Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 521–531

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of a RP-HPLC method for the determination of gentamicin sulfate and its related substances in a pharmaceutical cream using a short pentafluorophenyl column and a Charged Aerosol Detector

Arul Joseph*, Abu Rustum

Global Quality Services-Analytical Sciences, Schering-Plough, 1011 Morris Avenue, Union, NJ 07083, United States

ARTICLE INFO

Article history:
Received 17 June 2009
Received in revised form 26 August 2009
Accepted 2 September 2009
Available online 12 September 2009

Keywords:
Gentamicin sulfate
Charged Aerosol Detector
HPLC-CAD
High performance liquid chromatography

ABSTRACT

Gentamicin sulfate is a potent broad spectrum aminoglycoside antibiotic which is used as an active pharmaceutical ingredient (API) against both Gram-positive and Gram-negative bacteria. A reversed-phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated to determine the composition of gentamicin sulfate and to estimate its related substances (without any pre- or post-column derivatization) in a pharmaceutical cream. As gentamicin has a weak UV chromophore, it is not possible to detect low levels of known and unknown related substances of gentamicin using a UV detector. In this method, a Charged Aerosol Detector (CAD) was used to obtain high sensitivity that was necessary for the intended purpose of the method. This method can separate all the analogues of gentamicin including all known and unknown related substances of the API. A short (5 cm × 4.6 mm) pentafluorophenyl HPLC column from Restek (Allure PFP) was used with an ion-pair gradient mobile phase consisting of (A) heptafluorobutyric acid:water:acetonitrile (0.025:95:5, v/v/v) and (B) trifluoroacetic acid:water:acetonitrile (1:95:5, v/v/v).

1. Introduction

Gentamicin sulfate is an aminoglycoside based broad spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. Drug products containing gentamicin sulfate are used to combat various infections, for instance, the ophthalmic solution is commonly used to treat eye infections. Gentamicin was originally isolated from the microorganism *Micromonospora purpurea* in the research laboratories of Schering-Plough Corporation [1,2]. Gentamicin API is derived via fermentation and is a mixture that contains four major compounds: gentamicin C1, gentamicin C1a, gentamicin C2, gentamicin C2a, and a minor compound gentamicin C2b. Gentamicin and its related substances are highly polar compounds with high water solubility, very low solubility in most organic solvents and all of them have a weak UV chromophore. 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 API in the drug product. The second challenge is to retain and separate all the gentamicin related compounds under RP-HPLC conditions.

The absence of a strong UV chromophore in gentamicin and its related compounds makes conventional UV detectors not sensitive enough to detect low levels of related compounds of gentamicin. Therefore, developing a RP-HPLC method using direct UV detection to estimate low levels of related compounds of gentamicin is not feasible. Other detection techniques such as Refractive Index (RI) Detection, Evaporative Light Scattering Detection (ELSD), pre-column derivatization followed by UV Detection, and Electrochemical Detection (ECD) have major challenges and limitations for use in the routine analysis of gentamicin. RI detection is incompatible with gradient methods which are necessary to separate the related substances of gentamicin. The sensitivity of ELSD is relatively low; the precision and linear dynamic range is also narrow and is unsuitable for the intended purpose of this method. Pre-column derivatization procedures are typically cumbersome, difficult to reproduce and are not suitable for quantitative methods to estimate low levels of compounds in a sample. ECD is less rugged as the electrodes are frequently poisoned and require time-consuming cleaning. In addition, ECD response is highly sensitive to small changes in temperature, pump pulsations, and any extraneous electrical current. Because of all the challenges of the conventional HPLC detectors for gentamicin, a Charged Aerosol Detector (CAD) was used as the detector for this new method.

* Corresponding author. Tel.: +1 908 820 6694; fax: +1 909 820 6359.
E-mail address: arul.joseph@spcorp.com (A. Joseph).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.09.002

CAD is a relatively new universal detection technology, which is sensitive and has a broad dynamic range [3,4]. The principle of operation of the CAD involves nebulization of the eluent from an HPLC system followed by evaporation of the mobile phase, the resulting stream of non-volatile particles is ionized and the charged particles are detected using an electrometer. There are several implications of this principle of operation. All non-volatile eluents from the HPLC system will be detected by the CAD. Thus, even minor non-volatile impurities originating from the mobile phase, samples, or HPLC system will be visible as peaks in a chromatogram. In addition, CAD due to its principle of operation has a quadratic response versus concentration, and has a linear response over only a short concentration range and at lower analyte concentrations. An important implication of the nebulization of the eluent in the operation of the CAD is that the response of the CAD is dependent on the organic content (concentration of organic modifier) of the eluent from the HPLC. As a result the response of the CAD varies as a function of the change in organic modifier concentration in gradient methods.

The second major challenge is to retain gentamicin (C1, C1a, C2, C2a, and C2b) and its related substances (i.e., deoxystreptamine, garamine, and sisomicin) on typical RP-HPLC columns and obtain the selectivity that is necessary to get baseline separation of all analytes of interest. Also, the insolubility of gentamicin and most of its related compounds in many organic solvents limits the use of high proportions of organic solvents in aqueous mobile phases and sample diluents.

HPLC methods for the analysis of gentamicin using both direct and indirect methods have been reported in the literature [5–27]. The indirect methods involve either pre- or post-column derivatization with o-phthalaldehyde or dansyl chloride (5-dimethylamino-1-naphthalene sulfonyl chloride) with UV or fluorescence detection [5–16]. Direct detection methods using Refractive Index (RI) [17], Mass Spectrometry (MS) [18–22], Evaporative Light Scattering Detection (ELSD) [23,24], and Electrochemical Detection (ECD) [25–27] have been reported. A recent report compares ECD and ELSD detection methods using polymer and C-18 columns [28]. The methods reported in the literature lack both selectivity and sensitivity to separate and quantitate gentamicin and all of its related compounds.

This manuscript describes the development and validation of a sensitive, selective, and robust HPLC method to determine the composition of gentamicin and to estimate its related substances in a pharmaceutical cream using a short pentafluorphenyl stationary phase and Charged Aerosol Detector (CAD). To the best of our knowledge, this is the first report of an analytical method that can quantitate all the individual components (composition) of gentamicin sulfate and can also estimate its related substances in a drug product without derivatization using charged aerosol detection and a pentafluorophenyl column.

2. Experimental

2.1. Materials

All the organic solvents used were of HPLC grade. Acetonitrile and dichloromethane were purchased from Sigma (St. Louis, MO, USA). Heptafluorobutyric acid (HFBA) ≥99% and trifluoroacetic acid (TFA) ≥99.5% were purchased from Alfa Aesar (Ward Hill, MA, USA). Water (18.2 MΩ cm) was obtained using a Milli-Q system (Millipore, Billerica, MA 01821, USA). Gentamicin sulfate was provided by the Global Quality Services-Analytical Sciences group in Schering-Plough (Union, NJ, USA). Sisomicin sulfate, USP reference standard was purchased from Fisher Scientific (USA). Celestoderm-V cream with gentamicin samples and placebo were provided by Schering-Plough Labo (Heist-Op-Den-Berg, Belgium). Deoxystreptamine sulfate and garamine sulfate were obtained through a contract research organization, Syncom BV (Groningen, the Netherlands).

