Validation of Thin-Layer Chromatography-Bioautographic Method for Determination of Streptomycin

Isnaeni*, Andri Astuti, Muhammad Yuwono
Fakultas Farmasi, Universitas Airlangga, Surabaya

*Corresponding author: isna.yudi@gmail.com

Abstract

Background*: A simple bio-assay for determination of streptomycin hyphenated with planar chromatography techniques was developed.* ***Objective****: This study aims to validate the method for identification and determination of streptomycin in injection preparations with TLC-bioautography.* ***Methods****: Thin Layer Chromatography (TLC) was performed on the silica Gel GF-254 using KH_2PO_4 solution as mobile solvent. The visualization was performed by spraying 2% resorcinol. Direct bi autography was developed using Escherichia coli ATCC 25922 as a bacterial test, grown on the nutrient agar medium at 37^oC for 24 hours. The method was validated corresponding to linearity, limit of detection (LOD), intra day precision, and accuracy parameters. The accuracy was measured using streptomycin injection as a sample.* ***Results****: The Results showed that the KH_2PO_4 solution at 7.5% concentration was found to be the optimized solvent with Rf value of 0.5. The linear equation was y = 10.176x + 4.046 at 150 - 350 µg/mL concentration range with the linearity coefficient, Limit of Detection, accuracy, and variation coefficient were 0.9907; 40 ppm; 96.37 $\pm$ 2.22% (with an RSD value of 2.31%); and 1.63 respectively.* ***Conclusion****: The prospective TLC-bioautographic method was applied for the identification and determination of streptomycin in a preparation using a single eluent KH_2PO_4. The eluent system optimization remains necessary for the identification and determination of the mixture of streptomycin with other antibiotics, such as aminoglycoside groups.*

Keywords*: validation, TLC-bioautography, streptomycin*

INTRODUCTION

TLC-bioautography is a method consisted of chromatographic separation and biological activity determination (Choma, 2005). The method is widely applied for detection of antimicrobial (Choma & Edyta, 2011), antioxidant (Marston, 2011; Cheng & Wu, 2013) and enzyme inhibitory activities (Gu *et al.*, 2015). The TLC-bioautography of antimicrobial substances was performed based on the detection of compound in the chromatogram by using microorganisms as an indicator. Clear zone on the spot position indicates antimicrobial activity of the substances. Validation of the TLC-bioautography method with characteristic parameters namely selectivity, sensitivity, linearity, precision, recovery, and stability is performed by optimizing factors affected the analysis results such as plate type, time and temperature of incubation. The TLC-bioautography method of commonly used for bioactivity screening purposed in natural products (Choma & Edyta, 2011). The method validation for determination of streptomycin by TLC-bioautography has not been reported, although chromatogram profile of the aminoglycoside antibiotics using several solvent system has been reported by Claes & Vanderhaeghe (1982).

Streptomycin belongs to aminoglycoside antibiotic that widely used to treat infectious diseases in human, animal as well as in plant agriculture (Shafqat *et al.*, 2012). The antibiotic administrates in injection dosage form or powder for solution preparations (Wills, 2005). Determination of streptomycin and its derivatives by HPLC (Whall, 1981), LC-MS/MS (Pendela *et al.*, 2009) and LC-MS (Hormazabal & Ostensviko, 2013) TLC-Densitometry (Urszula *et al.*, 2009) have been reported. The potency testing in the quality control laboratories is used for determination of aminoglycoside antibiotics such as streptomycin, kanamycin and gentamycin in the pharmaceutical dosage form. The method gives very simple, accurate, and reproducible results. Drug monitoring sometimes is needed to evaluate of effectiveness and side effect after the drug administrated to patient. In case, the drug exists in a mixture with other substances, a valid method is needed to obtain responsible of analysis results. The streptomycin is one of aminoglycoside antibiotics that still used as a first line anti-tuberculosis drug with side effect of nephrotoxicity and ototoxicity (Toman, 2004). Where possible, serum level should be

P-ISSN: 2406-9388
E-ISSN: 2580-8303

monitored periodically. The aim of this research are to validate the TLC-bioautography method using single solvent system KH_2PO_4 solution for determination of streptomycin in the dry powder/small volume parenteral dosage form (injection). The result could be implemented for separation of the active compound (streptomycin) from its mixture not only in the dosage form but also in the specimens, like plasma and urine. The use of bacteria test for detecting streptomycin would be specific to distinguish from non-antibiotic compounds. The single solvent (KH_2PO_4 solution) used is relatively safe and cheaper compared to organic solvent.

