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Development and Validation of High Performance Planar Chromatography for Estimation of Gentamicin by Fluorophore Generation

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ABSTRACT: This study presents the development and validation of an HPTLC method for estimating Gentamicin in bulk powder, pharmaceutical dosage forms, and blood plasma samples. The chromatography separation was conducted on pre-coated aluminum plates using silica gel 60 F_{254} as the stationary phase and a mobile phase comprising chloroform, methanol, and 25% ammonia solution. The developed method was able to successfully separate and detect the various components of Gentamicin, despite the challenges of optimizing the separation and detection parameters. The method produced compact and well-resolved bands, which is important for accurate analysis. By demonstrating excellent linearity (r^2 = 0.9996) in the calibration plots, the method enables the quantification of Gentamicin within the concentration range of 60-200 ng/band at 436 nm. Furthermore, the method's precision, accuracy, robustness, specificity, limit of detection, and limit of quantification were validated according to the International Conference on Harmonization (ICH) guidelines. The results of the study confirm that the developed method is suitable for routine quality control testing of Gentamicin in bulk drug, marketed formulations, and plasma samples. This means that the method can be used to reliably ensure the quality of Gentamicin and guide its therapeutic use.

Keywords: Gentamicin, Planar-chromatography, Derivatization, HPTLC, Flurophore, Optimization.

INTRODUCTION

In the early 1940s, streptomycin was discovered by Waksman, marking the initial breakthrough in developing antibacterial drugs. Gentamicin, classified as an aminoglycoside, belongs to this group (Chackalamannil *et al.*, 2017). Ever since their discovery, aminoglycoside antibiotics have played a crucial role in our arsenal against serious gram-negative infections.

Gentamicin, a significant aminoglycoside antibiotic, was first isolated in 1963 by Weinstein (Florey, 1981). It is a mixture of water-soluble antibiotics produced through fermentation of *Micromonospora echinospora*. The composition includes aminocyclitol 2-deoxystreptamine and two tailoring sugars: purpurosamine and garosamine. Different substitution patterns of purpurosamine give rise to major components: C1, C2, and C1a (Fig. 1). The common sugar unit in gentamicin is garosamine, while the dissimilar 2,6-diamino sugars are known as purpurosamine A, B, and C, corresponding to gentamicins C1, C2, and C1a, respectively (Yoshizawa *et al.*, 1998; Florey, 1981). Gentamicin B is a minor component in the biosynthetic pathway of gentamicin (Wu *et al.*, 2017). Each major component of the gentamicin complex contains five basic amino functions (Benveniste and Davies 1973). It is worth noting that gentamicin sulfate, a typical antibiotic in this class, is obtained as a hydrated amorphous solid without a characteristic melting point or UV absorption (Florey, 1981).

The assay of gentamicin sulphate has been developed using various methods such as high performance liquid chromatography (HPLC) (Freeman *et al.*, 1979; Joseph and Rustum 2010; Bosch *et al.*, 2017), high-performance thin-layer chromatography (HPTLC) (Hubicka *et al.*, 2009; Bhogte *et al.*, 1997), and microbiological assay (Zuluaga *et al.*, 2009; Eissa *et al.*, 2021). Microbiological methods are recommended by the European, British, and U.S. pharmacopeias for determining aminoglycosides in substances and drug

forms (Hubicka *et al.,* 2009). HPLC is frequently recommended for analyzing aminoglycoside antibiotics in pharmaceutical preparations and biological materials, often utilizing pre-column derivatization and detection methods such as fluorescence, spectrophotometry, evaporative light-scattering, pulsed amperometric, and LC/MS. HPTLC commonly employs 4-chloro-7-nitro-2,1,3-benzoxadiazole and ninhydrin as derivatizing agents for the assay of gentamicin sulphate, as indicated in the literature (Hubicka *et al.,* 2009; Bhogte *et al.,* 1997).