2.2. Instrumentation and chromatographic conditions

Waters 2695 Alliance HPLC system (Milford, MA, USA) was used for method development. The HPLC system was equipped with a column compartment with temperature control and an on-line degasser including a CAD detector (ESA Biosciences, Chelmsford, MA, USA). Data acquisition, analysis, and reporting were performed using Millennium^{32} and or Empower chromatography software (Milford, MA, USA). Waters HPLC systems in different laboratories of Schering-Plough Corporation were used for method validation. The HPLC column used in this method (Restek Allure PFP Propyl, 50 mm × 4.6 mm I.D., 3 μm particle size) is manufactured by Restek Corporation (State College, PA, USA) and distributed by Fisher Scientific.

2.3. Chromatographic conditions of the final method

The solvents and proportions used in mobile phase A was heptafluorobutyric acid:water:acetonitrile (0.025:95:5, v/v/v) and that used in mobile phase B was trifluoroacetic acid:water:acetonitrile (1:95:5, v/v/v). The gradient program is listed in Table 1. As discussed above the response of the CAD varies as a function of the change in organic modifier concentration in gradient methods. This method minimizes the impact of this response change by not using a traditional gradient method which changes the organic modifier concentration but by using isocratic condition for the main segment which elutes the API and most of its related substances and by using an ion-pair gradient over a smaller concentration range for two related substances. In detail, this method employs an isocratic mobile phase of trifluoroacetic acid:water:acetonitrile (1:95:5, v/v/v) from retention time 16–33 min for eluting the gentamicins (C1, C1a, C2, C2a, and C2b) and most of the related substances of gentamicin. For eluting deoxystreptamine and garamine in the initial 16 min, it uses an ion-pair gradient varying the proportion of the ion-pairing agents heptafluorobutyric acid (HFBA) and trifluoroacetic acid (TFA) over a very small range from 0.025 to 1 (v/v) compared to the much larger water acetonitrile proportion of 95:5 (v/v) which remains a constant through the entire gradient. Thus, the resulting ion-pair gradient involves relatively minor changes in the total organic content of the mobile phase and thereby minimizes the impact of change in the nebulization efficiency (and response) of the CAD due to change in organic content.

In addition, the hydrophilic nature of gentamicins necessitates the use of highly aqueous mobile phases (containing 95% water). Using highly aqueous mobile phase (>80% water) on typical RP-HPLC column results in a decrease in retention of analytes due to dewetting (loss of mobile phase from stationary phase pores) [31]. To maintain the retention and performance of the reversed phase column despite use with highly aqueous mobile phase, the column was washed with mobile phase C, water:acetonitrile (20:80, v/v), for 10 min. To ensure re-equilibration after each gradient elution, the column was re-equilibrated at the gradient initial condition for 4 min. The column temperature was maintained at 35 °C. The Charged Aerosol Detector was set at 100 pA gain and a medium noise filter. The sample injection volume was 20 μL.

2.4. Sample preparation

Samples were prepared by following the procedure described below. 6.0 ± 0.05 g of cream sample was weighed into a suitable centrifuge tube. 6.0 mL of water (diluent) was added to the cream using a pipette and mixed well by vortexing for 2 min. 5.0 mL of

Table 1
Gradient program of the method.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Gradient curve	Comment
0.00	100.0	0.0	0.0	Linear	First isocratic ion-pair reagent (HFBA) elution
8.00	100.0	0.0	0.0	Linear	
12.00	80.0	20.0	0.0	Linear	
16.00	0.0	100.0	0.0	Linear	Second isocratic ion-pair reagent (TFA) elution
33.00	0.0	100.0	0.0	Linear	
Column wash and equilibrate to initial conditions					
33.50	0.0	0.0	100.0	Linear	Column wash
43.50	0.0	0.0	100.0	Linear	
44.00	100.0	0.0	0.0	Linear	Column equilibration
48.00	100.0	0.0	0.0	Linear	

Note:
(1) Use of highly aqueous mobile phases (95% water) on a reversed phase column can cause loss of mobile phase from the stationary phase pores and/or stationary phase collapse resulting in a reduction in retention of the analytes. A column wash step with water:acetonitrile (20:80, v/v) is used to replenish the mobile phase in the stationary phase pores and decrease the reduction in retention.
(2) The gradient time from 33 min to 48 min is for column wash and equilibration only, and, thus, no data acquisition is necessary.

dichloromethane was added using a pipette and mixed well by vortexing for 2 min to dissolve the cream completely. The solution was centrifuged to separate the aqueous and organic phases (for example, 3200 rpm for 5 min). A 5 mL aliquot of the aqueous top layer was carefully transferred using a pipette into a 10-mL volumetric flask and diluted to volume with water. The diluted solution was transferred into an HPLC vial for HPLC determination of composition of the components in gentamicin sulfate (C1, C1a, C2, C2a, and C2b) and for the estimation of gentamicin sulfate related substances. The analytical concentration of gentamicin sulfate in sample solution was approximately 0.5 mg/mL, which corresponds to an analytical concentration of gentamicin sulfate C1a of approximately 0.135 mg/mL.

The concentration is expressed in terms of gentamicin sulfate C1a because individual gentamicin sulfate C1, C1a, C2, C2a, and C2b standards were unavailable, an alternative approach to validation using the gentamicin sulfate reference standard, a mixture of C1, C1a, C2, C2a, and C2, was pursued. The certificate of analysis of the gentamicin sulfate reference standard lists gentamicin sulfate C1 as 26%, C1a as 27% and gentamicin sulfate C2 and C2a as a mixture of 47%. The certificate of analysis uses a UV derivatization method to determine the composition of gentamicin sulfate to be 100%, and does not include C2b as a separate peak. As the percent composition of gentamicin C1a (27%) listed in the certificate of analysis is similar to that observed with the current CAD based analytical method, the gentamicin sulfate reference standard was used as a 27% pure standard of gentamicin sulfate C1a. Expressing the gentamicin sulfate concentration in terms of C1a enables use of the reference standard without introducing any confusion as to the percent composition values of the peaks (C1, C2, C2a and C2b) based on the differing detection techniques employed in the current method and that of the certificate of analysis. In addition, for determining the signal to noise (S/N) values used for limit of detection (LOD) and limit of quantitation (LOQ) a single peak needs to be used. Gentamicin sulfate is a mixture of five compounds (C1, C1a, C2, C2a, and C2b) in varying proportions depending on the batch of the standard. This complicates the choice of peak to be used for S/N determination and relating the S/N of that peak with the total concentration of gentamicin sulfate. Expressing the concentration in gentamicin sulfate C1a allows a comparison of the S/N from the gentamicin sulfate C1a peak with its concentration.

Gentamicin sulfate standard solutions were prepared by dissolving the reference standard in water to have an analytical concentration of gentamicin sulfate of approximately 0.5 mg/mL (gentamicin sulfate C1a of approximately 0.135 mg/mL). Standard mixture solutions containing approximately 1% each of deoxystreptamine, garamine, sisomicin, in gentamicin were prepared by dissolving deoxystreptamine sulfate, garamine sulfate, and sisomicin sulfate in gentamicin sulfate standard solution (approximately 0.135 mg/mL of gentamicin sulfate C1a).

