



Development and Validation of LC/MS Method for the Estimation of Gentamicin Sulfate and its Impurities in Injections

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ABSTRACT

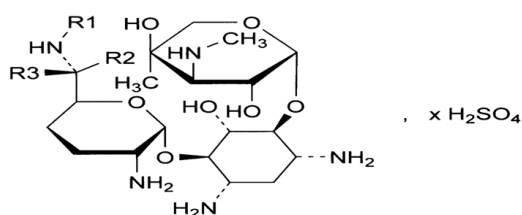
A reversed-phase liquid chromatographic method linked to the Mass Spectrometry (MS), using the pattern of electrospray ionization (ESI) source in the positive ion mode, has been developed and validated for the determination of Gentamicin sulfate and its impurities in injectable dosage form. Chromatographic separation of Gentamicin sulfate components and impurities was achieved on a reverse phase C18 column and 50mM trifluoroacetic acid (TFA), pH 2 adjusted with ammonium solution, as mobile phase. The mobile phase was pumped at a flow rate of 0.25 mL /min. Nitrogen was used as a drying gas at a flow rate of 5 L/min. The developed method provides a complete base line for the separation of the components C1, C1a, C2, C2a and C2b of Gentamicin sulfate. The method was successfully validated in accordance to ICH guidelines acceptance criteria for (specificity, limit of detection). The method was proven to be appropriate for the determination of both the active compounds of the Gentamicin sulfate, and its related impurities in injectable dosage form.

Keywords: Gentamicin Sulfate, Liquid Chromatography with Mass Spectrometer LC/MS, validation, injection.

INTRODUCTION

Gentamicin is an aminoglycoside antibiotic (AG) produced by the fermentation of *Micromonospora purpurea*. It is widely used for treatment of serious infections caused by both Gram-negative and Gram-positive bacteria as it has a broad-spectrum antibacterial activity. It is a mixture consisting of five major components, designated C1, C1a, C2, C2a and C2b, Fig.1.^{1,2}

Due to the multicomponent nature of gentamicin; it is a routine analysis requirement in pharmaceutical industry to measure the relative percentage of its major constituents.^{3,4}



Gentamicin	Mol. Formula	R1	R2	R3
C1	C ₂₁ H ₄₃ N ₅ O ₇	CH ₃	CH ₃	H
C1a	C ₁₉ H ₃₉ N ₅ O ₇	H	H	H
C2	C ₂₀ H ₄₁ N ₅ O ₇	H	CH ₃	H
C2a	C ₂₀ H ₄₁ N ₅ O ₇	H	H	CH ₃
C2b	C ₂₀ H ₄₁ N ₅ O ₇	CH ₃	H	H

Figure 1: Chemical structure of Gentamicin⁵

Gentamicins and its related substances are basic non-volatile, water soluble, very low solubility in most organic solvents, highly polar compounds, relatively stable,

structurally closely related compounds without UV absorbing chromophores.^{6,7}

These physicochemical properties of gentamicin and its related compounds create two major challenges to develop a RP-HPLC method that can be routinely used in quality control laboratories. The first challenge is to obtain high sensitivity that is necessary to detect and estimate low levels of impurities and degradation products of active pharmaceutical ingredient (API) in the drug product. The second challenge is to retain and separate all the gentamicin related compounds under RP-HPLC conditions.⁷

Several HPLC methods have been developed for the analysis of gentamicins using derivatization of amino groups with *o*-phthalaldehyde; 2, 4, 6 trinitrobenzen sulfonic acid; and 1-fluoro-2, 4-dinitrobenzene. The derivatization improves the chromatographic behavior of gentamicins, making them less polar and introducing a chromophore in the molecule to enable fluorescence or UV detection.⁸

But the UV detectors are not sensitive enough to detect low levels of related compounds of gentamicin⁷, and the derivatization step is a time-consuming process and needs well-controlled experimental conditions to produce repeatable results.⁴ Therefore, the developing of the RP-HPLC method which uses direct UV detection to estimate low levels of related compounds of gentamicin is not feasible.⁷

In addition to spectrophotometric, some other detections were used for AG. Aminoglycoside antibiotics were detected in culture broth with refractive index detection.