The literature suggests that both microbiological assay and HPLC methods have certain limitations. Microbiological assays are unable to quantify substances other than the active pharmaceutical ingredient in the same matrix, such as metabolites or impurities (Zuluaga *et al.,* 2009). Another drawback is the requirement for sufficient incubation time to determine bacterial growth or inhibition (De Louvois, 1974). On the other hand, a comprehensive analysis has revealed that HPTLC offers several advantages over HPLC. These include minimal sample preparation, reduced solvent usage, and improved resolution and sensitivity, making it a more efficient and cost-effective analytical method. Additionally, HPTLC's non-destructive nature allows for further analysis of separated compounds, making it versatile and capable of providing comprehensive results. Ninhydrin is frequently employed in bioanalytical techniques, particularly for amino acid analysis (Perrett and Nayuni 2014). Nonetheless, the stability of ninhydrin solution and its derivatives poses a significant limitation for its use (Zacharis and Tzanavaras 2013).

4-Chloro-7-nitrobenzofurazan (NBF-Cl) reacts with nucleophilic compounds (amines, mercaptans etc.), altering the polarization of its chromophore system and resulting in corresponding 7-substituted-4-nitrobenzofurazan derivatives emitting intense fluroscence (Staneva and Grabchev 2021)**.** In this study we utilized NBF-Cl as an derivatizing agent.

The objective of this study was to develop and validate (as per the ICH guidelines ICH Q2-R1) an HPTLC method. Silica gel 60 F_{254} TLC plates were utilized, and the mobile phase consisted of a mixture of chloroform, methanol, and 25% ammonium hydroxide. After derivatization with NBF-Cl, quantitative estimation was carried out using densitometric scanning at a wavelength of 436 nm. This method can be employed for routine analysis in the pharmaceutical industry, particularly when a large number of samples need to be analyzed within a single day to meet healthcare requirements.

Instrumentation. In this study, the CAMAG HPTLC system played a pivotal role, comprising several essential components. The system included the WINCATS software, LINOMAT V automatic sample applicator, automatic development chamber, scanning densitometer CAMAG scanner 3, and photo documentation apparatus CAMAG reprostar 3. To ensure precise separation and analysis, an aluminum-based silica gel plate 60 F_{254} (Merck, Mumbai) was chosen as the stationary phase, with dimensions of 20 cm x 20 cm and a particle size of 5-10 µm. Sample application on the HPTLC plates was meticulously performed using a 100 µl syringe from HAMILTON, Switzerland. Furthermore, hydrophilic nylon syringe filters with a pore size of 0.22 µm were employed for the filtration of both samples and standards, ensuring optimal purification.

MATERIALS AND METHODS

For this study, Gentamicin sulphate (GMS) standard was procured from Sisco Research Laboratories Pvt. Ltd., based in Mumbai, India. The 4-Chloro-7 nitro benzofuran (NBF-Cl) was obtained from Sigma Aldrich. To ensure the highest quality, analytical grade methanol and chloroform were purchased from Merck, a reputable supplier in Mumbai, India. Additionally, the 25% ammonia solution used in the experiment was sourced from Finar Chemicals, located in Ahmedabad, India.

Standard Preparation. A standard marker for quantification, around 1 mg (calculated as free base) of GMS, were placed into a separate eppendorf tube and dissolved in 2 mL of water (0.5 mg/mL). The calibration curve was created in accordance with the requirements of the ICH (Panchal *et al*., 2017).

Calibration. The standard curve was created in accordance with the ICH recommendations. Each concentration was spotted on a plate (10 × 10 cm) in triplicates with bands that were each 6 mm wide and separated by 10 mm. Both the bottom and side edges of the plate were 12 and 8 millimetres away, respectively. The application rate was applied 10 µL/s, the bands were developed using chloroform, methanol, and 25% ammonium hydroxide in a 1:1:1 volume ratio after saturation for 20 min. The length of the chromatogram run was 9 cm. The plate was dried at 120°C for 20 min, dipped in methanolic NBF-Cl (0.26 mg/ml) for 2 sec using an automated Camag dipping chamber and heated again at 120°C for 10 min. The cooled plate was re-chromatographed in methanol in the same direction as the first development. Densitometric analysis of the separated material was carried out using the Camag TLC Scanner II, in the fluorescence/ reflectance mode at an excitation wavelength of 436 nm (Hg lamp as a source of radiation) and a sharp cut-off K-500 filter (Camag). Scanning speed was kept at 1 mm/s. Using peak area, linear regression analysis was used for evaluation.