MATERIALS AND METHODS

Chemicals and reagents

Streptomycin sulphate (Sigma) and injection of streptomycin were commercially obtained. Potassium phosphate mono basic (Sigma), distilled water, Nutrient agar (Oxoid), *Escherichia coli* ATCC 25922 (Health Laboratory, Surabaya), and saline (Sodium chloride 0,9%) solution.

Thin layer chromatography bioautography

A standard stock solution of streptomycin (100 mg/100 mL) was prepared by dissolving 100 mg streptomycin powder in 100 mL distilled water. Concentration of the standard 1000 µg/mL was diluted with distilled water to obtain 30, 40, 50, 60, 70, and 80 µg/mL for determination of LOD, while for determination of linearity was performed by dilution of the standard solution to obtain 150, 200, 250, 300, and 350 µg/mL concentration. The sample solution was obtained from the dry powder dosage form (2-gram streptomycin powder in vial), prepared, diluted, and analyzed by the same method as a standard solution. The mobile solvent was prepared in several concentrations for optimization and to choose concentration of the KH_2PO_4 solution 5% or 7.5%.

Suspension of *Escherichia coli* ATCC 25922 was prepared by growing it in a slant medium (nutrient agar, at 35 ± 2 ºC for 24 h). The growth cells were suspended in a saline sterile solution and diluted to give a suspension with 25 ± 2% transmittance at 580 nm using a 1 cm absorption cell and 0.9% NaCl sterile as a blank solution. Seed layer medium was prepared by inoculating 5 µL cell suspension in 7 mL of nutrient agar medium at 48 ºC (Susanti *et al.,* 2009); which then overlaid on the surface of nutrient agar based layer medium.

The chromatography of antibiotic standard and samples solution was performed on silica gel F_{254} (Merck), with the KH_2PO_4 solution as the mobile solvent (Isnaeni, 2005). The chromatogram of a developed TLC plate was contacted on a surface of the nutrient agar media inoculated by *Escherichia coli* ATCC 25922 as a test bacterium. (Susanti *et al.,* 2009). The mobile solvent was 7.5% KH_2PO_4 solution. An amount of 10 µL of each sample and standard solution were applied on the TLC plates in a spot and developed using the mobile solvent system. The plates were dried to remove solvent residue on the plates (Suleimana *et al.,* 2010**).** Resorcinol solution (2%) was used as detection reagent to observe position of purple color spot on the chromatogram plate.

Furthermore, the different chromatography plates free from spray reagent was placed on the surface of test medium containing the suspension of *Escherichia coli* ATCC 25922 and then storage in the refrigerator for 1 hour to allow diffusion of active substances on the test media. The growth of bacterium was appeared on the surface of the test media after incubation overnight, excluding spots of the streptomycin. The diameters (mm) of the clear zone around the spots were measured by calibrated digital caliper (Susanti *et al.,* 2009)**.**

Method validation

The method was validated according to the International Conference on Harmonization (ICH, 2005) for evaluation of the performance attributes like LOD, linearity, accuracy and precision. The LOD is the minimum amount of analyte that can reliably inhibit the test microorganisms. This attribute was done by assaying a serial of standard solution at 30 - 80 µg/mL range of concentration. This characteristic is applicable for Minimum Inhibition Concentration (MIC) determination. The linearity was evaluated through three independent assays using linear regression analysis and calculated by a least-squares method for five doses of the reference substance. The accuracy means the ability of the method to measure the actual or true value of the analyte. The test was repeated in three consecutive days. Three concentration levels, covering 80% to 120% of the selected range of 250, 300, and 350 µg/mL, were tested each day. The precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenized sample. This parameter was assessed through the repeatability and

P-ISSN: 2406-9388
E-ISSN: 2580-8303

intermediate precision and expressed as the relative standard deviation (RSD).

