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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF GENTAMICIN SULFATE REFERENCE STANDARDS AND INJECTION USP

CHUONG M.C.*, CHIN J., HAN J.W., KIM E., ALHOMAYIN W., AL DOSARY F., RIZG W., MOUKHACHEN O. AND WILLIAMS D.A.

College of Pharmacy, MCPHS University, Boston, Massachusetts, USA.
*Corresponding Author: Email- monica.chuong@mcphs.edu

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Abstract- The USP-NF 2013 describes 4 aminoglycoside chromatography peaks with an elution order of C1, C1a, C2a and C2. The method calculates gentamicin sulfate from a single standard concentration. The USP method for Gentamicin Sulfate Injection is a microbial assay, not a HPLC method. Therefore, the purposes were to evaluate the performance of different C18 columns for separating the aminoglycosides in the USP reference standard, a gentamicin sulfate powder met USP testing specifications, and a commercial injection product, and to construct a multi-point standard curve for calculating each of aminoglycosides rather than from a single concentration. Three LC columns from the same manufacturer were selected: (1) AquaÒ C18, 5 micron, (2) LunaÒ C18, 5 micron, and (3) Nuc leosilÒ C18, 3 micron (all in 4.6 x 150 mm). When samples prepared from a gentamicin sulfate powder met the USP test specifications were injected, only one peak at 8.8 min was displayed in the 35-min runtime chromatograms for Aqua C18 column, while three peaks were displayed for the Nuc leosil C18 column, and four peaks for the Luna column. Standard linearity was obtained from 0.2 to 2 mg/mL. Using the Luna column, the aminoglycosides eluded from the samples prepared from the USP Reference Standard and from a commercial injection USP were noted as three.

Keywords- o-phthalaldehyde solution, Aqua C18, Luna C18, Nuc leosil C18, pre-column derivatization, sodium 1-heptanesulfonate

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Introduction

Gentamicin, isolated in 1958 and reported in 1963 by Weinstein, et al., is active against many common Gram negative pathogens [1]. This drug is obtained commercially from *Micromonospora purpurea*, and four aminoglycosides have been identified in the USP-NF 2013 (cUSP) as C1, C1a, C2a and C2 [2] with three structures were listed in the compendium [Fig-1]. Cream and ointment dosage forms are used in the treatment of skin infections and burns complicated by *Pseudomonemia* while an injectable solution including 10- and 40-mg/mL gentamicin sulfate is used for serious systemic and genitourinary tract infection [3]. The USP-NF 2013 describes a single-point content 0.65 mg/mL determination by dissolving Gentamicin Sulfate USP in water prior to the addition of other ingredients for HPLC. The cUSP method describes the elution order as gentamicin C1, gentamicin C1a, gentamicin C2a, and gentamicin C2 with a 5-mm × 10-cm column that contains 5-µm packing L1 [2]. L1 is defined as octadecyl silane chemically bonded to porous or nonporous silica or ceramic microparticles, 1.5 to 10 µm in diameter, or a monolithic silica rod [2]. The purposes of this project were (a) to compare the performance of different LC columns for conducting gentamicin sulfate assay, (b) to explore whether a multi-point standard curve was feasible to establish so that samples of unknown concentrations may be determined, and (c) to examine whether the mobile phase might be simplified or modified under the modern LC column technology.

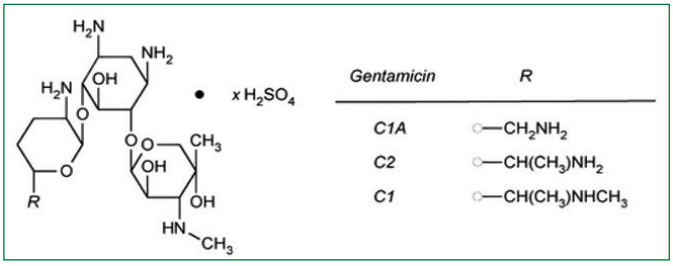

Fig. 1- Structures of gentamicin sulfate [2,5]

Materials and Methods

Materials

USP Gentamicin Sulfate Reference Standard (RS) (Lot M1J001) was purchased from USP, Rockville, MD. Gentamicin Sulfate powder met the USP test specifications (Amresco LLC, Lots 2562C059 and 2712C327, Solon, OH), ammonium hydroxide, boric acid, chloroform, glacial acetic acid, isopropyl alcohol, methanol, o-phthalaldehyde, potassium hydroxide, sodium 1-pentasulfonate, sodium 1-hexanesulfonate, sodium 1-heptanesulfonate, thioglycolic acid were purchased from VWR (Bridgeport, NJ). Gentamicin Sul-

fate for Injection USP (APP, Lots 03012DK, 6102719) was obtained from Cardinal Health.