Electrochemical and evaporative light scattering detections were used in the analysis of AG and gentamicin components with ion pairing chromatography.¹

Numerous analytical methods have been used to quantify aminoglycosides such as TLC, LC with spectroscopic and fluorescence detections, electrochemical detection, evaporative light scattering detection, and also capillary electrophoresis (CE).³

For detection of gentamicins also mass spectrometry can be used. Perfluorinated carboxylic acids (PA) were used as pairing ions to improve the separation of gentamicins in HPLC analysis. The behavior of trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) as pairing ions for the chromatography of AG antibiotics was studied showing that higher concentration of AP increases the retention and the resolution of gentamicin components on non-polar column.⁸

Mass Spectrometry seems to be the technique of choice for aminoglycosides' detection (including gentamicin) because of its high sensitivity and positive identification. By using Mass Spectrometry no derivation steps will be required.³

The aim of this study is to develop a sensitive and specific reverse phase HPLC method with MS detector for the estimation of gentamicin sulfate and its impurities in injectable bulk samples.

The method was validated in accordance to International Conference of Harmonization (ICH) guidelines acceptance criteria and the United States Pharmacopoeia (USP) for category (2): Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products.^{9,10}

MATERIALS AND METHODS

Instrumentation

The analysis of drug was carried out on a SHIMADZU Lc / Ms – 2020 system equipped with a reverse phase C18 column (250x4.6mm, 5µm in particle size), a 20µl injection loop and ESI-MASS spectrometer detector.

Reagents and solutions

The reference sample of gentamicin sulfate was supplied by National Drug Quality Control & Research Labs, Ministry of Health (Damascus – Syria).

The reference sample of sisomicin sulfate was supplied by Sigma-Aldrich Chemical Co. (Hamburg, Germany).

The reference sample of G-418 sulfate was supplied by Sigma-Aldrich Chemical Co. (Hamburg, Germany).

HPLC grade water and TFA: trifluoroacetic acid and acetonitrile were purchased from Merck (India) Ltd., Mumbai.

Methods were tested on three commercially available injections containing 80 mg of gentamicin sulphate in 2 mL.

Chromatographic Conditions

Chromatographic separation of Gentamicin sulfate components and impurities was achieved on a reverse phase C18 column in a mobile phase of 50mM TFA, Ph = 2 adjusted by using ammonium solution as mobile phase. The mobile phase was pumped at a flow rate of 0.25 mL /min. The detector was Mass Spectrometry (MS) with an electrospray ionization (ESI) source in the positive ionmode.

Stock solution

Accurately weighed 0.9mg of sisomicin was transferred to an amber vial of 1.5 ml, and it was solved in water. Standard stock solution of sisomicin (600 µg/ml) was prepared. Working solutions were prepared daily by appropriate dilution of this stock solution.

Accurately weighed 0.9mg of G-418 was transferred to an amber vial of 1.5 ml, and it was solved in water. Standard stock solution of G-418 (600 µg/ml) was prepared. Working solutions were prepared daily by appropriate dilution of this stock solution.

Accurately weighed 7.5mg of Gentamicin sulfate was transferred to an amber vial of 1.5 ml, and it was solved in water. Standard stock solution of Gentamicin sulfate (5000 µg/ml) was prepared. Working solutions were prepared daily by appropriate dilution of this stock solution.

Preparation of sample solutions

Sample solutions of injections containing 80 mg/2mL of gentamicin sulphate, were prepared by diluting the sample with water to a working concentration of 100 µg /mL of total gentamicin.

Preparation of mobile phase

0.95 ml of the TFA was transferred to a 250 ml volumetric flask, and volume was made up to the mark by water. The solution was filtered through 0.45µm filter under vacuum.

Optimization of the chromatographic Conditions

Proper selection of the stationary phase depends up on the nature of the sample molecular, weight, and solubility. The drug Gentamicin is polar. Between C8 and C18, C18 column was selected. The elution of the compounds from the column was achieved by a very polar mobile phase. Therefore, TFA was selected and the effect of the concentration and the composition of mobile phase on the retention time of Gentamicin was thoroughly investigated. The chromatographic conditions were optimized through several trials to achieve good resolution and peak shapes. It was found that a mobile phase of TFA 50mM (pH = 2) without acetonitrile was appropriate. Using Nitrogen as a drying gas at a flow rate of 5 L/min was necessary.