RESULTS AND DISCUSSION

Thin layer chromatography-bioautography

The chromatogram of streptomycin standard solution gave Rf value of 0.50 and 0.33 at 7.5% and 5% concentration of KH_2PO_4 solvent, respectively. It was found that clear zone was appearance sharply at 10 µL and 15 µL containing 0.8 µg and 1.2 µg streptomycin respectively (Figure 1) for both before and after the plate development (Table 1). It should be noted that the clear zone was not detected on the chromatogram at 5 µL sample solution for all concentrations of the eluents.

Table 1. Optimization of sample tested volume on contact bioautography qualitatively with *E.coli* ATCC 25922

Vol. of Samples (µL)	Appearance of clear zone inhibition (qualitatively) at concentration (µg/mL) of				
	80	100	120	140	160
	Before and after the plate development				
5	negative				
10	positive				
15	positive				

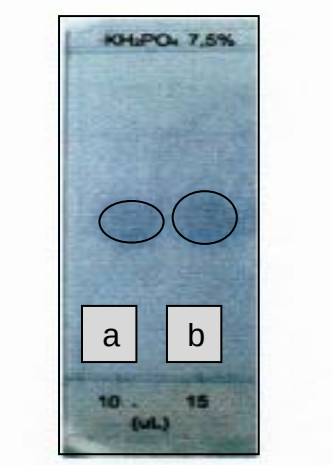

Figure 1. Bioautogram of Streptomycin sulphate on silica gel GF_{254}plate; Eluted by7.5% KH_2PO_4 solution with *E. coli* ATCC 25922 as a test bacteria; (a) 10 µL sample and (b)15 µL sample

Method validation

LOD

It was found that the LOD of streptomycin was 40 µg/mL. This value was reflected as Minimum Inhibition Concentration (MIC) of the streptomycin (Table 2).

Table 2. The results of LOD evaluation on the bioautography of streptomycin sulphate with *E.coli* ATCC 25922

Conc. of Samples (µg/mL)	Diameter of clear zone growth inhibition (mm)
< 40	-
40	***7.862**
50	8.281
60	8.883
70	10.062
80	10.241
* Diameter of hole/reservoir = 7 mm	

Linearity

The standard solution at 150, 200, 250, 300, and 350µg/mL concentration was used as linearity test. The intra-day precision was determined by loading 10 µL three standard solutions. (n = 3). The mean of the recorded clear zone diameter (mm) was taken for calibration curve; which obtained by plotting against log concentration (Figure 2).

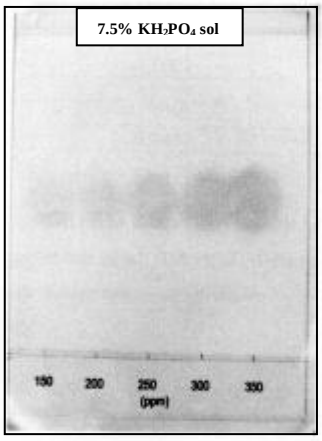

Figure 2. Linearity observed by bioautography on silica gel F_{254} plate; eluted by 7.5% KH_2PO_4 solution with *E. coli* ATCC 25922 at concentration range of streptomycin 150 - 350 µg/mL.

The method resulted a good linearity at 150 - 350 µg/mL range. The linear equation was y = 10.176x + 4.046. The correlation coefficient (r = 0.9907) and determination of coefficient (r^2 = 0.9819) were highly significant.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Accuracy and precision

The accuracy was evaluated by the recovery determination of streptomycin sulphate in the injection dosage form and visualized in Figure 3. The mean accuracy was 96.37 $\pm$ 2.22%, with an RSD value of 1.63%.