High Performance Liquid Chromatography Method

Sample Preparation

Solutions of Gentamicin Sulfate in water at 0.2, 0.4, 2, 4, 8 and 20 mg/mL were prepared using the USP Gentamicin Sulfate RS and the gentamicin sulfate powder met the USP test specifications, respectively. Ten mL of each solution was transferred to a 25 mL volumetric flask. Five mL of isopropyl alcohol and 4 mL of o-phthalaldehyde solution were then added and mixed [2]. The o-phthalaldehyde solution was prepared by dissolving 1.0 g of o-phthalaldehyde in 5 mL methanol, and adding 95 mL of 0.4 M boric acid which had been adjusted with potassium hydroxide (8 N) to pH 10.4. After two mL of thioglycolic acid was added, the resulting solution was adjusted again with potassium hydroxide (8 N) to pH 10.4 for pre-column derivatization. The flask was then filled with isopropyl alcohol to the mark of 25 mL. Flasks were heated in a 60°C water bath (Precision Scientific, Model 183, Chicago, IL) for 15 minutes, and then cooled to room temperature [2].

LC Conditions

Mobile phase was prepared by mixing methanol, water, and glacial acetic acid (70:25:5). Sodium 1-heptanesulfonate was next dissolved. Its composition in the mobile phase was 0.5% w/v. Filtration with deaeration was followed. Three LC columns were selected for the study. They were (a) Aqua, 5 micrometers [6], (b) Luna, 5 micrometers [7], and (c) Nuc leosil, 3 micrometers (all were 4.6 × 150 mm, Phenomenex, CA). The Agilent Series 1100 (Hewlett Packard) LC system contained vacuum degasser, binary pump, auto sampler, column thermostatic compartment, and variable wavelength detector. The detection wavelength was set at 330 nm with flow rate at 1.1 mL/min and run time 37 min/cycle. Mobile phase was methanol/water/glacial acetic acid/sodium 1-heptanesulfonate in a ratio of 700/250/50/5 (v/v/v/w) [2]. For testing Aim (c), sodium 1-heptanesulfonate was either removed from the mobile phase or replaced with sodium 1-pentanesulfonate or sodium 1-hexanesulfonate.

Results

Using the assay method described in the cUSP [2] and the standard concentration of 2 mg/mL prepared from the gentamicin sulfate powder met the USP test specifications, by the end of 35 min, there were only one remarkable peak at 8.8 min was displayed in the chromatogram of Aqua C_{18} column [Fig-2a], while there were three peaks at the retention time of 18.3, 24.0 and 27.7 min were eluted from a Nuc leosil column (in three repetitions, [Fig-2b]), and the same number of peaks at 10.5, 13.5 and 15.2 min were eluted from the commercial Injection USP [Fig-2c]. The third column, Luna, separated the USP Gentamicin Sulfate RS into three peaks with retention time of 18.3, 24.0 and 30.0 min [Fig-3a] and also separated the commercial Injection USP into three peaks at 14.8, 19.2 and 23.7 min [Fig-3b]. But this Luna column was able to separate the gentamicin sulfate powder which met the USP test specifications into four aminoglycoside peaks with the retention time of 19.0, 25.0, 28.3 and 31.0 min [Fig-3c]. The linearity of the gentamicin sulfate standard curve ranged from 0.2, 0.4 and 2 mg/mL were presented in all peaks eluted by either the Nuc leosil column [Fig-4a] or the Luna column [Fig-4b]. In the derivatization temperature of 60°C all standard samples were in clear solution form but expressing different colors. The sample of 0.2 mg/m was slightly tinted yellow; 0.4 mg/mL tinted yellow; 2 mg/mL dark wine red. The color at 4 mg/mL or higher still to reduce the redness with 4 mg/mL in bright wine red, 8 mg/mL slight reddish peach, and 20 mg/mL in peach color. After all samples were allowed to cool to room temperature and stored in 4°C overnight, needle shape of crystal was found in the solution of 4 and 8 mg/mL samples [Fig-5], while the sample of 20 mg/mL or higher formed powder shape precipitate in clear color solution. The content % of each component listed in the product information of a distributor, cUSP-NF and the column specific results from our study are summarized in [Table-1].

