growth media

Eighteen grams of agar and 8 g of nutrient broth were dissolved in 1000 mL distilled water, mixed and heated with stirred until homogeneous. The media was sterilized by autoclave at 121ºC for 15 minutes.

Preparation of bacterial inoculum

Escherichia coli ATCC 8739 were inoculated on nutrient agar slant media and incubated at 35-37ºC for 24 hours. The bacterial suspension was prepared by adding 10 mL of sodium chloride 0.9% solution to the 24 hours culture and shaking with vortex until the entire colony was removed from the surface of the agar media. The optical density of colony suspension was measured at 580 nm and adjusted to obtain 25% transmitant.

Preparation of standard solutions

An accurately weighed 0.1 g of kanamycin sulfate and dissolved in 100 mL distilled water for obtaining 1000 µg/mL stock of standard solution. A serial dilution was made to obtained 10, 250, and 500 µg/mL of standard solution.

Preparation of sample solution

An amount of kanamycin sulfate vial content equivalent to 0.025 g kanamycin sulfate was dissolved in 10 mL aqua pro injections.

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Optimization of mobile phase

Optimization of mobile phase was carried out using 10% and 12% of potassium dihydrogen phosphate solution respectively. The Kanamycin sulfate standard solution of 500 µg/mL was spoted as much as 10 µL on the TLC plate, and then developed in the mobile phase. The eluated plate was dried.

TLC-contact bioautography

Ten microliters of standard and test solution were spotted on the TLC plate, then developed with selected eluent of potassium dihydrogen phosphate solution. After the eluent front was reached, the TLC plate was dried aceptically to remove the eluent from the silica gel 60 F_{254}. The TLC plate was placed on the inoculated agar surface with 5 µL *Escherichia coli* ATCC 8739 inoculum and storaged in refrigerator for 2 hours to allow diffusion process. Furthermore, the plate was removed and the petri dish was incubated at 37ºC for 16 hours. The growth inhibitory zone diameter was measured.

Method validation

Selectivity

The specificity of TLC-contact bioautography was established by analyzed of streptomycin sulfate and kanamycin sulfate standard solutions simultaneously 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 and LOQ

The LOD and LOQ of TLC-contact bioautography was determine by analyzed of kanamycin sulfate standard solutions on the silica gel 60 F_{254} TLC plate. Elution was carried out with 10% potassium dihydrogen phosphate solution. The Minimum Inhibitory Concentration (MIC) was limit of detection.

Linearity

The linearity of TLC-contact bioautography was determine by analyzed of kanamycin sulfate standard solutions on the silica gel 60 F_{254} TLC plate to obtain concentration 100, 150, 200, 250, 300 and 350 µg/mL. Elution was carried out with 10% potassium dihydrogen phosphate solution. Inhibitory zone plotted against corresponding amount to obtain the calibration plot.

Accuracy

The recovery of kanamycin sulfate at different levels in sample, pre-analyzed sample was spiked with 80%, 100%, and 120% extra kanamycin sulfate standard and analyzed by use of the TLC-contact bioautography.

Precision

The precision of TLC-contact bioautography was assessed by repeatability (intraday precision). The repeatability was performed by analyzed of three different concentration levels of kanamycin sulfate (150, 175, and 200 µg/mL) under the same operating conditions over a short interval of time (the same day). The precision was evaluated as the coefficient of variation (CV).

Potency ratio of antibiotic

Five microliters of test bacteria inoculum 25% transmitant was inoculated on 8000 µL of melted nutrient agar media, vortexed homogenous. The cylinders placed from each other 20 - 25 mm apart. A standard solutions (S1:S2:S3) and sample solutions (U1:U2:U3) were dropped 20 µL. Incubation at 35 - 37ºC for 24 hours. The growth inhibitory zone diameter was measured. Statistical analysis of data using the formula equation (1).

Potency ratio = Antilog Mu x 100 %.....(1)

RESULTS AND DISCUSSION

Optimization of mobile phase

The results of optimization of the mobile phase showed by Figure 1. If the mobile phase is able to separate the analyte from the sample matrix. This is indicated by the value of Rf = 0.2-0.8 and spot is not tailing (Sherma & Fried, 2013). A 12% potassium dihydrogen phosphate solution has Rf value 0.7 and has a tailing spot, while 10% potassium dihydrogen phosphate solution has Rf value 0.4 and has not tailing spot. The results of obtained Rf values confirm that, of all concentration of mobile phases, the mobile phase which containing 10% potassium dihydrogen phosphate solution was suitable for identification of kanamycin sulfate.