Sample Preparation

Bulk drug. To determine the gentamicin (GM) content of the bulk drug, an aqueous solution equivalent to 20 µg/mL of GM prepared. The solution was filtered through hydrophilic nylon syringe filters (0.22 µm pore size).

Marketed formulation

Specific amount of GMS Cream USP 0.1% w/w marketed formulation (equivalent 1 mg of GM), was taken with 50 mL of ether in a separating funnel and shaken, the combination was extracted by four 20 mL portions of phosphate buffer solution (PBS) pH 8.0. The aqueous extracts were collected and filtered

through hydrophilic nylon syringe filters (0.22 µm pore size).

Plasma sample. Porcine plasma samples were collected from a slaughterhouse. Aliquots of plasma (0.5 mL) were added to glass centrifuge tubes with stoppers. Then, 1 mL of GMS from concentrated stock solutions of GMS in water was added to each tube to give gentamicin concentrations of 20, 30, and 40 µg/mL. The tubes were vortexed for 3 minutes to dissolve the drug in the plasma. A blank was prepared simultaneously. Trichloroacetic acid (0.5 mL) was added to each tube to precipitate the plasma proteins. The tubes were then centrifuged at 1125 g for 30 minutes.

Sample Assay Preparation. As mentioned earlier, the sample and standard solutions were prepared. They were then spotted three times on a TLC plate and developed under the same conditions as those used for the standard. After development, the plates were derivatized and dried in a hot air oven. The analyte was completely separated from other components. When the linear and compact zones were scanned at 436 nm, peak regions for GMS were observed. Different concentrations of bulk drug, marketed formulation, and plasma samples were spotted on the TLC plate. The study was conducted in triplicate.

RESULTS AND DISCUSSION

Silica gel TLC plates were used to evaluate different mixtures of chloroform, methanol, and 25% ammonium hydroxide as the mobile phase. The lower organic layer of the mobile phase was used to separate the components of gentamicin. Different ratios of the lower organic layer were used to find the optimal mobile phase for the separation of gentamicin. A ratio of 1:1:1 (v/v/v) yielded a satisfactory resolution. Following derivatization, the standards exhibited a well-resolved and symmetrical band for GMS under optimized conditions. The application of NBF-Cl reagent resulted in yellow fluorescent spots for GMS. After the initial development of the TLC plate, it was rechromatographed in methanol in the same direction as the first development. This resulted in the visualization of more yellow fluorescent spots (Fig. 2). The standard compound, GMS (R_f = 0.21, 0.26 and 0.30), exhibited three sharp peak showed in the HPTLC chromatogram (Fig. 3). The most favorable scanning was achieved at wavelength of 436 nm (Hg lamp as a source of radiation) and a sharp cut-off K-500 filter (Camag). The test conducted on the mobile phase solvent revealed that the stability of the mobile phase depends on the presence of ammonium hydroxide solution. To ensure proper retention of ammonia in the mobile phase, it is essential to store the prepared solution in a closed container. Figure 4 exhibits the spectra scanning of GMS.

Linearity. Linearity, which refers to the ability to obtain test results directly proportional to the analytes concentration, was determined. A series of reference GMS concentrations were applied in triplicate for analysis. The average peak areas of selected R_f = 0.21, 0.26 and 0.30, were plotted against the corresponding concentrations. The standard curve for GMS exhibited a satisfactory linear relationship across the concentration range of 60–220 ng/spot, with the linear regression equation y = 10.65x - 1.818, where y represents the spot area and x represents the analyte concentration. The correlation coefficient (r^2) was 0.999 (Fig. 5).