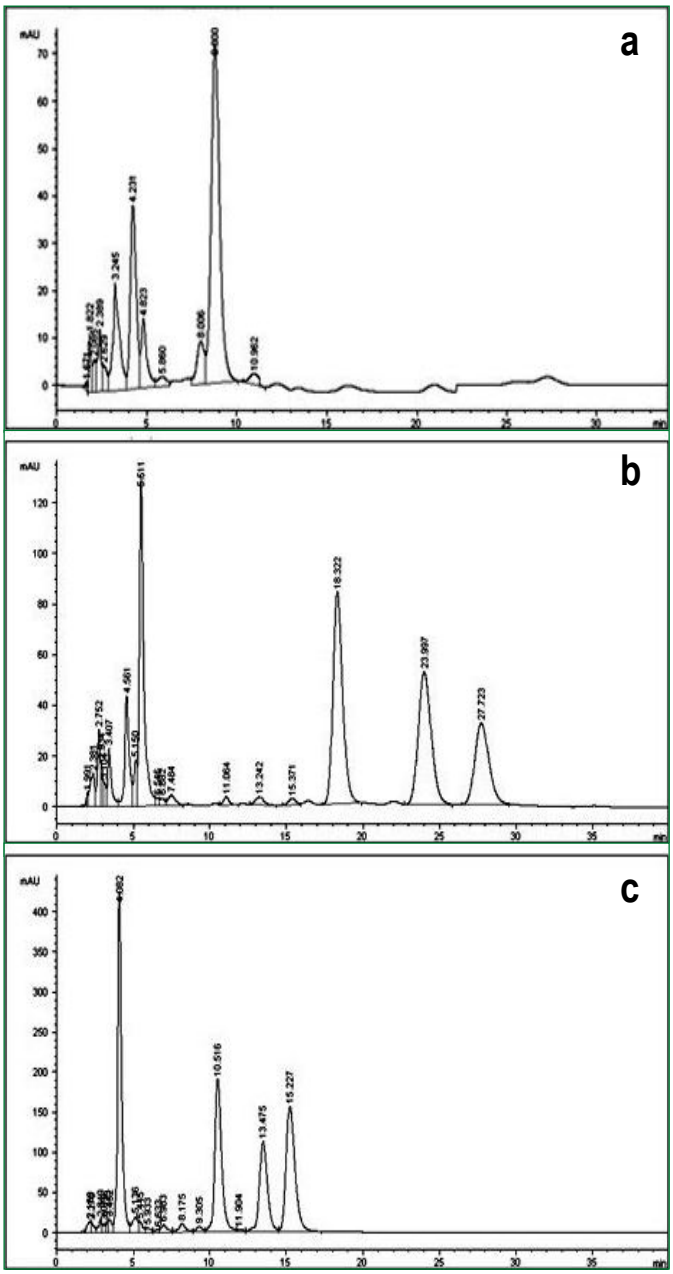

Fig. 2- The chromatograms of the first two study LC columns: (a) Aqua C_{18}, (b) the Nuc leosil C_{18} at the concentration of 2 mg/mL. The sample in chromatograms a) and (b) was prepared from the gentamicin sulfate powder met the USP test specifications, but eluded through an Aqua C18 and a Nuc leosil column, respectively. Figure (c) was the chromatogram of the commercial Gentamicin Sulfate for Injection USP eluded by a Nuc leosil C_{18} column.

