International Journal of Pharmacy and Biological Sciences-IJPBS™ (2020) 10 (3): 355-360
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Pharmaceutical Sciences | OA Journal | MCI Approved | Index Copernicus

Validated Advanced Analytical Method for Related Substances Quantification Using Liquid Chromatographic Ultraviolet Technique in Gentamicin.

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Received: 10 Mar 2020 / Accepted: 9 Apr 2020 / Published online: 1 Jul 2020
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Abstract
Gentamicin sulfate is a potent broad-spectrum aminoglycoside antibiotic which is active against Gram positive and Gram-negative bacteria. Gentamicin sulfate is a mixture containing four major components, namely Gentamicin C_1, Gentamicin C_{1a}, Gentamicin C_2, and Gentamicin C_{2a}. Aminoglycosides (e.g., Gentamicin) are originally obtained from the fermentation of a stain of *Micromonospora purpurea* [1,2] and they contain other unknown substances like Sisomicin. Gentamicin molecule is basic in nature, water-soluble and relatively stable. All major components and related substances have weak UV absorbing chromophores [3]. Owing to the lack of UV chromophores in molecular structure, the quantification of these molecules has always been challenging.

Keywords
Gentamicin sulfate,

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INTRODUCTION
Gentamicin sulfate is a potent broad-spectrum aminoglycoside antibiotic which is active against Gram positive and Gram-negative bacteria. Gentamicin sulfate is a mixture containing four major components, namely Gentamicin C_1, Gentamicin C_{1a}, Gentamicin C_2, and Gentamicin C_{2a}. Aminoglycosides (e.g. Gentamicin) are originally obtained from the fermentation of a stain of *Micromonospora purpurea* [1,2] and they contain other unknown substances like Sisomicin. Gentamicin molecule is basic in nature, water-soluble and relatively stable. All major components and related substances have weak UV absorbing chromophores [3]. Owing to the lack of UV chromophores in molecular structure, the quantification of these molecules has always been challenging. Various liquid chromatographic detection techniques such as Refractive Index (RI), Charged Aerosol Detector (CAD) and Electrochemical Detection (ECD) were used to quantify these related substances. All these detection methods have some limitations for use [4]. RI detection slightly varies from gradient methods which are required to separate the related substances from its main components; CAD has very low sensitivity [5]. ECD detectors are very sensitive to small changes in a specified temperature [6]. Sensitivity and selectivity related problems occurs while using liquid chromatographic technique coupled with tandem mass spectroscopy (LC-MS/MS) [8,9] and fluorescence detector [10]. However, this method is inferior in terms of reproducibility, separation and robustness. To counter all these challenges faced by conventional detectors, an attempt was made to develop a derivatization technique with UV detector. Derivatization technique helps the substances to form a derivative and improve its physicochemical properties which can be used for the separation of the related substances from its original component [11]. Derivatization technique makes the substances

more UV active and improves the peak shape, elution time, peak symmetry, plate count and other indicators of chromatographic performance [12, 13]. As per the literature review, no such official liquid chromatographic techniques are available for the quantitative analysis of related substances for these aminoglycosides' antibiotic. Hence, the aim of this study was to develop a simple, precise, accurate and validated HPLC method, using UV detection to quantify the related substances present in Gentamicin.

2 MATERIALS AND METHODS

2.1. *Reagents and materials*

HPLC grade of organic solvents was used. An HPLC grade of methanol was supplied by Sigma Aldrich. Glacial acetic acid, boric acid, and potassium hydroxide of ACS grade was procured from Merck, and Sodium hexane sulfonate of ACS grade from Sigma was procured. *Ortho* phthalaldehyde (OPA) was procured from Sigma Aldrich. Milli-Q water was obtained from Milli-pore system. Gentamicin and Sisomicin were provided by the Sigma Aldrich (Bangalore).

2.2. *Instrumentation and chromatographic conditions*

Waters e2695 Alliance HPLC system was used during the analysis. The HPLC system was equipped with a column compartment with temperature control and on line degasser including UV detector. Data acquisition analysis and reporting were performed using Empower 3.0 software (Waters). The HPLC analytical column used in this method was Thermo Scientific Hypersil Gold column having the dimensions 150 x 4.6 mm and 5µm particle size. This column produced well-separated peaks and was considered as the suitable column to run for further analysis. Micropipette (Eppendroff), Analytical weighing balance (Metrohm, Model XP205 and XP26), and pH meter (Metrohm model 780) were used. The mobile phase was filtered under the vacuum through 0.45 µm membrane filters (Merck Millipore).