Intermediate Precision (Reproducibility). To assess the precision of the proposed method, standard solutions of GMS was evaluated at two different concentrations (80 and 160 ng/spot for GMS). The analysis was conducted three times on the same day and repeated on the next day to determine intraday and interday precisions. The results, presented as relative standard deviations (RSD) in Table 1, revealed that the intraday precision for GMS ranged from 0.4501-0.5006. Regarding interday precision, which assesses variability across different days, GMS exhibited a range of 0.4968-0.9501. The RSD values below 2 percent indicate consistent and reproducible results, indicating good precision in the analysis.

Accuracy (Percentage Recovery). The accuracy of the methods was assessed by calculating the recovery of GMS using the standard addition approach. Three sets of recovery trials were conducted, including 50%, 100% and 150%, additions of GMS. The peak area values were used in the regression equations of the calibration curves to determine the levels of the analytes (Table 1). The proposed method showed percentage recovery rates between 100.158–101.795%, which were within the acceptable range of 100 ± 2% accepted (Kleinschmidt, 2005).

System Specificity. To evaluate the precision of the equipment, reference standard solutions of GMS at different concentrations were injected repeatedly (n = 6). The repeatability of the HPTLC instrument was assessed by applying the same sample solution six times to a plate using an automatic spotter with the same syringe. The sample spot was scanned six times for GMS without changing the plate's position. The % RSD range for GMS was found to be 1.3974. These results indicate the high precision of the equipment in generating consistent measurements for both analytes.

Robustness. A deliberate change in various parameters as mentioned in Table 1, produced % relative standard deviations of the peak area of less than 2%, indicating the robustness of the method.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The following equations were used to calculate the limit of detection (LOD) with a S/N of 3:1 and the limit of quantification (LOQ) with a S/N of 10:1 for both compounds in accordance with ICH regulations.

$LOQ = 3.3 \times \sigma/SD$

$LOQ = 10 \times \sigma/SD$

where the response's standard deviation is σ and SD stands for the standard deviation of the y-intercept of the regression line. GMS had an LOD of 3.79 ng/spot and an LOQ of 11.49 ng/spot.

Sample analysis report. Under optimal conditions, using silica gel TLC plates and a mobile phase consisting of chloroform, methanol, and 25% ammonium hydroxide in a ratio of 1:1:1 (v/v/v),

satisfactory results were obtained. After derivatization, a distinct and symmetrical band for GMS was observed in test samples. Specifically, the percentages were 99.92 % for bulk drug, 98.93% for marketed formulation and 98.76, 99.24 and 98.73 % for plasma samples spotted 80, 120 and 160 ng/ band respectively. The % RSD value was found to be less than 2%. The calibration curve equations mentioned earlier, with x representing the amount of marker and y representing the area under the curve, were used to determine these amounts. In the HPTLC chromatogram, the standard GMS (R_f = 0.21 ± 0.02, 0.26 ± 0.01 and 0.30 ± 0.01) exhibited three sharp peak, which was also found in the test samples. To ensure specificity, the R_f values of the standard and sample were compared. Further, the peak purity of the drug in pharmaceutical dosage form was confirmed by comparing the overlaid spectra at the peak start, peak apex and peak end positions of the band, result indicated the specificity of method in the presence of various excipients.