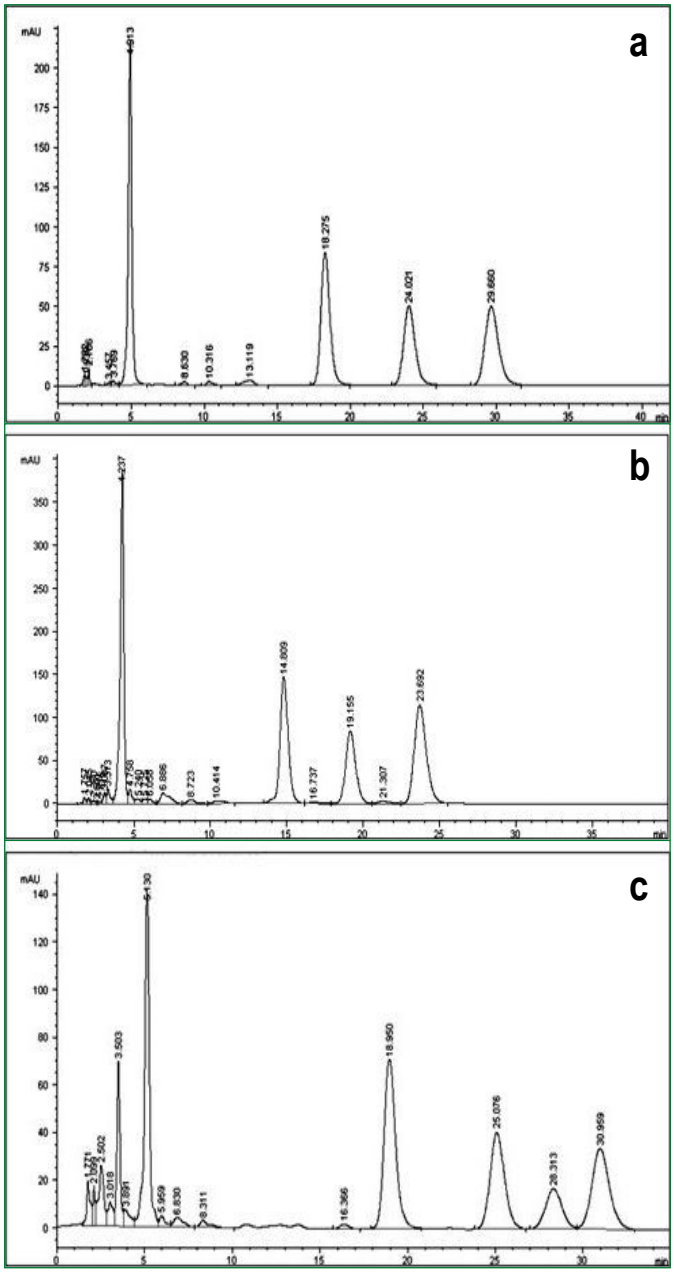

Fig. 3- The chromatograms of the third columns, Luna C_{18}. Figure (a) was the sample prepared from the USP Gentamicin Sulfate RS and Figure (b) was the commercial Injection USP. Both displayed three aminoglycoside peaks. Figure (c) was the sample prepared from the gentamicin sulfate powder met the USP test specifications. All concentrations were at 2 mg/mL.

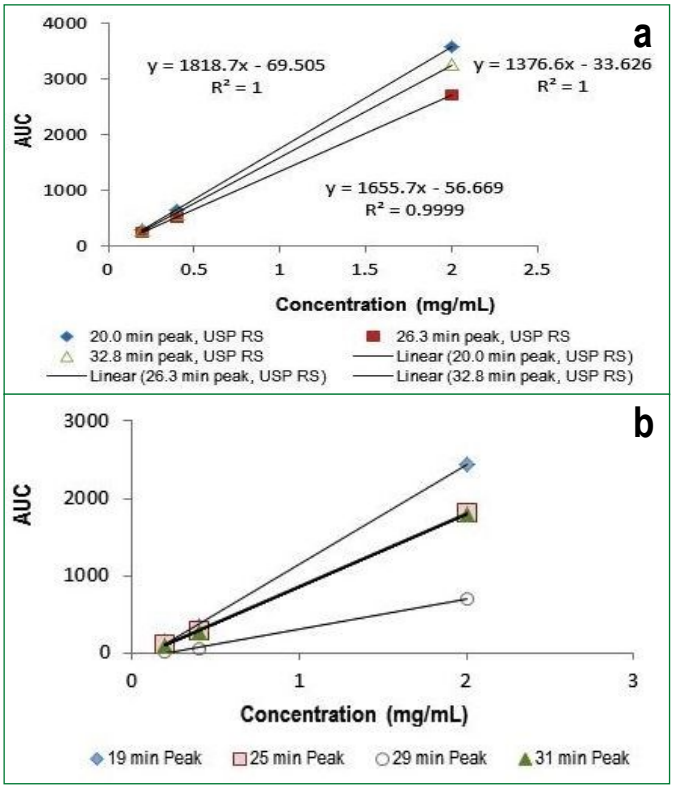

Fig. 4- Standard Curves of Gentamicin Sulfate and Precipitates at higher concentrations. Using the Luna column, the linearity of the gentamicin sulfate standard curve ranged from 0.2, 0.4 and 2 mg/mL: (a) the sample prepared from the USP RS; (b) the sample from the gentamicin sulfate powder met the USP test specifications.