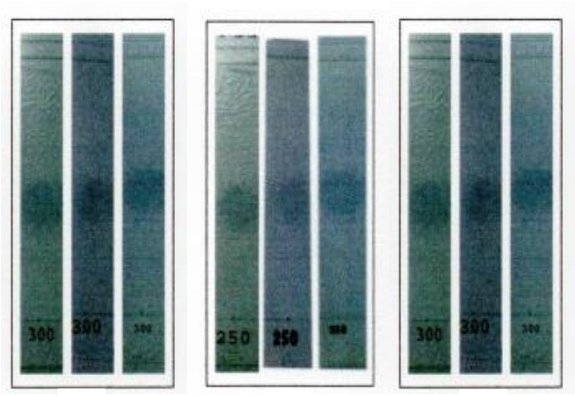

Figure 3. Inter day precision observed by bioautography on silica gel F_{254} plate; developed by 7.5% KH_2PO_4 solution with *E. coli* ATCC 25922; streptomycin concentration of 250, 300, and 350 µg/mL performed by three (A, B, C) independent assays.

The analytical method is usually selected based on analysis purposes, such as qualitative, semi-quantitative or quantitative. On the other hands, equipments and reagents should be considered for development of accessible and useful the method (Suleimana *et al.,* 2010). In this research, KH_2PO_4 solution was used as a single mobile solvent since it is safe, simple and cheaper compare to the organic solvent, such as butanol and methanol.

The KH_2PO_4 solution at 7.5% was then chosen for plate development on the TLC-bioautographic system as recommended by Isnaeni (2005). The lower concentration of the solvent was originally reported by Claes & Vanderhaeghe (1982), by which the streptomycin could be separated from eight other aminoglycoside antibiotics using 15% and 10% aqueous solution of KH_2PO_4 at pH 4.4 and 4.5 respectively. This solvent system gave Rf value of 0.66 and 0.56 respectively. In case of single compound analysis, 7.5% of KH_2PO_4 solution is recommended, but the higher and various concentration are observation needed for mixed compound analyzed.

The agar diffusion or contact bioautography chosen for simplicity reasoning, the technique is carried out in the same manner as common detection of antimicrobial activity or potency by using two layers of agar media. The chromatogram is placed face down onto the inoculated agar layer incubated by test microorganism (Dewanjee *et al.*, 2015) for a specific period to enable diffusion. Pre-incubation is needed to allow diffusion of the analyte in the chromatogram spot on the surface of agar medium. Then the chromatogram plate was removed from the agar after incubation for certain time.

The *Escherichia coli* ATCC 25922 was selected as the test microorganism because of its susceptibility to streptomycin, yielding sharply and clearly defined zones of growth inhibition, by which more precise measurements achieved (Susanti *et al.*, 2009).

The validation method was performed according to Wills (2005) for parameter evaluation. Current method is valid and accurate; which appropriate acceptance criteria of $\leq$ 5% (ICH, 2005). Thus, the results obtained of the method were close to the true concentration values of the tested samples. The TLC-Bioautography is a analysis method provided for components exhibiting antimicrobial activity, that performed in situ, in comparison with other commonly used antimicrobial susceptibility activity or potency tests. The bio-assay precision of intra-day repeatability determined on the same days with three different test solutions of streptomycin sulphate was gave good results.

CONCLUSION

Various methods have been developed for the streptomycin determination, but have some disadvantages of being time-consuming and very expensive. The proposed method was found to be rapid, accurate, and repeatable in a hasty manner and techniques. It can be concluded that the simple bioautography detection in thin-layer chromatography with a single solvent system of 7.5% KH_2PO_4 solution pH 4.3 using *Escherichia coli* ATCC 25922 permitted determination of streptomycin in the injection sample validly.

ACKNOWLEDGEMENTS

The authors wish to thank Health Laboratory, Surabaya for providing *Escherichia coli* ATCC 25922 as a test bacterium.