The CAD is a quadratic response detector and has a linear response over only a short concentration range and at low analyte concentrations. For the intended purpose of this method, using a linear fit using a single 100% level gentamicin sulfate standard (0.5 mg/mL) was preferred to using a quadratic fit using multiple concentration level standards and calibration curves. Also, analysts in QC analytical labs routinely use a single 100% level standard based analytical methods and are familiar with such methods. To use the CAD for quantitation of components using a linear fit over the wide concentration range of 0.2% (LOQ) to 150% of 0.135 mg/mL requires much broader method acceptance criteria than that required for a typical HPLC method (using a linear response UV detector). Thus, this method has a broader acceptance criteria compared to typical UV detector based HPLC methods.

To determine the linearity of gentamicin sulfate C1a, triplicate preparations of gentamicin sulfate C1a in celestoderm-V with gentamicin cream placebo at each of six levels of sample concentration were prepared and tested. The six levels of sample concentration were 0.2%, 50%, 75%, 100%, 125%, and 150% of the gentamicin sulfate analytical concentration (0.5 mg/mL). The linearity of gentamicin sulfate related compound, sisomicin sulfate, in celestoderm-V cream with gentamicin placebo at each of five levels of sample concentration were prepared and tested. The five levels of sample concentration for sisomicin sulfate were 0.2%, 0.4%, 1.25%, 2.5%, and 5.0% compared to the gentamicin sulfate C1a analytical concentration of 0.135 mg/mL. The limit of detection (LOD) and limit of quantitation (LOQ) of the tested compounds, gentamicin sulfate and sisomicin sulfate, are 0.1% and 0.2% respectively of the gentamicin sulfate C1a analytical concentration of 0.135 mg/mL.

2.5. Calculation

The composition of gentamicin sulfate C1, gentamicin sulfate C1a, gentamicin sulfate C2, gentamicin sulfate C2a, and gentamicin sulfate C2b is calculated as a percent area by area normalization. The percent area of each individual gentamicin sulfate and total percent area of gentamicin sulfate can be calculated using the equations shown below.

$$\text{Percent area of gentamicin sulfate compound i} = \frac{\text{Peak area of gentamicin sulfate compound i}}{\text{Total peak area of gentamicin sulfate}} \times 100$$

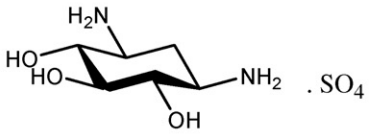
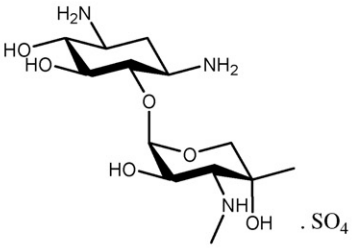
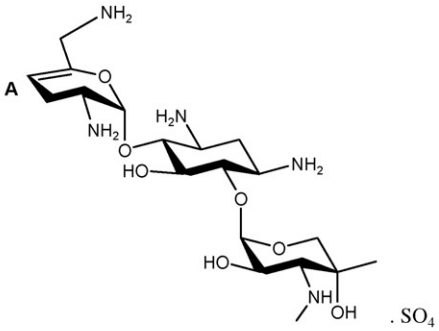
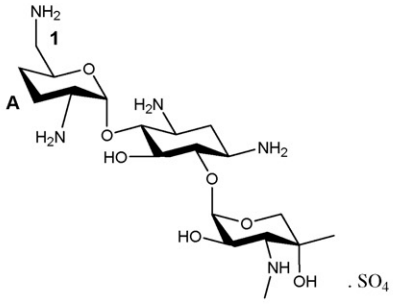
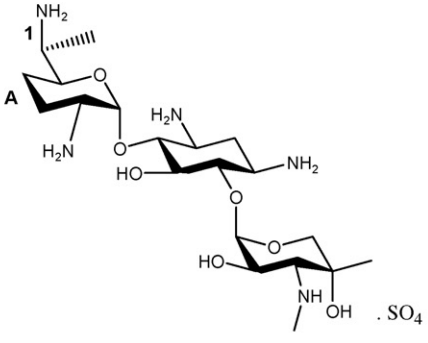
Compound Name	Structure	Identity	Properties
Deoxystreptamine Sulfate		Impurity/ Degradation product	UV inactive Water soluble
Garamine Sulfate		Impurity/ Degradation product	UV inactive Water soluble
Sisomicin Sulfate		Impurity/ Degradation product	UV inactive Water soluble
Gentamicin Sulfate C1a		API	UV inactive Water soluble
Gentamicin Sulfate C2		API	UV inactive Water soluble

Fig. 1. Compound name, chemical structure, identity, and physical properties of gentamicin sulfate, their degradation products/impurities, and unknowns in celestoderm-V cream with gentamicin.

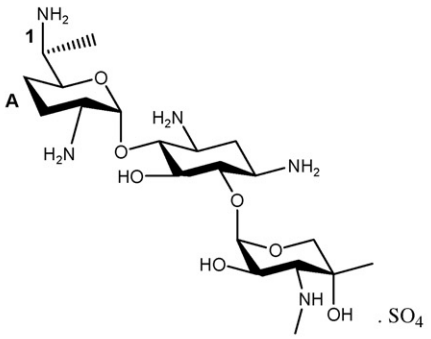
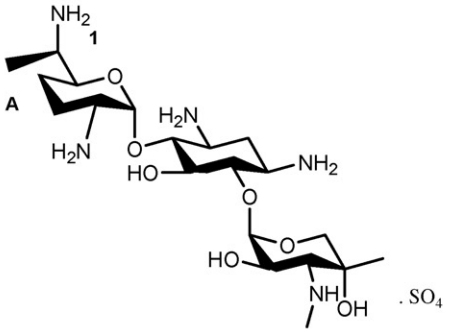
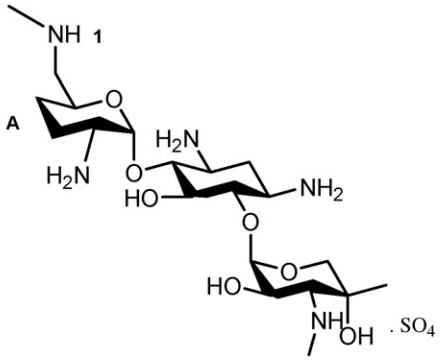
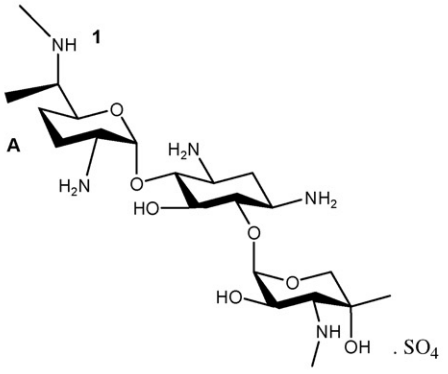
Gentamicin Sulfate C2		API	UV inactive Water soluble
Gentamicin Sulfate C2a		API	UV inactive Water soluble
Gentamicin Sulfate C2b		API	UV inactive Water soluble
Gentamicin Sulfate C1		API	UV inactive Water soluble

Fig. 1. (*Continued*).