The HPTLC method was validated following the guidelines of the International Conference on Harmonisation (ICH) (Ngamkhae *et al.*, 2022). Precision studies, showed % RSD values within acceptable limits (≤2), indicating good intra-day and inter-day variations for the simultaneous determination of GMS. The correlation coefficients (r^2) obtained for the calibration curves ≥ 0.995, meeting the requirement set by the USP for linearity. The linearity curves are illustrated in Fig. 5. Table 1 displays the percent recovery for GMS, falling within the acceptable accuracy range (97.0-103.0%) (Rahman *et al.*, 2014). Moreover, additional validation parameters such as the LOQ and LOD were determined. Overall, the results from these validation parameters confirm the reliability and suitability of the analytical method for the accurate analysis of both compounds. The HPTLC method was successfully employed to confirm the concentrations of GMS in the bulk drug, marketed formulation, and plasma samples. The developed HPTLC method accurately quantified GMS compound in test samples, offering a versatile and time-saving approach for their analysis. This method holds significant importance in ensuring the quality and consistency of GMS in various applications.

Table 1: Validation: Parameters and observations

Parameters	**Observations**	
Specificity	**No interference observed**	
System Specificity (%RSD)	1.3974	
Linearity		
R^2 [A]	0.9996	
Slope (± S.E.)	10.6569 (±0.0790)	
Confidence limit of Slope[B]	-26.2492 to 22.6114	
Intercept (± S.E.)	-1.8188 (±10.3316)	
Confidence limit of Intercept[B]	10.4699 to 10.8433	
Sensitivity		
LOD (ng/ band)	3.7938	
LOQ (ng/ band)	11.4965	
Accuracy (studied in triplicate)[D]		
50% (120 ng/ band)	100.925±1.852532051	
100% (160 ng/ band)	101.7958±0.949040085	
150% (200 ng/ band)	100.1583±0.946081098	
Precision (%RSD)		
Intra-day Precision	0.4501-0.5006	
Inter-day Precision	0.4968-0.9501	
Robustness[C] (100ng/band)	**Area ± SD (ng/band)**	**RSD**
Chromatograph run length	**100 ng**	
8.5 cm	100.6733 (± 1.0524)	1.0454
9.5 cm	99.7266 (± 1.5340)	1.5382
Chamber saturation time		
30 mins	101.373 (±0.9683)	0.9552
40 mins	99.7833 (±1.18 86)	1.1912
NBD Cl amount		
0.255 mg mL^{-1}	101.2667 (±0.9280)	0.9164
0.265 mg mL^{-1}	99.71667 (± 1.1657)	1.1690

S.E. = standard error, % RSD = relative standard deviation,
[A]Average of five determinations.
[B]Confidence interval at 95% confidence level and 8 degree of freedom
[C]R_f values for the three peaks were found to be 0.21, 0.26 and 0.30, similar to standard.
[D] Studied in triplicate.

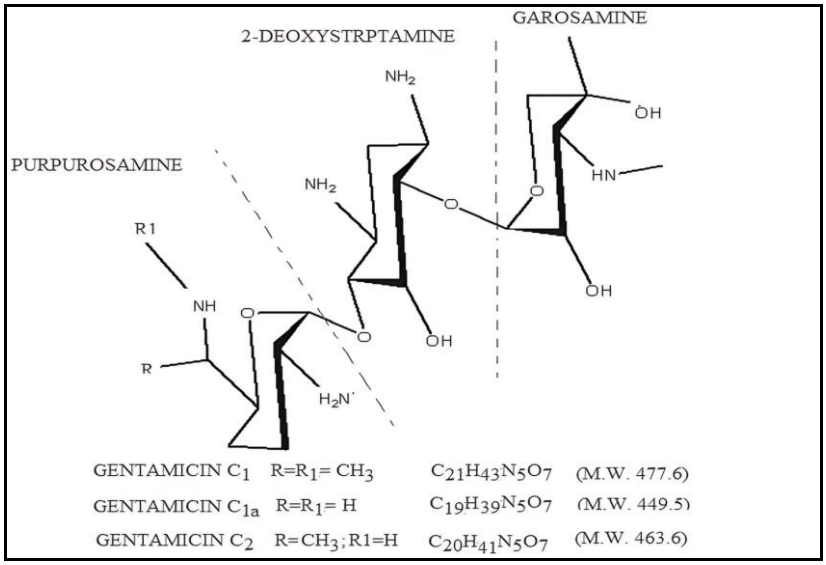

Fig. 1. Variations in the substitution pattern of purpurosamine result in major components: C1, C2, and C1a.