Procedure

A mobile phase of TFA was found to be suitable for ideal separation of Gentamicin compounds and impurities.

The mobile phase was filtered through a 0.45 μ membrane filter before use. It was pumped through the column at a flow rate of 0.25 ml/min. The column was equilibrated by pumping the mobile phase through the column for at least 30 min prior to the injection of the drug and impurities solutions. The run time was set at 90 min. A chromatogram showing the separation of the gentamicin components is given in Fig. 2.

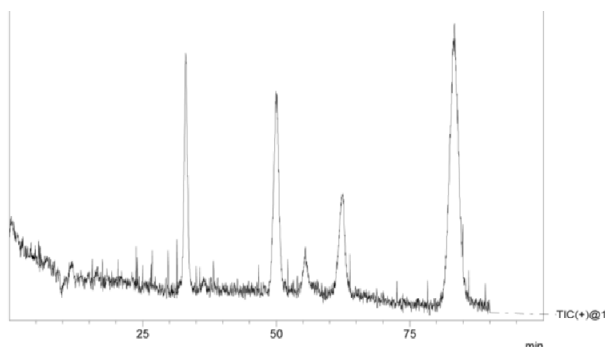


Figure 2: Chromatogram of Gentamicin

A chromatogram showing the separation of the gentamicin's impurities is given in Fig. 3.

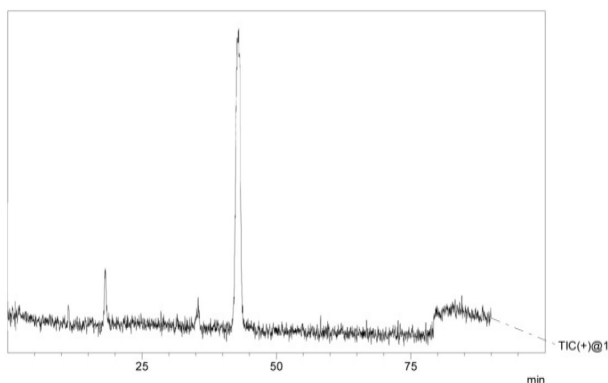


Figure 3: Chromatogram of Gentamicin's impurities
(Mixture of sisomicin and G-418 at 5 μ g/ml for each one)

Validation Procedure

The objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines. The method was validated for specificity and sensitivity (LOD: limit of detection).

Specificity of the method was found out through non-interference between the peaks of active compounds of Gentamicin and impurities in the analyzed samples and also in the mixture of the two standard impurities.

Sensitivity of the method was expressed through LOD: the limit of detection which was calculated from the peak information (signal to noise ratio of 3).

RESULTS AND DISCUSSION

In the proposed method, the retention times of gentamicin active compounds were found to be:

- 33.2min for gentamicin C1a (m/z = 450)
- 50 min for gentamicin C2 (m/z =464)
- 55.49 min for gentamicin C2b (m/z =464)
- 62.4 min for gentamicin C2a (m/z =464)
- 83.3 min for gentamicin C1 (m/z =478)

And the retention times of gentamicin's impurities were found to be:

- 35.4 min for G-418 (m/z =497)
- 43 min for sisomicin (m/z =448)

The limit of detection was found to be:

- 1.5 μ g/ml for the impurity: sisomicin
- 5 μ g/ml for the impurity : G-418

That indicates the sensitivity of the method.

The specificity of the method was guaranteed by MS transitions of the analyzed compounds. No interfering peaks were found in the chromatograms of the standards and injections solutions within the run time that indicates that excipients used in injectable formulations did not interfere with the estimation of the gentamicin active components and impurities by the proposed HPLC method.

The proposed HPLC/MS method is sensitive and specific for the detection of gentamicin active compounds, and impurities and can be reliably adopted for routine quality control analysis of gentamicin and its impurities in injectable dosage forms.

CONCLUSION

This article describes an HPLC/MS method for impurities identification in gentamicin. ESI was successfully applied for the ionization of polar aminoglycosides and no ionization suppression was observed at high (50mM) TFA concentration.

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