Total percent area of gentamicin sulfate i from sample$_{n-A}$

$$= \sum P_i$$

For recovery, the sample solutions were bracketed between two gentamicin sulfate reference standard solutions and the experimental concentration was determined using the following equation: Experimental Concentration = (P2 × C1)/(P1 × RRF).where P1 = average peak area of gentamicin sulfate C1a in adjacent bracketing standards; P2 = peak area of gentamicin sulfate or related substance in recovery sample solution; RRF: Relative Response Factor is the ratio between the

response factor of each individual gentamicin sulfate compound i or related substance and the response factor of gentamicin sulfate C1a. The RRF was obtained by dividing the slope of the linear regression curve of gentamicin sulfate C1a by the slope of the linear regression curve of the gentamicin sulfate compound i or individual related substance. The recovery of each concentration level was then determined by the following equation:

$$\text{Percent recovery} = \left(\frac{\text{experimental concentration}}{\text{theoretical concentration}}\right) \times 100$$

3. Results and discussion

3.1. HPLC method development

The main objective was to develop a sensitive and rugged RP-HPLC method that can separate gentamicin C1, C1a, C2, C2a, and C2b from each other and from the known (deoxystreptamine, garamine, and sisomicin) and unknown related substances of gentamicin. Despite a protracted search of commercial sources, the related substance gentamicin B1 was not available and hence was not included in the development and validation of the method. Gentamicin C1, C1a, C2, C2a and C2b differ from each other only in the degree of alkyl substitution or the position of a methyl group on or adjacent to the amine (labeled 1) on ring A of the structure (Fig. 1). Ion-pairing agents can form an ion-pair with the amine functional groups of gentamicin sulfate compounds and enable retention of these compounds on RP-HPLC columns, which is otherwise unfeasible due to their physicochemical properties. In addition, ion-pairing agents could possibly provide the required selectivity for the resolution of these compounds on RP-HPLC columns. As non-volatile additives are incompatible with the Charged Aerosol Detector, it was necessary to explore volatile ion-pairing agents (e.g., carboxylic acid and perfluorocarboxylic acids) during method development activities. The use of alkanesulfonates [29] and perfluorocarboxylic acids [30] as ion-pairing agents to separate aminiglycoside antibiotics has been described in the literature. The alkanesulfonate method [29] describes the separation of streptomycin, dihydrostreptomycin and their related substances, and not the gentamicins. In addition, due to the relative lower volatility, alkanesulfonates cannot be used as effective mobile phase additives for charged aerosol detection of gentamicins. The perfluorocarboxylic acid method [30] describes the separation of a mixture of aminoglycoside antibiotics on a reversed phase C18 column. However, this method does not separate gentamicin C2, C2a and C2b, which elute as a single peak (gentamicin C2), and sisomicin and gentamicin C1a were also not baseline separated.

Method development activities were initiated using a C18 column with mobile phases containing ion-pairing agents. The composition of the mobile phase was modified to investigate different ion-pairing agents e.g., trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA). In addition, various acids (e.g., methane sulfonic acid, formic acid, acetic acid, etc.) and organic modifiers (e.g., methanol, isopropanol, THF, etc.) in combination with water at different proportions were also investigated. Gentamicin C1, C1a, C2, C2a and C2b were successfully separated from each other using a mobile phase of 50 mM TFA in water. The retention and resolution of the gentamicin compounds were found to progressively improve with increasing concentrations of TFA and concentrations from 1% to 2% were found to best retain and resolve the five gentamicin compounds. TFA concentration of 1% was selected as being optimal for routine analysis. As expected, even low concentrations of the higher fluorocarboxylic acid homologs, PFPA (0.2%) and HFBA (0.05%), increased the retention of gentamicins considerably. Thus, two limitations of using PFPA and HFBA as ion-pairing agents is the very long

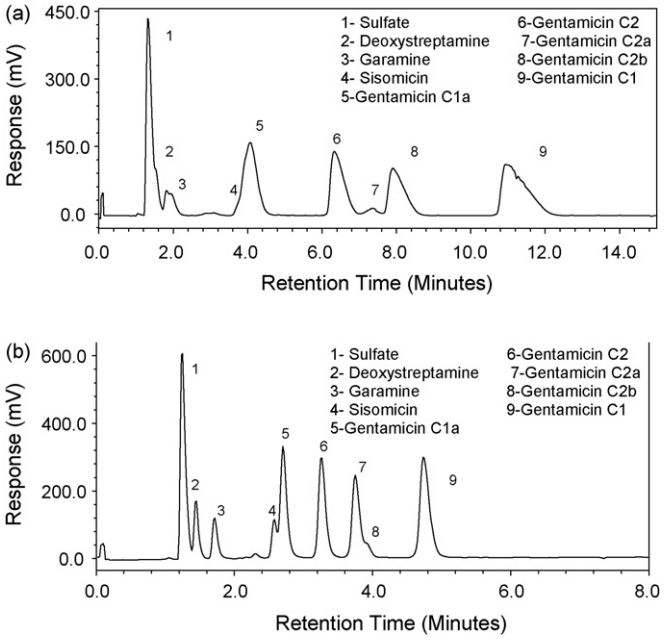


Fig. 2. (a) Representative chromatogram of a standard mixture containing gentamicin sulfate, deoxystreptamine sulfate, garamine sulfate, and sisomicin sulfate on an Ace-Aq C-18 column using a mobile phase of 0.6% TFA in water. (b) Representative chromatogram of a standard mixture containing gentamicin sulfate, deoxystreptamine sulfate, garamine sulfate, and sisomicin sulfate on an Ace phenyl column using a mobile phase of 1% TFA in water.

run times needed to elute all gentamicin compounds and the poor chromatographic behavior of the late eluting compounds. The separation of gentamicin on C18 columns using perfluorocarboxylic acids has been reported in the literature [27,30]. However, these methods do not adequately resolve sisomicin from gentamicin C1a due to the inherent structural similarity between gentamicin C1a and sisomicin. These two compounds differ only in the presence of a double bond in ring A of sisomicin, which is absent in gentamicin C1a (Fig. 1) and therefore is the most challenging pair to obtain a baseline resolution. A representative chromatogram of a standard mixture containing gentamicin, deoxystreptamine, garamine, and sisomicin on an Ace-AQ C-18 column is shown in Fig. 2a. The chromatogram presented in Fig. 2a clearly demonstrates the inability of C18 stationary phase to separate all the components that are present in a gentamicin product.

Theoretically, sisomicin should exert much stronger pi–pi interaction with a phenyl stationary phase compared to a C18 stationary phase as it contains the additional pi-bond in the molecule. Therefore, a phenyl stationary phase should provide better discrimination (i.e., higher alpha value) between gentamicin C1a and sisomicin. However, the phenyl stationary phase would provide less retention and separation of all other analogues of gentamicin. Hence the challenge was to find a condition to achieve the separation of the sisomicin and gentamicin C1a peak without sacrificing the retention and separation of all other analogues of gentamicin sulfate. A number of stationary phases were selected for column screening on the basis of their known physicochemical characteristics. The objective of this study was to identify column(s) that would provide the best selectivity for all the peaks of interest for the intended purpose of this method. The list of columns that were screened during method development is presented in Table 2. Experiments conducted using phenyl stationary phases confirmed the poor retention and separation of other gentamicin sulfate peaks as shown in Fig. 2b.

Besides pi–pi and hydrophobic interaction, an additional interaction with the stationary phase via different retention mechanism

Table 2
C-18, phenyl, cyano, HILIC, and cyclodextrin columns screened during method development.