2.3. *Analytical conditions of the proposed method*

The proposed mobile phase comprises a ratio of Methanol: Water: Glacial acetic acid: Sodium hexane sulfonate as 70:25:5:3 v/v/v/w. Mobile phase composition was constant throughout the HPLC separation run for better separation of the peaks. This mobile phase provides a steady baseline with less noise and good separation between the related substances and gentamicin components. The column temperature varied from 25˚C to 30˚C. When the column temperature was set at 30˚C, a significant decrease in the retention time of the components was observed, which was further reduced by decreasing the flow rate from 1.0 mL/min to 0.5 mL/min and improvement in resolution. Injection volume was kept as 20 µL throughout the run to get the better appearance of the chromatogram.

2.4. Derivatization Procedure

Gentamicin and its related substance (Sisomicin) lack the presence of any active moiety in their molecular structure; hence, peaks were not detected in the absence of any derivatizing agents. Various derivatizing reagents were tried and finally *Ortho*-phthalaldehyde (OPA) was considered as suitable derivatizing agent. Preparation of derivatizing agent is given as follows.

2.4.1. *Preparation of 8N Potassium hydroxide Solution*

Readily available 8N potassium hydroxide solution from Sigma was used to adjust the pH.

2.4.2. *Preparation of 0.4M Boric acid Solution*

Accurately weighed 2.47 g of boric acid was dissolved in 100 mL of milli Q Water, and then the pH of the solution was adjusted to 9.5 with potassium hydroxide solution.

2.4.3. *Preparation of Ortho*-Phthalaldehyde *(OPA) reagent*

Accurately weighed 1.5 g of *Ortho*-Phthalaldehyde was transferred to 200 mL volumetric flask and was dissolved in previously prepared boric acid solution; to which 3 mL of thioglycolic acid and 5 mL of methanol was added. Then the pH of the solution was adjusted with potassium hydroxide solution to 9.5. The solution was stored at 2-8℃.

3. DEVELOPMENT ROUTE

3.1. *Mobile Phase Selection*

Several mobile phase combinations were tested during optimization of the method. Various combinations of mobile phases such as Methanol: Water: Acetic acid: sodium hexane sulfonate (70:25:5:5 v/v/v/w), Methanol: Water: Acetic acid: sodium octane sulfonate (70:25:5:5 v/v/v/w), Methanol: Water: Glacial acetic acid: sodium hexane sulfonate (70:25:5:4 v/v/v/w), and Methanol: Water: Glacial acetic acid: sodium hexane sulfonate (70:25:5:3 v/v/v/w) were checked to improve the baseline of chromatogram.

It was observed that the baseline of the chromatogram was good in mobile phase column with the combination, Methanol: Water: Glacial acetic acid: Sodium hexane sulfonate (70:25:5:3 v/v/v/w). It might be due to the presence of less ionic buffer in the mobile phase combination.

3.2. *Analytical column selection*

The related substance of Gentamicin was injected in various column entities such as Waters Symmetry C_{18}

(250x 4.6 mm,5µ), Phenomenex luna C_{18}(250x 4.6 mm,5µ), Agilent Zorbax C_{18}(250 x 4.6 mm,5µ), and Thermo Scientific hypersil Gold C_{18}(150 x 4.6 mm, 5 µ). Lastly, it was found that Thermo Scientific hypersil Gold column was more suitable column for the separation and elution of Gentamicin and its related substances.

3.3. *Column temperature*

The column temperature was varied between 25˚C and 30˚C. It was observed that the column temperature at 30˚C was found to be more suitable for this chromatographic condition.

3.4. *Flow rate and injection volume*

The flow rate of the mobile phase was considered as 0.5 mL/min. Preliminary chromatographic condition was started without derivatization of the sample to detect the elution of the peaks by using various combinations of mobile phase with different analytical columns. But no elution of peaks was observed.

Therefore, it was decided to derivatize the sample and inject the sample into various columns to improve the separation of the Gentamicin components and its related substance.

4. EXPERIMENTATION

4.1. *Sample Preparation*

Samples were prepared by following the procedure described below.

4.1.1. *Preparation of Solution*

4.1.1.1. *Preparation of Standard Stock Solution*

Gentamicin and Sisomicin stock solutions were prepared using 2 mg/mL and 0.5 mg/mL concentrations respectively.

4.1.1.2. *Preparation of Standard Solution*

Further, the respective stock solutions of Gentamicin and Sisomicin were diluted, and standard solution was prepared at the concentration level of 0.5 mg/mL and 0.0075 mg/mL respectively. All the dilution was made by using the diluent.