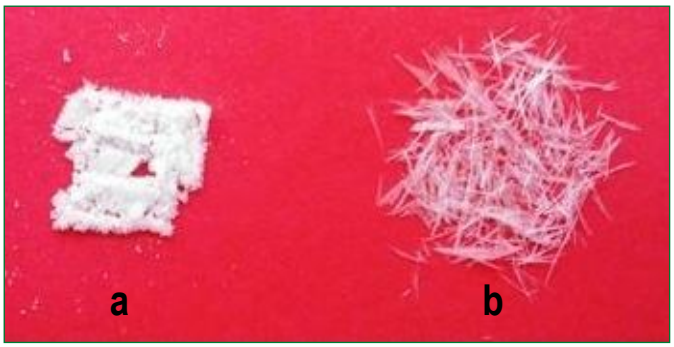

Fig. 5- Recrystallization of Gentamicin Sulfate USP. In the left is powder met the USP test specifications. In the right is the regular acicular crystal. These needles were collected from the three derivatization samples of 4 and 8 mg/mL each after being stored in 4°C overnight, filtered by gravity and air dried.

Table 1- Using three LC columns to study the percent of peak area of each particular gentamicin component in the sample prepared from the gentamicin sulfate powder met the USP test specifications at the concentration of 2 mg/mL compared to the product information from a distributor and the cUSP-NF [2]

Column Type (Sample Size)	Peak 1 (%)	Peak 2 (%)	Peak 3 (%)	Peak 4 (%)	Sum of Peak 3 and Peak 4 (%)
Aqua C_{18}	100	ND	ND	ND	ND
Nucleosil C_{18} (n=3)	38.63±2.87	30.55±4.76	-	-	30.82±7.59
Luna C_{18} (n=5)	32.87±5.88	28.41±1.96	13.32±8.16	25.41±3.19	38.70±3.52
Distributor Info[a]	< 45	< 30	-	< 30	-
USP Guidelines[b]	25 - 50	10 - 35	Mentioned, but % not specified	Mentioned, but % not specified	25 - 55

[a] from Reference 4; [b] 5-mm × 10-cm column containing 5-µm packing L1

The pH of the aforementioned studies mobile phase was 3.04. In order to examine whether the mobile phase might be simplified under the modern LC column technology, 0.5%w/v sodium 1-heptanesulfonate was removed from the mobile phase. The pH of such a modified mobile phase was 3.2. When the same sample was injected into the LC system with Luna bonded phase C_{18} (which was able to separate gentamicin sulfate standard preparation at 2 mg/mL) into four peaks in the aforementioned study) there was only a small peak was observed at 10.5 min (data not shown). This suggests that for using Luna bonded phase C_{18}, a recently developed LC column, sodium 1-heptanesulfonate was still required in the mobile phase to assay gentamicin sulfate. Among sodium 1-pentanesulfonate, sodium 1-hexanesulfonate, sodium 1-heptanesulfonate, the peak elution of a 2 mg/mL sample prepared from USP RS changed from four into three when the same samples were injected with the one of the first two reagent in the mobile phase within the same day but a couple hours later, while sodium 1-heptanesulfonate eluded the USP RS into three aminoglycoside peaks consistently.