Column	Dimensions	Manufacturer/vendor	Comments
Ace-Aq C18	50 mm × 4.6 mm, 3 μM	MacMod	Good separation between gentamicin C1,C1a, C2, C2a and C2b
Ace 3 C18 (300A°)	50 mm × 4.6 mm, 3 μM	MacMod	Poor separation of C2 and C2b, elution order of C2a and C2b reversed
Hydrobond Aq	50 mm × 4.6 mm, 3 μM	MacMod	Poor retention and separation of gentamicin C1,C1a, C2, C2a and C2b
Hydrobond PS	50 mm × 4.6 mm, 3 μM	MacMod	Good separation between gentamicin C1,C1a, C2, C2a and C2b
YMC Pack Pro C18	50 mm × 4.6 mm, 3 μM	YMC	Good separation between gentamicin C1,C1a, C2, C2a and C2b
YMC ODS Aq	50 mm × 4.6 mm, 3 μM	YMC	Good separation between gentamicin C1,C1a, C2, C2a and C2b
YMC ODS Aq (300A°)	50 mm × 4.6 mm, 3 μM	YMC	Poor separation of C2 and C2b
YMC hydrosphere C18	50 mm × 4.6 mm, 3 μM	YMC	Poor separation of C2 and C2b
TSK gel Super ODS	50 mm × 4.6 mm, 3 μM	Tosoh Biosciences	Moderate baseline separation of C2, C2a and C2b
X-Bridge Shield RP 18	75 mm × 4.6 mm, 2.5 μM	Waters	Poor separation of C2a and C2b
Develosil C30	50 mm × 4.6 mm, 3 μM	Phenomenex	Good separation between gentamicin C1,C1a, C2, C2a and C2b
XTerra RP	50 mm × 4.6 mm, 3 μM	Waters	Compounds are not retained
Atlantis HILIC silica	50 mm × 4.6 mm, 3 μM	Waters	Poor chromatographic behavior
Cyclobond I 2000 DMP	150 mm × 4.6 mm, 3 μM	MacMod	Poor retention and separation of gentamicins
Ace 3 CN	50 mm × 4.6 mm, 3 μM	MacMod	Poor retention and separation of gentamicins
Ace 3 Phenyl	50 mm × 4.6 mm, 3 μM	MacMod	Mediocre retention and separation of gentamicins

is needed to obtain adequate separation of gentamicin C1a from sisomicin and at the same time, maintain the separation between the analogues (C1, C1a, C2, C2a, and C2b) and related substances (deoxystreptamine and garamine) of gentamicin. To achieve this goal, a pentafluorophenyl stationary phase was selected because it can provide a dipole–dipole interaction between the analyte and the stationary phase. The dipole–dipole interaction comes from the C–F bonds of pentafluorophenyl stationary phase with the C–F bonds of the ion-paired gentamicin and its related compounds. Therefore, the C–F based stationary phase can be used advantageously to obtain better retention and separation between the molecules whose structures are closely related to each other. Pentafluorophenyl stationary phase also provides much stronger pi–pi interaction than the classical phenyl stationary phase and therefore would help to distinguish sisomicin from gentamicin C1a. Hence, pentafluorophenyl stationary phase was selected for the method described in this report. Using this column, successful separation was achieved between gentamicin sulfate C1a and sisomicin sulfate without sacrificing the retention and separation of all other gentamicin related compounds. Screening of all the commercially available pentafluorophenyl columns was conducted to identify the best column for this method. The result of column screening for pentafluorophentyl stationary phase is presented in Table 3. All of the pentafluorophenyl columns provide good retention and separation of the gentamicins. However, differences in the columns due to the nature of the silica and the chain length of the alkyl group (e.g., propyl) tethering the pentafluorophenyl group to the silica lead to slight differences in their retention behavior. The Restek Allure PFP column was found to provide excellent separation of the gentamicin peaks. Based on the results of the column screening studies, the Restek Allure PFP column (50 mm × 4.6 mm, 3 μm particles) was selected as the primary column for this method and the Thermo Fluophase column (50 mm × 4.6 mm, 5 μm particles) was selected as an alternate column to the primary column.

The excipient peaks from gentamicin drug products elute as broad peaks near the void volume. It is preferable to increase the retention time of the first compound to approximately 4 min or longer to avoid potential overlap with the excipient peaks near the void volume. The first compound, deoxystreptamine, is eluted close to the excipient peaks (at about 2 min) on all columns including pentafluorophenyl columns under isocratic conditions. Modifying the concentration of TFA and acetonitrile did not provide the desired improvement in retention of deoxystreptamine. As discussed earlier, HFBA and PFPA can be used to retain deoxystreptamine to 4 min or longer, but the later eluting gentamicins C2a, C2b and C1 are retained for undesirably long periods on the column and exhibit poor peak shape. To achieve the goal of retaining deoxystreptamine and eluting all gentamicin peaks within a reasonable time period, a shallow ion-pair gradient was employed. The ion-pair gradient uses 0.025% HFBA from time-zero to 8 min to retain deoxystreptamine. Then 1.0% TFA is gradually introduced to elute the other gentamicin peaks (Table 1). The HFBA concentration (0.025%) used in the ion-pair gradient was selected to elute the deoxystreptamine at about 5 min and prevents its overlap with excipient peaks near the void volume. The TFA concentration of 1% was ideal to resolve the other gentamicin compounds within a reasonable run time.

Data obtained during method development showed a decrease of retention time for gentamicin and its related compounds with each successive gradient cycle including sample injections. The decrease of retention time with each successive gradient run was due to the use of a highly aqueous mobile phase (95% water) on a RP-HPLC column which causes the loss of ability by the stationary phase to maintain hydrophobic interaction with the analytes. To mitigate and reduce the negative impact of the highly aqueous mobile phase, a column wash step using a wash solvent with high organic content (80% acetonitrile) was introduced at the end of each gradient cycle. This wash step replenishes the

Table 3
Pentafluorophenyl (PFP) columns screened during method development.

Column	Dimensions	Manufacturer/vendor	Comments
Allure PFP	50 mm × 4.6 mm, 3 μM	Restek	Excellent separation between gentamicin C1,C1a, C2, C2a and C2b and between C1a and sisomicin
Fluophase PFP	50 mm × 4.6 mm, 5 μM	Thermo	Good separation between gentamicin C1,C1a, C2, C2a and C2b and between C1a and sisomicin, column can be used as alternate column
Pursuit PFP	50 mm × 4.6 mm, 3 μM	Varian	Moderate separation between C2a and C2b
Hypersil Gold PFP	50 mm × 4.6 mm, 3 μM	Thermo	Good separation of all compounds
Curosil PFP	50 mm × 4.6 mm, 3 μM	Waters	Good separation of all compounds but strong retention leading to longer run times
Discovery HS F5	50 mm × 4.6 mm, 3 μM	Supelcosil	Good separation of all compounds
Fluorosep RP phenyl	50 mm × 4.6 mm, 3 μM	ES Industries	Good separation of all compounds