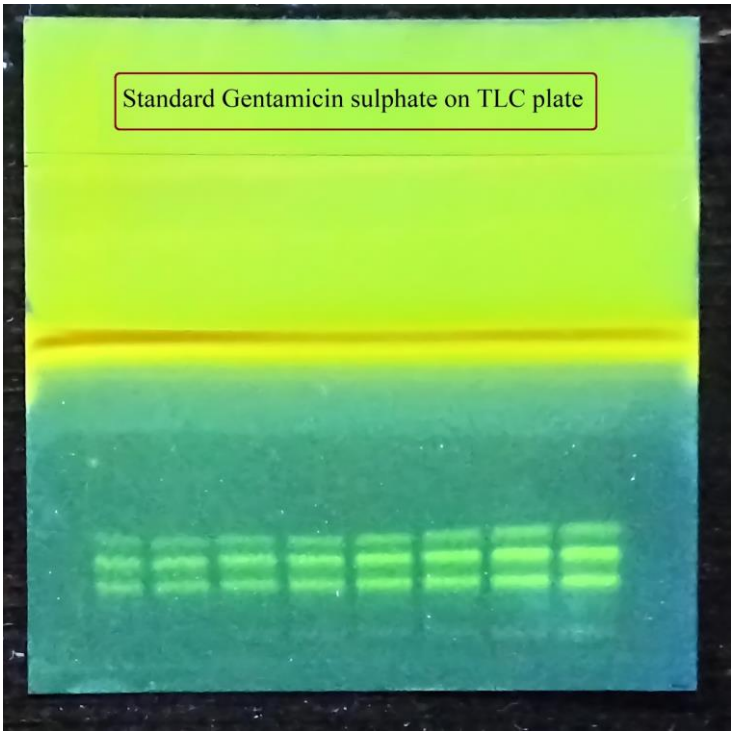

Fig. 2. After derivatization standard Gentamicin sulphate on TLC plate.

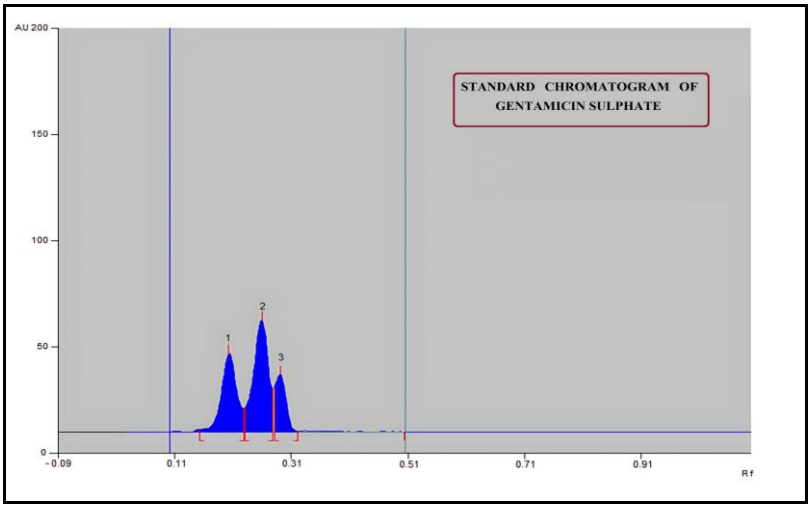

Fig. 3. Standard Chromatogram of Gentamicin sulphate.