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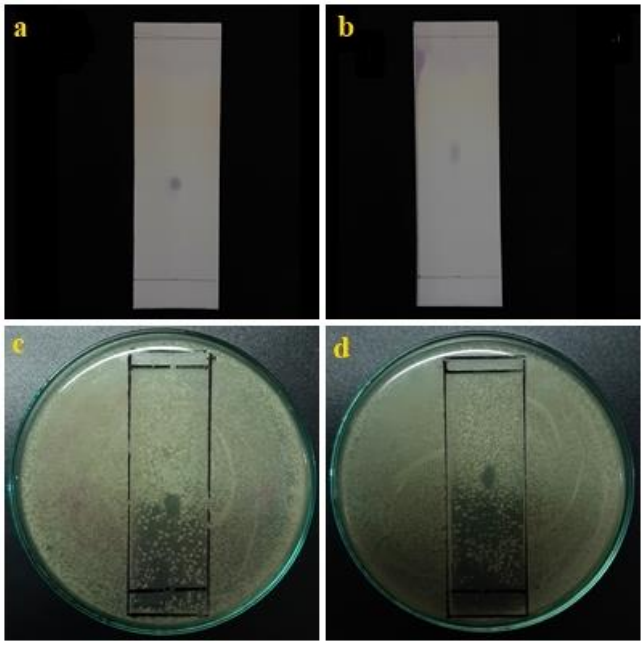

Figure 1. Result of chromatogram of mobile phase using (a) 10 % potassium dihydrogen phosphate solution and (b) 12 % potassium dihydrogen phosphate solution. TLC-bioautogram of mobile phase using (c) 10 % potassium dihydrogen phosphate solution and (d) 12 % potassium dihydrogen phosphate solution

Validation of the proposed method

The proposed TLC-contact bioautography for determination kanamycin sulfate in injection preparations was validated according to USP guidelines with respect to selectivity, LOD, LOQ, linearity, accuracy and precision.

Selectivity results showed Rs value was 3.47. The method satisfies the selective requirement if value of Rs $\geqslant$ 2.00, which means that the two analytes has been separated well. The results are summarized in Table 1.

Table 1. Result of resolution (Rs) value of streptomycin (STR) and kanamycin (KAN)

Sample	Rf (STR)	Rf (KAN)	Rs
Kanamycin sulfate	-	0.4	-
Kanamycin sulfate + streptomycin sulfate	0.7	0.4	3.47

LOD was determined by minimum inhibitory concentration of kanamycin sulfate analyzed using TLC-contact bioautography was 0.70 µg while LOQ was 2.31 µg.

Linearity of the TLC-contact bioautography method was determined by plotting concentration of kanamycin sulfate standard (x) versus growth inhibitory zone diameter (y), as shown in Figure 2. The plot (n = 5) was linear for kanamycin sulfate in the range from 100 to 350 µg/mL with correlation coefficients (r) was 0.9993.

Accuracy of the method is determined by recovery. The recovery from addition of blank samples at three different concentrations of kanamycin sulfate was obtained 101.40% and SD = 2.02. The results are summarized in Table 2. The precision of the method (% CV) was assessed by repeatability. If the method gives a coefficient of variation < 2%, then it gives good precision (Gandjar & Rochman, 2007). In this method the coefficient of variation was 0.080%. From these data the TLC-contact bioautography method meets the requirements.

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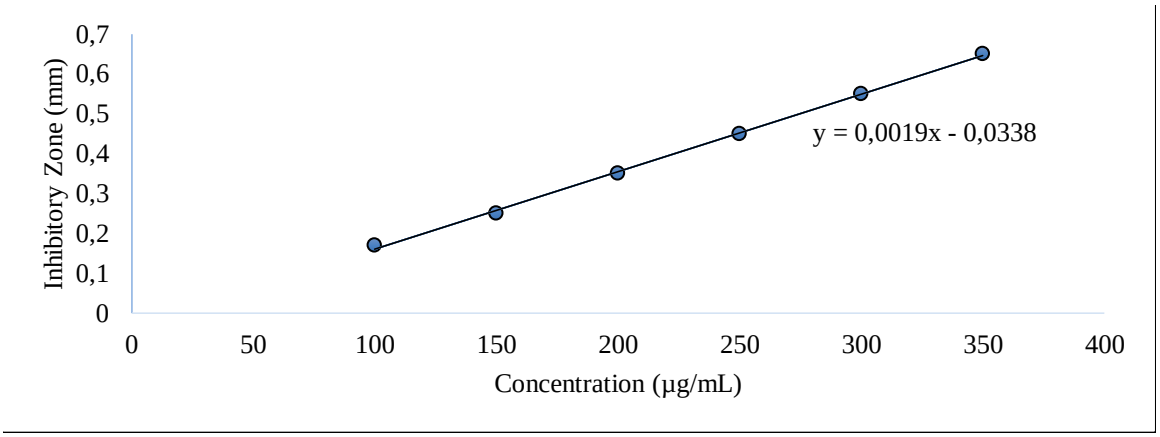