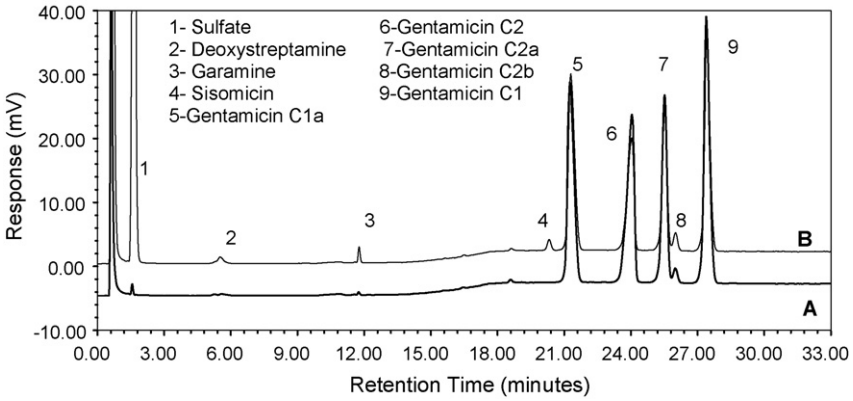


Fig. 3. Representative chromatograms of (A) gentamicin sulfate reference standard and (B) standard mixture containing gentamicin sulfate, deoxystreptamine sulfate, garamine sulfate, and sisomicin sulfate.

silica particle pores with acetonitrile and enables the highly aqueous mobile phase to access the pores of the silica particle and thereby maintain the interactions between the analytes and stationary phase. No reduction of retention time was observed after the addition of this extra step at the end of each gradient run. An experimental study of this phenomenon of dewetting and the use of an organic mobile phase (such as 80% acetonitrile) wash step to maintain the retention time is described by Nagae et al. [31]. Representative chromatograms of gentamicin sulfate reference standard and a standard mixture containing gentamicin sulfate, deoxystreptamine sulfate, garamine sulfate, and sisomicin sulfate are shown in Fig. 3. The chromatograms were obtained using 0.025% HFBA in water:acetonitrile (95:5) as mobile phase A and 1.0% TFA in water:acetonitrile (95:5) as mobile phase B. The chromatographic run time was 33 min and is followed by a column wash step with water:acetonitrile (80:20). The method described in this report along with the sample preparation procedure described earlier was applied to a pharmaceutical cream (celestoderm-V cream) containing gentamicin sulfate. Representative chromatograms of diluent, placebo, and celestoderm-V cream sample are shown in Fig. 4. As can be seen from the placebo chromatogram in Fig. 4 there is no interference from the celestoderm-V cream matrix. Celestoderm-V cream matrix contains an additional API, betamethasone 17-valerate, and the following excipients chlorocresol, sodium dihydrogen phosphate, paraffinum liquidum, cetostearyl alcohol, cetomacrogol 1000, white petrolatum, sodium hydroxide, phosphoric acid, and water. The sample preparation procedure involves dissolving the cream in water and extraction with dichloromethane which removes betamethasone 17-valerate and all the hydrophobic excipients leaving behind only sodium hydroxide, phosphoric acid, and sodium dihydrogen phosphate in the aqueous phase. All three excipients in the aqueous phase are eluted at the void volume and do not interfere with gentamicin and its related substance.

3.1.1. Analytical method validation

Two analysts from different laboratories performed the method validation of celestoderm-V cream with gentamicin using testing parameters such as response linearity, precision, specificity, robustness, sample stability, limit of quantitation (LOQ) and limit of detection (LOD).

Due to the limited availability of the gentamicin sulfate related compounds listed in Fig. 1, validation was conducted using reference standards of gentamicin sulfate (a mixture of C1, C1a, C2, C2a, and C2b) and sisomicin sulfate. The percent composition of gentamicin sulfate analogues listed in the certificate of analysis of the reference standard is C1 (26%), C1a (27%), and C2 and C2a as a mixture (47%). As the percent composition of gentamicin sulfate C1a (27%) listed in the Certificate of Analysis was similar to that observed with the current analytical method, the gentamicin sulfate reference standard was used as a 27% pure standard of gentamicin sulfate C1a. The rationale for expressing the concentration in terms of gentamicin sulfate C1a has been explained above.

3.1.2. Method specificity

The method specificity was demonstrated by a peak identification test and by testing three representative celestoderm-V cream with gentamicin samples from different manufacturing dates. The

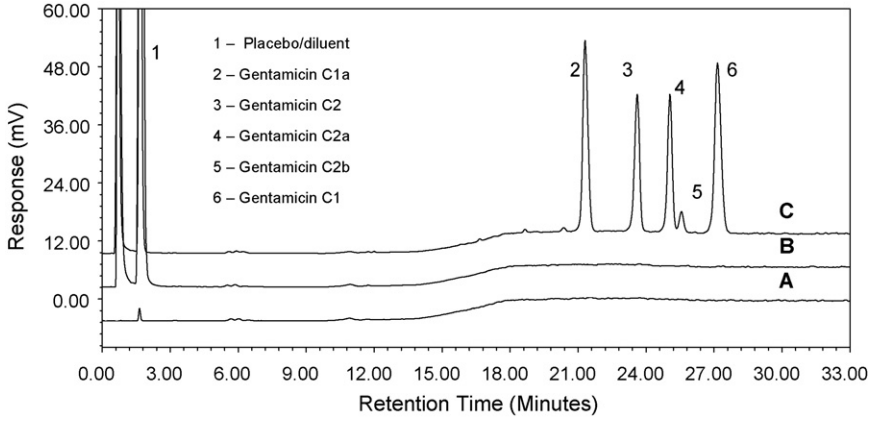


Fig. 4. Representative chromatograms of (A) diluent (B) celestoderm-V cream placebo, and (C) celestoderm-V cream sample.

Table 4
The percent recovery of gentamicin sulfate C1a from triplicate preparations.

Percent label strength	Preparation	Percent recovery of gentamicin sulfate C1a[a]	
		Analyst 1	Analyst 2
50%	1	106.4	105.7
	2	106.7	105.4
	3	107.9	104.1
75%	1	102.0	101.8
	2	102.3	101.8
	3	101.3	102.9
100%	1	99.4	98.4
	2	99.4	96.8
	3	99.4	99.7
125%	1	98.8	95.9
	2	98.0	96.9
	3	97.2	95.4
150%	1	95.7	95.7
	2	96.0	95.9
	3	94.6	92.2
Average % recovery		101.3	99.2

[a] % recovery = experimental concentration/theoretical concentration × 100%.

identification test was performed to show that the method is capable of resolving gentamicin sulfate and key related substances from each other in a specificity test mixture using the method to determine the resolution and relative retention of each gentamicin sulfate related compound. To demonstrate method specificity, ICH guidelines require stress studies for new drug candidates in order to predict real-life degradation chemistry of the drug substances and formulated products under specific stability storage conditions. Celestoderm-V cream with gentamicin has been commercially available for many years and its degradation chemistry is well characterized. Drug development processes related to celestoderm-V cream with gentamicin, such as drug formulation design, selection of storage conditions and packaging are well-established. Based on all the aforementioned facts, therefore, stress studies under conditions such as heat, photo-stability, acid, base, and oxidation were not performed. Instead, a specificity test mixture was analyzed using the method to determine the resolution factor and the relative retention of each gentamicin sulfate related substance. The specificity test mixture consisted of an expired celestoderm-V with gentamicin cream sample spiked with approximately 1% each of deoxystreptamine sulfate, garamine sulfate, and sisomicin sulfate. The relative retention of gentamicin sulfate C1, C1a, C2, C2a, C2b, and related substances were compared with the retention times and relative retention listed in the analytical method. Also, three representative celestoderm-V cream with gentamicin samples from different manufacturing dates (expired and unexpired) were tested by Analyst 1 for method specificity in the method validation. These stability samples provided a true reflection of the degradation chemistry under the real-life scenario. Gentamicin sulfate was adequately separated from its related substances demonstrating that the method prescribed in this report is a stability indicating method.