REFERENCES

Cheng, Z. & Wu, T. (2013). TLC Bioautography: High Throughput Technique for Screening of Bioactive Natural Products. *Combinatorial Chemistry & High Throughput Screening; 16;* 531-49.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Choma, I. (2005). The Use of Thin-Layer Chromatography with Direct Bioautography for Antimicrobial Analysis. *Lc Gc Europe; 18;* 482-488.

Choma, I. M. & Edyta, M. G. (2011). Bioautography Detection in Thin-Layer Chromatography. *Journal of Chromatography* A; 1218; 2684-2691.

Claes, P. J. & Vanderhaeghe, H. (1982). Thin-layer Chromatographic Identification of Aminoglycoside Antibiotics. *Journal of Chromatography; 248;* 483-487.

Dewanjee, S., Moumita, G., Niloy, B., Ritu, K. & Tarun, K. D. (2015). Bioautography and Its Scope in the Field of Natural Product Chemistry. *Journal of Pharmaceutical Analysis; 5;* 75-84.

Gu, L. H., Liao, L. P., Hu, H. J., Annie, S. W. B., Wang, C. H., Chou, G. X. & Wang, Z. T. (2015). A Thin-Layer Chromatography-Bioautographic Method for Detecting Dipeptidyl Peptidase IV Inhibitors in Plants. *Journal of Chromatography A; 1411;* 116-22.

Hormazabal, V. & Ostensviko. (2013). Determination of Streptomycin and Dihydrostreptomycin in Milk and Meat by Liquid Chromatography-Mass Spectrometry. *Journal of Liquid Chromatography & Related Technologies; 23;* 2756-64.

ICH Harmonized Tripartite Guideline. (2005). Validation of Analytical Procedures: Text and Methodology - Q2(R1)-ICH Steering Committee. Geneva: Commission of the European Communities, Geneva.

Isnaeni. (2005). Bioautografi Antibiotika Hasil Fermentasi Mutan Streptomyces Griseus ATCC 10137. *Jurnal Farmasi Airlangga; 5;* 12-16.

Marston, A. (2011). Thin-Layer Chromatography with Biological Detection in Phytochemistry. *Journal of Chromatography A; 1218;* 2676-83.

Pendela, M., Jos, H., Ann, V. S. & Erwin, A. (2009). LC-MS of Streptomycin Following Desalting of a Nonvolatile Mobile Phase and pH Gradient. *Journal of Separation Science; 32;* 3418-3424.

Shafqat, U., Arshad, H., Asad, U., Waseem, H. & Khaliq-ur, R. (2012). Simple and Rapid Method on High Performance Liquid Chromatography (HPLC) for Estimation of Streptomycin Sulphate. *World Applied Sciences Journal; 19;* 645-49.

Suleimana, M. M., McGaw, L.J., Eloff J. N. & Naidoo, V. (2010). Detection of Antimicrobial Compounds by Bioautography of Different Extracts of Leaves of Selected South African Tree Species. *African Jorunal of Traditional, Complementary and Alternative Medicines; 7;* 64-78.

Susanti, M., Isnaeni & Poedjiarti, S. (2009). Validation of Bioautographic Method for the Determination of Chloramphenicol. *Indonesian Journal of Medicine; 1;* 15-24.

Toman, K. (2004). Toman's tuberculosis Case Detection, Treatment, and Monitoring: Questions and Answers.2nd ed. Geneva: World Health Organization.

Urszula, H., Krzek, J., Woltyska, H. & Stachacz, B. (2009). Simultaneous Identification and Quantitative Determination of Selected Aminoglycoside Antibiotics by Thin Layer Chromatography and Densitometry. *Journal of AOAC International; 92;* 1068-1075.

Whall, T. J. (1981). Determination of Streptomycin Sulfate and Dihydrostreptomycin Sulfate by High-Performance Liquid Chromatography. *Journal of Chromatography A; 219;* 89-100.

Wills, K. (2005). A Practical Guide to validation. In Easter MC. (Eds.) Rapid Microbiological Methods in the Pharmaceutical Industry. New York: *Interpharm/CRC Press*.

P-ISSN: 2406-9388
E-ISSN: 2580-8303