Figure 2. Linear regression of kanamycin sulfate standard analyzed by TLC-contact bioautography using *Escherichia coli* ATCC 8739 as test organism

Table 2. Evaluation of the accuracy of TLC-contact bioautography method

Sample	Conc (µg/mL)	Inhibition zone diameter (cm)	% Recovery
Kanamycin sulfate	150	3.02	98.53
		3.02	98.53
		3.02	98.53
	175	3.60	101.40
		3.60	101.40
		3.60	101.40
	200	3.75	97.50
		3.75	97.50
		3.75	97.50

Application to pharmaceutical dosage forms

TLC-contact bioautography method developed for the determination of kanamycin sulfate was reliable by only using single solvent, specific and very sensitive method for identification kanamycin sulfate. The results of identification of kanamycin sulfate in injection preparations showed positive containing kanamycin sulfate. The results are summarized in Table 3.

Table 3. Evaluation of the precision of TLC-contact bioautography method

Sample	Conc (µg/mL)	Inhibition zone diameter (cm)	% CV
Kanamycin sulfate	150	3.05	0.006
		3.02	
		3.02	
	175	3.12	0.080
		3.60	
		3.60	
	200	3.45	0.050
		3.75	
		3.75	

Potency ratio of antibiotic

After measuring the diameter of the inhibitory zone showed by Table 4, the percentage of potential antibiotic test can be calculated by Table 5. The results of ratio of kanamycin sulfate standard and kanamycin in injection preparations showed by Figure 3. Kanamycin in injection preparation is considered good if it has the potential to inhibits bacterial growth in the range of 90% to 115% (Ministry of Health of the Republic of Indonesia, 2014). The results of the calculation of the percentage of antibiotic potential in this study was 100.6%. Therefore, kanamycin in injection preparations is appropriate because it is in the range listed in accordance with Indonesia Pharmacopoeia fifth edition.

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Table 4. Evaluation of identification kanamycin sulfate by TLC-contact bioautography method

Sample	Replication	Inhibition zone diameter (mm)	Conc (µg/mL)
Kanamycin injection	1	1.25	640.10
	2	1.25	640.10
	3	1.05	534.84
	Mean	1.18	605.01

Table 5. The result of measurement inhibitory zone of kanamycin injection using *Escherichia coli* ATCC 8739 as test organism

Petri dishes	Inhibition zone diameter (mm)					
	S_1	S_2	S_3	U_1	U_2	U_3
1	20.50	21.40	22.10	20.05	21.05	22.40
2	20.75	21.45	22.70	20.25	21.70	22.05
3	20.80	21.05	22.70	20.55	21.50	22.10
4	20.30	21.50	22.20	20.80	21.50	22.10
∑	82.35	85.40	89.70	81.65	85.75	88.65
Mean	20.59	21.35	22.43	20.41	21.44	22.16

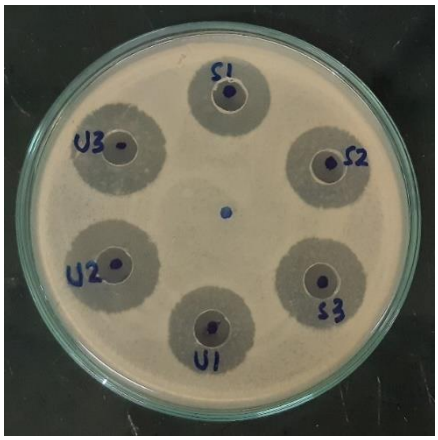

Figure 3. Result of ratio of kanamycin sulfate standard (S) and kanamycin in injection preparations (U) using *Escherichia coli* ATCC 8739 as test organism on nutrient agar media. S1 and UI = 40 µg/mL; S2 and U2 = 60 µg/mL; S3 and U3 = 90 µg/mL.

CONCLUSION

The TLC-contact bioautography for determination kanamycin sulfate in injection preparations was validated according to USP guidelines meet the method was validated to meet requirements which include selectivity, LOD, LOQ, linearity, accuracy and precision. The TLC-contact bioautography method was supported by determination of kanamycin potency ratio in the injection preparation. Moreover, this method could be applied to the quality control analysis of the investigated drugs.

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