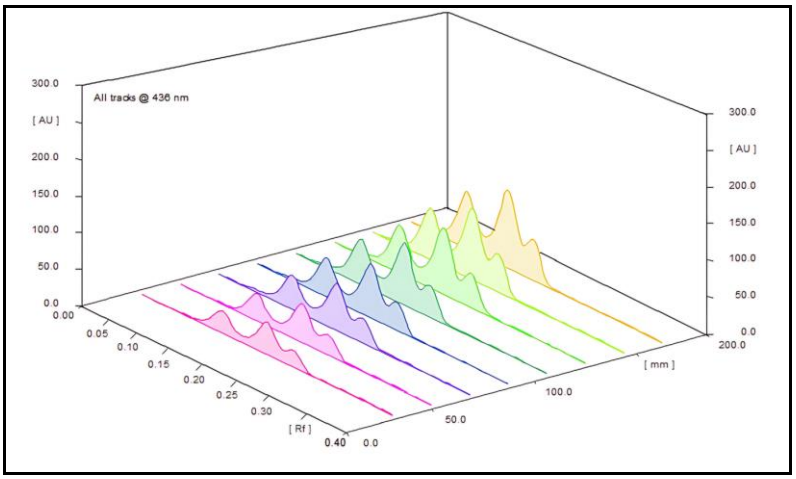

Fig. 4. HPTLC 3D Chromatogram of Gentamicin sulphate.

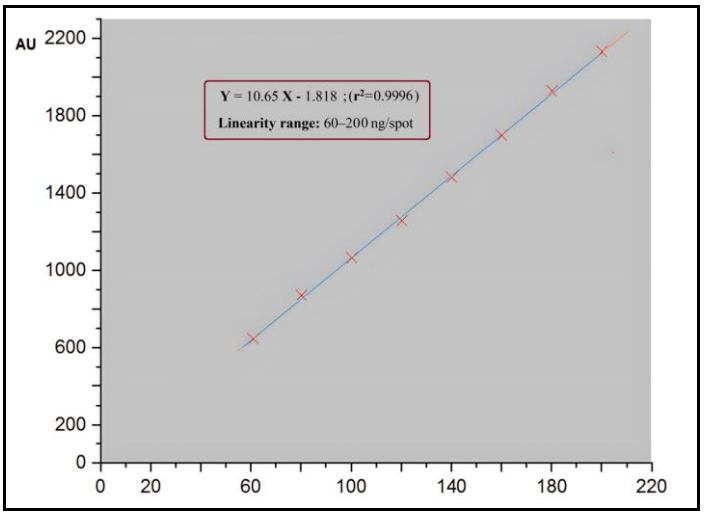

Fig. 5. Gentamicin sulphate standard calibration plot.

CONCLUSIONS

The developed HPTLC technique enables the quantitative analysis of GMS in bulk drug, marketed formulation, and plasma samples. With RSD values of 2%, the method demonstrates satisfactory accuracy. The recovery percentages for GMS (100.925–101.795%) further confirm the efficiency and reliability of the method. In addition, the fingerprint profiling of chromatograms obtained from GMS can be used for comparing and evaluating commercial samples. Compared to HPLC, HPTLC offers advantages such as shorter processing times, lower sample requirements, optimized extractions using cost-effective chemicals, and smaller mobile phase volumes. This quick, easy, and sensitive HPTLC procedure serves as a valuable quality control tool for assessing the aerial GMS in different test samples.

FUTURE SCOPE

Potential applications in quality control of raw materials, and finished formulations, exploring synergistic effects with other compounds, and investigating the correlation between chemical composition and biological activity for optimized therapeutic use.

Author contributions. Conceived and designed the analysis: Arpan Chakraborty, Arka Bhattacharjee, Nitai Chand Chaulya, Goutam Mukhopadhyay. Collected the data: Arka Bhattacharjee, Arpan Chakraborty, Baishakhi Mondal, Alpana Majumder. Contributed data or analysis tools: Arka Bhattacharjee, Arpan Chakraborty, Baishakhi Mondal, Alpana Majumder. Performed the analysis: Arpan Chakraborty, Arka Bhattacharjee, Baishakhi Mondal. Wrote the paper: Arpan Chakraborty, Arka Bhattacharjee, Baishakhi Mondal.

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Conflict of Interest. None.

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