Standard solution was considered as system suitability solution.

4.1.1.3. *Preparation of Sample Solution*

Sample solutions were prepared at 0.5 mg/mL concentration level and injected separately to identify any inherent known peak.

4.1.1.4. *Preparation of Linearity Solution*

Sisomicin solution was spiked into the sample concentration of Gentamicin sample and prepared at various concentration levels to perform the linearity. Linearity solution was prepared at 0.5 mg/mL test concentration level and injected at various concentration levels of 0.0075 mg/mL to 0.0225 mg/mL for Sisomicin. For each concentration of Linearity solution preparation, the required amount of Gentamicin and Sisomicin stock solutions were transferred into 25 mL volumetric flask and then 4.0 mL of OPA reagent and 5.0 mL of methanol were added and heated at 60 °C for 15 min. The final volume was made up to the mark with diluent.

4.2. METHOD VALIDATION

The method was validated using the one described by the International Council for Harmonization (ICH). Various analytical method performance characteristic parameters viz Specificity, Precision, Linearity and Accuracy were evaluated to assess the method performance.

4.2.1. Specificity

The specificity was demonstrated to identify the analyte present in the components that may be expected. For ascertaining specificity, an injection of diluent, along with System Suitability solution and Sisomicin standard solution was done. No interfering peaks were found at the retention time of Gentamicin and its related substances. The retention time of related substance was confirmed by injecting the individual standard solution. Comparison of chromatogram was performed among the blank (diluent), System Suitability solution and Sisomicin standard solution. Specificity chromatograms indicate that there was no interference from the blank injection.

4.2.2. Precision/ Repeatability

Method repeatability was determined using the six replicates determination at 100% of the test concentration (0.0150 mg/mL). Six replicate injections of 0.0150 mg/mL solution concentration was carried out and then calculated the average Relative Standard Deviation (RSD) of experimental concentration values for related substance in Gentamicin component. Results are presented in Table 3.

4.2.3. Linearity

Linearity solutions were serially diluted from each stock solution of Gentamicin and its related substance to obtain various concentration levels covering QL to 150%. Concentration of Gentamicin and its related substance (Sisomicin) was considered as 0.0150 mg/mL. The linearity covered the concentration range from 0.0075 mg/mL to 0.0225 mg/mL, which corresponds to 150% of QL. The experimental concentration (mg/mL) of related substances was plotted against the theoretical concentration (mg/mL) for individual related substance, and Correlation coefficient was determined. Summary of results are presented in Table 3.

4.2.4. Limit of Quantification (LOQ)

LOQ was estimated by checking the signal to Noise (S/N) at the lowest concentration level. The concentration of Sisomicin at the limit of Quantification (LOQ) level was considered as 0.0075 mg/mL. Signal-to-noise (S/N) ratio at LOQ level were presented in Table 3.

4.2.5. Accuracy

The recovery experiment was conducted to evaluate the accuracy of the method. Sisomicin was spiked into the test solution of gentamicin at various concentration levels and assessed the accuracy. Recovery was calculated by using experimental concentration (mg/mL) of the related substances and theoretical concentration (mg/mL). The percentage of recovery values (accuracy) obtained for Sisomicin was presented in Table 3.

5. REPORTING TABLE

5.1. System Suitability results (SST)

System suitability solution was prepared and injected to check the correct performance of the system. The system suitability was injected six replicates and calculated the % RSD of Peak area, tailing factor and plate count.

Results and chromatogram for system suitability are presented below Table 1 and Figure 1.

Table 1: System Suitability results (SST)

Parameter	Acceptance Criteria	Results				
		C1	C1A	C2A	Sisomicin	C2
SST	Theoretical plates should be NLT 2000	2875	6770	7000	6786	7916
	Tailing Factor should be NMT 2.0	1.0	1.0	1.1	1.0	1.0
	% RSD of six replicate injection should be NMT 5.0%	0.5	0.8	0.1	0.2	0.5
	Resolution Between Sisomicin peak and adjacent peak should be NLT 1.5	1.9				