Discussion

There are four main reasons of using derivatization in HPLC: to improve detectability, to change molecular structure of polarity of analyte for better chromatography, to change the matrix for better preparation and to stabilize a sensitive analyte [8]. Post-column derivatization is commonly accomplished using a reaction detector where the analyte is derivatized after the separation but prior to detection. Pre-column derivatization requiring less equipment as it may be performed manually or automated. Pre-column derivatizing reagents most typically used for amino acid analysis are o-phthalaldehyde [8], phenyl isothiocyanate, fluorescamine, and dansyl chloride. Reaction can be either simply mixing at room temperature or requiring heating. There are at least eight publications in the topic of gentamicin HPLC since 1979 [9-17]. Freeman, et al [10]. reported that utilizing pre-column derivatization with an ophthaladelhyde/thioglycolic acid reagent on a Hypersil O.D.S column and UV detection, four major components of gentamicin were resolved (C1, C1a, C2 and C2a). The elution sequence of gentamicin components is different from that of the cUSP (1). But ion-paired chromatography was used to quantify the derivative. Barends, et al [11] published an HPLC method to quantify gentamicin in serum and identified two chromatographic peaks for C1a and C1 + C2. But peak height instead of area under the curve was reported, since both peaks did not return to the baseline at the end of elution. Cabanes, et al [12]. quantified gentamicin in biological fluids using a silica column. Pre-column derivatization with o-phthalaldehyde was done to form fluorescent products for detection. They reported standard curves in both plasma and urine at concentration ranging from 0 to 10 mg/L [12], which is much lower than our finding of 0.2 to 2 mg/mL. Graham, et al [13] tested degradation of gentamicin sulfate in dextrose solution. Pre-column derivatization was not incorporated. Instead, 0.11 M aqueous trifluoroacetic acid - methanol (95:5) was used to dilute the stock of gentamicin sulfate in water. They reported the elution order as C1a, C2, C2a and C1, with the composition being 10-12% for C2a and 1-2% for C2b (the fifth component) [13]. Since the mobile phase and sample preparation did not follow the cUSP-NF method, the results were not comparable. It is also interesting to find that gentamicin chromatogram only contains a single peak in addition the internal standard peak from tobramycin [14]. Grahek, et al [15]. identified 17 gentamicin impurities by using liquid chromatography tandem mass spectrometry. However, their mobile phase was different from that of the cUSP [2]. The Kowalczuk, et al [16]. determined the gentamicin components as G1, G2 and G3 by using o-phthalaldehyde in combination with N-acetycysteine (NAC). Like our endeavor, Joseph, et al [17]. also screened columns during method development. Since the detector of their study was a charged aerosol type, ion-paring agents was required to put into mobile phase to provide the required selectivity for gentamicin compound resolution on a RP-HPLC column. Three different ion-paring agents were investigated. Eventually trifluoroacetic acid was chosen. The authors believed that gentamicin sulfate is a mixture of C1, C1a, C2, C2a and C2b with C1 of 26%, C1a 27%, C2 and C2a as a mixture of 47% [17]. The AUC of Peak C1b was extremely small, < 1%.

Our project was the first endeavor of reporting that gentamicin sulfate HPLC assay is reference standard specific as well as the performance specific of each tested column. We found four peaks when Luna bonded phase C_{18} [7] and the gentamicin sulfate powder met the USP test specifications was used. This column is recommended for extremely high pH application, such as pH 10.4 in this project, or when longer retention is desired [7] in order to separate multiple components. Aqua's polar end capping produces a surface chemistry that is well suited for the analysis of small peptides [6]. Separation mechanism of a Nuc leosil column is based on hydrophobic (van der Waals) interactions. When o-phthalaldehyde solution (pH 10.4) reacts with primary amino group of a compound in an alkaline medium, it will give a soluble product. This compound has been exploited for spectrophotometric study by measuring the absorbance at 340 nm [9] and quantitatively estimating of compounds containing a primary amino group. At the concentration, the reaction produced the strongest color. It is important to maintain medium alkaline [9]. This explains the short linear standard curve found in this study (0.2 to 2 mg/mL). The elution order described in the cUSP is C1, C1a, C2a and C2. Based on the physicochemical property of gentamicin and column chemistry, Kowalczuk, et al [16] reported the correct elution order of C1a, C2 and C1. But their method failed to identify the fourth peaks. Joseph, et al [17]. suggested that the fourth peak, C2a, is the epimer of C2.

Conclusions

The cUSP listed gentamicin salt in three different structures [Fig-1], but describes the HPLC elution sequence as C1, C1a, C2a and C2 with the specification of C1 content ranging 25-50%, C1a 10-35%, C2a and C2 25-55%. The compendium only provides general information of the column used (a 5-mm×10-cm column that contains 5-µm packing L1 [2]) without further specifying which one to use. Among the three different LC columns studied in this project, the AquaÒ C18 and LunaÒ C18 are both labeled as 5 micrometers. Both their performances were totally different. The estimation of gentamicin major component made by a Nuc leosil column fulfilled the description in the product information of a distributor [4]. A Luna column was capable of identifying four chromatographic peaks consistent with the description of cUSP [2] when the samples were prepared from gentamicin sulfate powder met the USP test specifications, but not from the USP Gentamicin Sulfate RS nor from the commercial injections USP. Perhaps this was due to either different manufacturing process, column performance or other causes. This project also advanced the single standard concentration preparation in the cUSP to a standard curve from 0.2 to 2 mg/mL.

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