3.1.3. Linearity

The analytical concentration of gentamicin sulfate C1a was approximately 0.135 mg/mL (0.5 mg/mL of gentamicin sulfate). For gentamicin sulfate C1a the linearity range investigated covered the concentration range from 0.001 mg/mL to 0.2025 mg/mL, which corresponds to QL (0.2%) to 150% of gentamicin sulfate label claim, in the presence of placebo. For sisomicin sulfate the linearity range investigated covered the concentration range from 0.001 mg/mL to 0.025 mg/mL, which corresponds to LOQ (0.2%) to 5% of gentamicin sulfate label claim, in the presence of placebo and 0.5 mg/mL of gentamicin sulfate. Although the typical concentration of the related compounds was not greater than 0.5%, we tested a wider range to cover any future potential increase in concentrations of the related compounds in celestoderm-V cream with gentamicin samples. The slope, y-intercept, and coefficient of determination (r^2) were obtained from linear regression analysis performed using the software SAS System JMP® version 4. The peak areas of each individual compound were plotted against corresponding concentrations. Linear regression analysis yielded a coefficient of determination r^2 of greater than 0.99 ($n = 18$) for gentamicin sulfate and greater than 0.98 ($n = 15$) for the related substance sisomicin sulfate. Representative linear regression curves of gentamicin sulfate C1a and sisomicin sulfate from this linearity study are shown in Fig. 5.

3.1.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection and limit of quantitation of gentamicin sulfate C1a was 0.37% (0.00185 mg/mL gentamicin sulfate/0.0005 mg/mL gentamicin sulfate C1a) and 0.74% (0.0037 mg/mL gentamicin sulfate/0.001 mg/mL gentamicin sulfate C1a) respectively of the gentamicin sulfate analytical concentration of 0.5 mg/mL. The limit of detection and limit of quantitation of sisomicin sulfate was 0.1% (0.0005 mg/mL sisomicin sulfate) and 0.2% (0.001 mg/mL sisomicin sulfate) respectively of the gentamicin sulfate analytical concentration of 0.5 mg/mL. Signal to noise (S/N) values ranging from 11 to 142 were obtained for the LOQ and values ranging from 5 to 65 were obtained for

Table 5
Precision–repeatability and precision–intermediate of gentamicin sulfate C1a and sisomicin sulfate.

Analyst	% RSD gentamicin sulfate C1a	Difference in % RSD gentamicin sulfate C1a (Analyst 1 and Analyst 2)		
		50%	100%	150%
Analyst 1: 50%	0.7			
Analyst 1: 100%	0.0			
Analyst 1: 150%	0.8			
Analyst 2: 50%	0.8	0.1	1.5	1.4
Analyst 2: 100%	1.5			
Analyst 2: 150%	2.2			
Analyst	% RSD sisomicin sulfate	Difference in % RSD sisomicin sulfate (Analyst 1 and Analyst 2)		
		0.2%	1.25%	5%
Analyst 1: 0.2%	5.7			
Analyst 1: 1.25%	0.5			
Analyst 1: 5%	0.9			
Analyst 2: 0.2%	1.5	4.2	2.3	2.4
Analyst 2: 1.25%	2.8			
Analyst 2: 5%	3.3			

Table 6
The relative retentions (RRs) of gentamicin and related substances under selected HPLC parameter robustness conditions.

Compound name	Method condition	Column temp. = 30 °C	Column temp. = 40 °C	Flow rate = 0.9 mL/min	Flow rate = 1.1 mL/min	Injection volume = 18 μL	Injection volume = 22 μL
Deoxystreptamine sulfate	0.33	0.29	0.30	0.33	0.30	0.33	0.32
Garamine sulfate	0.57	0.56	0.58	0.56	0.58	0.57	0.57
Sisomicin sulfate	0.96	0.96	0.96	0.95	0.96	0.96	0.96
Gentamicin sulfate C1a	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Gentamicin sulfate C2	1.11	1.10	1.11	1.11	1.10	1.11	1.11
Gentamicin sulfate C2a	1.17	1.17	1.17	1.18	1.17	1.17	1.17
Gentamicin sulfate C2b	1.20	1.20	1.20	1.20	1.19	1.20	1.20
Gentamicin sulfate C1	1.29	1.29	1.27	1.30	1.27	1.29	1.28

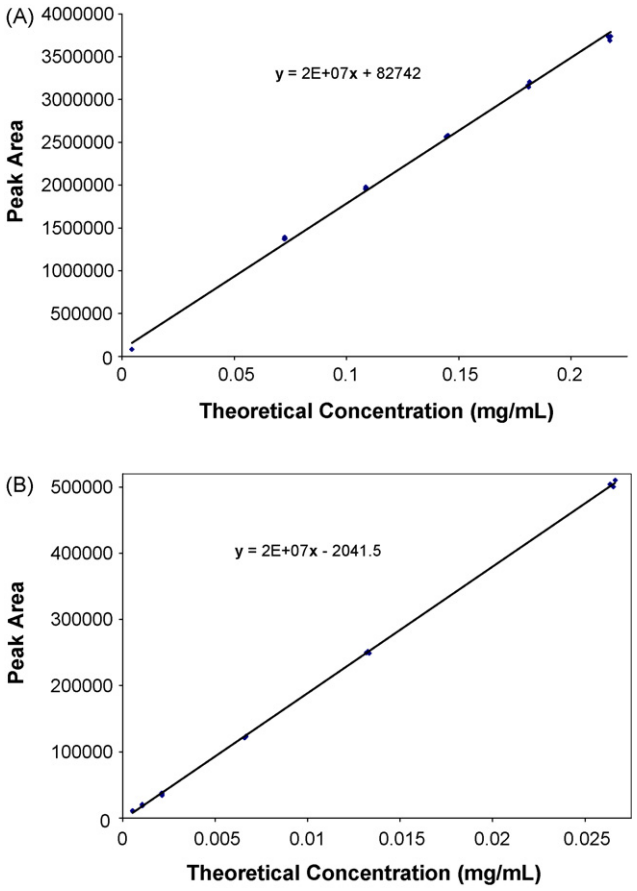


Fig. 5. Representative linear regression curves of (A) gentamicin sulfate C1a and (B) sisomicin sulfate from linearity studies.

LOD were observed by both analysts during validation depending on the HPLC-CAD system used for the analysis. The lowest values of 5 and 11 were observed in a CAD system with low sensitivity which was near its due date for annual performance maintenance cleaning and calibration.

3.1.5. Accuracy

The recovery and accuracy of the method was determined using the solutions used for the linearity study. This method determines the percentage composition by area normalization and is not a quantitative assay method. The percentage recovery values obtained for gentamicin sulfate C1a are listed in Table 4. The average % recovery from 15 preparations (three for each concentration level (50%, 75%, 100%, 125% and 150%)) was 101.3% for Analyst 1 and 99.2% for Analyst 2. The accuracy values also reflect the quadratic response of the CAD, i.e., a higher response at lower concentrations and lower response at higher concentrations. Thus, a higher average accuracy value of 107.0% for Analyst 1 and 105.1% for Analyst 2 is observed for the 50% concentration level and a lower average accuracy value of 95.4% for Analyst 1 and 94.6% for Analyst 2 is observed for the 150% concentration level. The percentage recovery of the related substance sisomicin sulfate for the 0.2–5% concentration level ranged from 109% to 72%.