Where: NLT: Not less than
NMT: Not more than

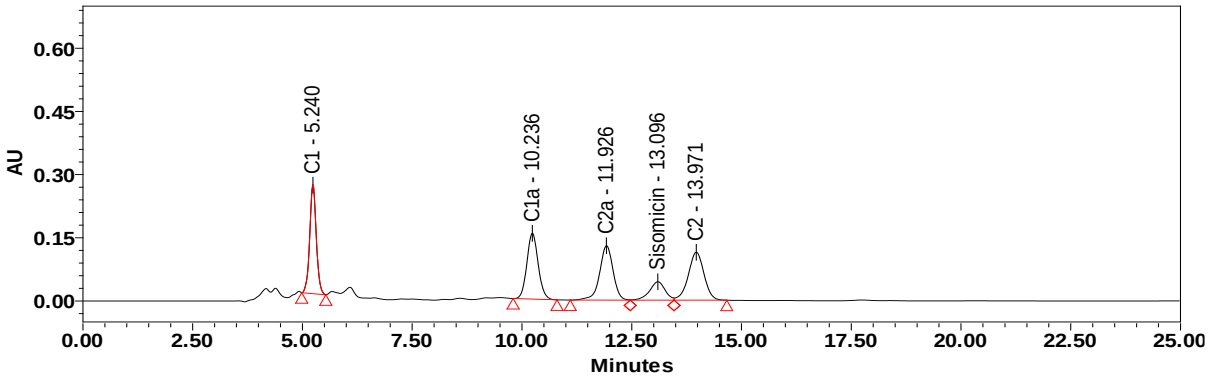

Figure 1: Zoomed chromatogram for SST injection

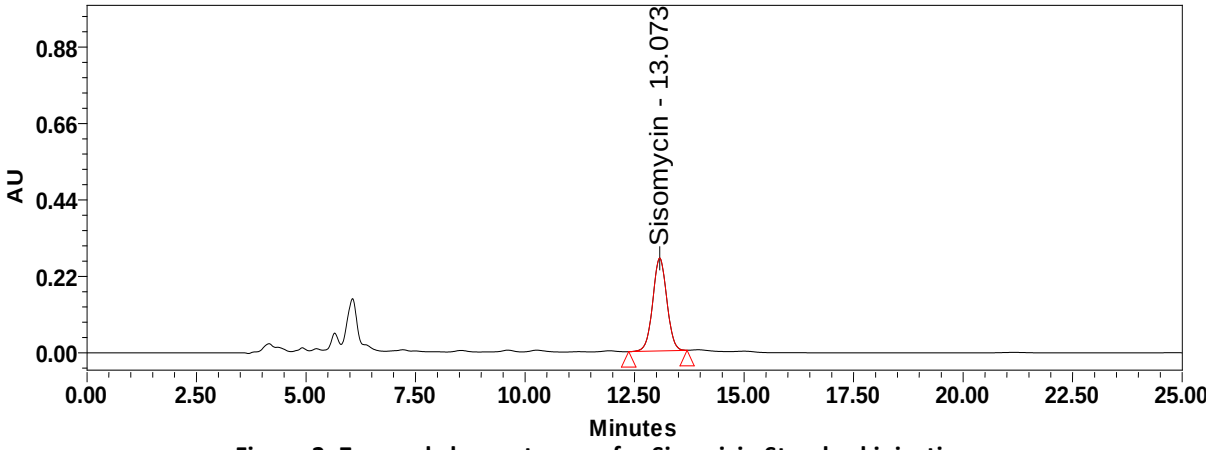

Figure 2: Zoomed chromatogram for Sisomicin Standard injection

5.2. Specificity results

Table 2: Results for Specificity parameter

Injection	Results
Blank(diluent)	No interference was observed at the RT of gentamicin and its related substances.
Acceptance criteria	No interference at the RT of gentamicin and its related substances. Even if it appears should be less than 50% of QL solution peak areas.