3.1.6. Method reproducibility and precision

The method reproducibility and intermediate precision were determined using the data obtained from the linearity study. The method reproducibility was determined from the percentage relative standard deviation (%RSD) of the recoveries obtained from samples prepared as triplicates at the low (50%), middle (100%),

and high (150%) gentamicin sulfate C1a concentration levels. For sisomicin sulfate, the method reproducibility was determined from the %RSD of the recoveries obtained from samples prepared as triplicates at the low (0.2%), middle (1.25%), and high (5%) concentration levels of the corresponding related compounds. The intermediate precision was determined from the difference in the average recoveries and the difference in the %RSD of recoveries between the two analysts. The results for gentamicin sulfate C1a and sisomicin sulfate are listed in Table 5, which reveals that this method has good reproducibility and intermediate precision.

3.1.7. Method robustness

Deliberate variations in HPLC parameters were made to demonstrate the robustness of the method. We evaluated the method robustness based on the changes in relative retentions (RRs), the resolution between gentamicin sulfate C1a and sisomicin sulfate, and the tailing factor of gentamicin sulfate C1a under the tested conditions. Gentamicin sulfate C1a peak was used for RR calculation. The RRs of all the peaks were calculated against the gentamicin sulfate C1a peak, and the resolution between sisomicin sulfate and gentamicin sulfate C1a was found to be $\geq$1.5 and the tailing factor was found to be $\leq$2.0 under the various chromatographic conditions tested. The RRs of the tested compounds obtained under a few representative HPLC conditions are summarized in Table 6. It can be seen that the RRs obtained under various chromatographic conditions remain fairly close.

4. Conclusion

The HPLC method described in this report is the first known method which can separate and accurately estimate all the individual analogues of gentamicin including all the related compounds by direct detection (i.e., no derivatization) using a Charged Aerosol Detector (CAD). This method was successfully validated by two analysts from two different laboratories and has been demonstrated to have good accuracy, linearity, precision, reproducibility, specificity, and robustness. The analytical method described in this paper has been successfully used to determine the composition of gentamicin sulfate and also to estimate its related substances in celestoderm-V cream. This method is also a stability indicating method for celestoderm-V cream because it can separate all the known and unknown degradation products of gentamicin from the API peaks and from each other and can accurately quantitate the content of gentamicin in the samples of celestoderm-V cream. Thus, this method can be used in quality control labs for stability studies and also for routine analysis of gentamicin in commercial lots of celestoderm-V cream including release testing.

Acknowledgements

The authors would like to thank all the analytical scientists in Schering-Plough Global Quality Services-Analytical Sciences group for their support of this study. The authors would also thank the analysts from Schering-Plough Quality laboratories in Heist, Belgium for their participation in the method validation.

References

[1] M.J. Weinstein, G.M. Luedemann, E.M. Oden, G.H. Wagman, Antimicrob. Agents Chemother. 1 (1963) 1–7.
[2] M.J. Weinstein, G.M. Luedemann, E.M. Oden, G.H. Wagman, J.P. Rosselet, J.A. Marquez, C.T. Coniglio, W. Charney, H.L. Herzog, J. Black, J. Med. Chem. 6 (1963) 463–464.
[3] R.W. Dixon, D.S. Peterson, Anal. Chem. 74 (2002) 2930–2937.
[4] P.H. Gamache, R.S. McCarthy, S.M. Freeto, D.J. Asa, M.J. Woodcock, K. Laws, R.O. Cole, LC–GC Eur. 18 (2005) 345–354.
[5] J.P. Anhalt, Antimicrob. Agents Chemother. 11 (1977) 651–655.
[6] S.K. Maitra, T.Y. Yoshikawa, J.L. Hansen, I. Nilsson-Ehle, W.J. Palin, M.C. Schotz, L.B. Guze, Clin. Chem. 23 (1977) 2275–2278.
[7] G.W. Peng, M.A.F. Gadalla, A. Peng, V. Smith, W.L. Chiou, Clin. Chem. 23 (1977) 1838–1844.
[8] G. Seidl, H.P. Nerad, Chromatographia 25 (1988) 169–171.
[9] P. Gambardella, R. Punziano, M. Gionti, C. Guadalupi, G. Mancini, A. Mangia, J. Chromatogr. 348 (1985) 229–240.
[10] L.O. White, A. Lovering, D.S. Reeves, Ther. Drug Monit. 5 (1983) 123–126.
[11] P.J. Claes, R. Busson, H. Vanderhaeghe, J. Chromatogr. 298 (1984) 445–457.
[12] J.H. Albracht, M.S. De Wit, J. Chromatogr. 389 (1987) 306–311.
[13] M. Freeman, P.A. Hawkins, J.S. Loran, J.A. Stead, J. Chromatogr. 2 (1979) 1305–1317.
[14] K. Kraisintu, R.T. Parfitt, M.G. Rowan, Int. J. Pharm. 10 (1982) 67–75.
[15] P.J. Claes, Y. Chaerani, H. Vanderhaeghe, J. Pharm. Belg. 40 (1985) 95–99.
[16] E. Kaale, S. Leonard, A. Van Schepdael, E. Roets, J. Hoogmartens, J. Chromatogr. A 895 (2000) 67–79.
[17] D. Samain, P. Dupin, P. Delrieu, G. Inchauspe, Chromatographia 24 (1987) 748–752.
[18] R.T. Parfitt, D.E. Games, M. Rossiter, M.S. Rogers, A. Weston, Biomed. Mass Spectrom. 3 (1976) 232–234.
[19] T.A. Getek, M.L. Vestal, T.G. Alexander, J. Chromatogr. 554 (1991) 191–203.
[20] M. Cherlet, S. De Baere, P. De Backer, J. Mass Spectrom. 35 (2000) 1342–1350.
[21] D. Loffler, T.A. Ternes, J. Chromatogr. A 1000 (2003) 583–588.
[22] C. Lecaroz, M.A. Campanero, C. Gamazo, M.J. Blanco-Prieto, J. Antimicrob. Chemother. 58 (2006) 557–563.
[23] N.C. Megoulas, M.A. Koupparis, J. Pharm. Biomed. Anal. 36 (2004) 73–79.
[24] I. Clarot, P. Chaimbault, F. Hasdenteufel, P. Netter, A. Nicolas, J. Chromatogr. A 1031 (2004) 281–287.
[25] E. Adams, W. Roelants, R. De Paepe, E. Roets, J. Hoogmartens, J. Pharm. Biomed. Anal. 18 (1998) 689–698.
[26] T.A. Getek, A.C. Haneke, G.B. Selzer, J. Assoc. Off. Anal. Chem. 66 (1983) 172–175.
[27] V. Manyanga, K. Kreft, B. Divjak, J. Hoogmartens, E. Adams, J. Chromatogr. A 1189 (2008) 347–354.
[28] V. Manyanga, O. Grishina, Z. Yun, J. Hoogmartens, E. Adams, J. Pharm. Biomed. Anal. 45 (2007) 257–262.
[29] T.J. Whall, J. Chromatogr. 219 (1981) 89–100.
[30] G. Inchauspé, D. Samain, J. Chromatogr. 303 (1984) 277–282.
[31] N. Nagae, T. Enami, S. Doshi, LC–GC 20 (2002) 964–972.