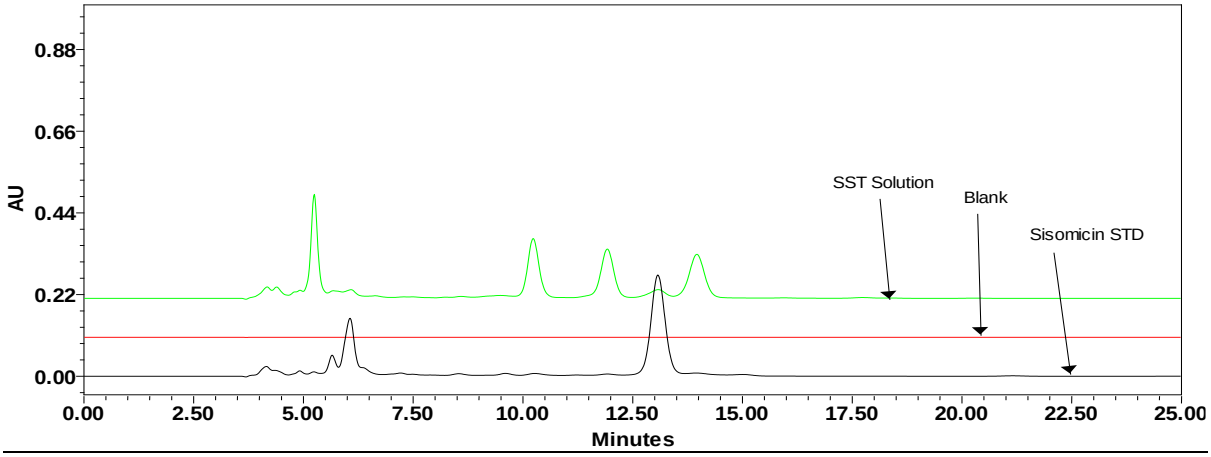

Figure 3: Zoomed Overlay chromatogram of Blank, SST Solution and Standard solution

5.3. Summary of results for Precision, Linearity, Accuracy and Limit of Quantification

Parameter	Acceptance criteria	Results
Precision	% RSD of Sisomicin for experimental concentration should not be more than 5 %	Table 3
Linearity	Plot a Linearity graph for Experimental concentration Vs. Theoretical concentration of Sisomicin. The correlation coefficient should be Not Less than (NLT) 0.99	Table 3 and Figure 4
Accuracy	% Recovery of Sisomicin from QL to 150% of specification limit should be 80-120%	Table 3
LOQ	S/N ratio should be NLT 10	Table 3

Table 3: Results for Precision, Linearity, Accuracy and LOQ solution of Sisomicin

Parameter	Results				
	TSR 50	**TSR 75**	**TSR 100**	**TSR 125**	**TSR 150**
%RSD of Experimental Concentration (Precision)	2	1	2	2	3
Accuracy	96	98	97	95	94
Signal to Noise at LOQ	20	Not Applicable			
Linearity	0.9987				

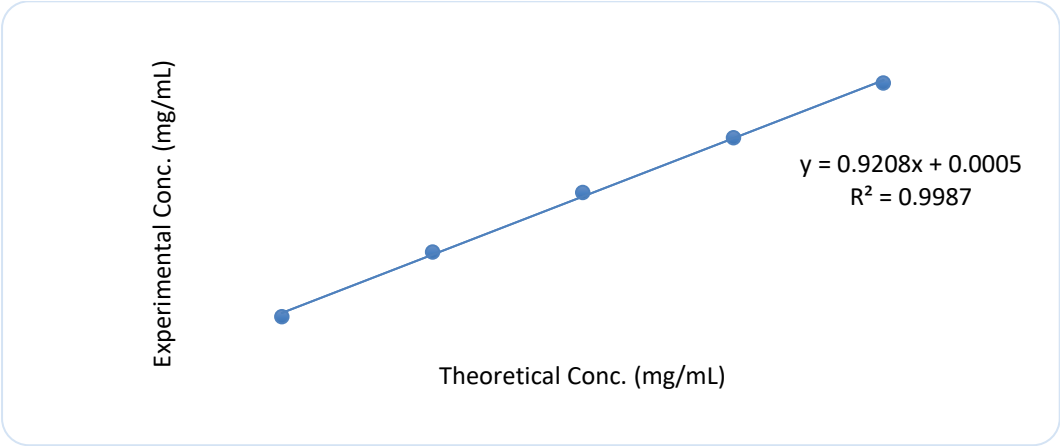

Figure 4: Linearity profile of Sisomicin

6. RESULTS AND DISCUSSION

Sisomicin is considered as the degradant product which might obtain during fermentation or storage condition. Structure of Gentamicin and its related substance are closely related to each other and do not possess UV absorbing chromophores which leads to the challenging quantification. Proposed chromatographic method can separate the analogue of Gentamicin and its related substances. The quantification was carried out using isocratic elution on an analytical column (Make: Thermo Scientific Hypersil Gold (150 X 4.6 mm, 5 µm particle size) at a flow rate of 0.5 mL/min and column temperature at 30 ˚C. The percentage recovery was found to be in between 94 % to 98 % and the % RSD of the method precision was found to be less than 5 %. The results of the proposed method were quite satisfactory and it is simple, precise and accurate.

7. CONCLUSION

The HPLC method was considered as the suitable method for the determination of related substance in Gentamicin, which can be separated and accurately estimated. The method was successfully validated in terms of good accuracy, linearity, precision/reproducibility, specificity and robustness. The proposed method successfully separated the related substances and the components of Gentamicin.

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