Development and Validation of a RP-HPLC Method for the Estimation of Netilmicin Sulfate and its Related Substances using Charged Aerosol Detection

Arul Joseph*, Shrina Patel, and **Abu Rustum**
Global Quality Services-Analytical Sciences, Schering-Plough, 1011 Morris Avenue, Union, NJ 07083

Abstract

Netilmicin is a semi-synthetic aminoglycoside antibiotic used against a broad spectrum of Gram-negative bacteria. A reversed-phase high-performance liquid chromatographic method has been developed to determine the composition of netilmicin sulfate and to estimate its related substances without pre- or post-column derivatization. A UV detector cannot be used to detect low levels of known and unknown related substances of netilmicin, as it has only a weak UV chromophore. A charged aerosol detector was used instead to obtain the high sensitivity that was necessary for the intended purpose of the method. This method can separate all related substances of netilmicin. A (10 cm × 4.6 mm) pentafluorophenyl high-performance liquid chromatographic column from Restek was used with a mobile phase consisting of (A) pentafluoropropionic acid–water–acetonitrile (0.1:96:4, v/v/v) and (B) trifluoroacetic acid–water–acetonitrile (1:96:4, v/v/v).

Introduction

Netilmicin is a semi-synthetic aminoglycoside antibiotic that is active against Gram-negative bacteria. Netilmicin is an ethyl derivative (at the 1-N position of the deoxystreptamine ring) of sisomicin and is synthesized by the alkylation of sisomicin. Netilmicin was first synthesized by Wright in 1976 (1). Netilmicin is effective against bacteria that are resistant to gentamicin, sisomicin, and tobramycin and has been found to be less toxic compared to these aminoglycoside antibiotics (2,3).

Netilmicin and its related substances have only a weak UV chromophore, are highly polar, are highly water soluble, and have very low solubility in most organic solvents. These properties of netilmicin and its related compounds create several challenges in developing a reversed-phase high performance liquid chromatography (RP-HPLC) method. The first challenge is to obtain high sensitivity, which is necessary to detect and estimate low levels of impurities and degradation products of netilmicin. The second challenge is to retain and separate netilmicin and all its related substances under RP-HPLC conditions.

As netilmicin and its related compounds possess only a weak UV chromophore, conventional UV detectors are not sensitive enough to detect low levels of netilmicin related substances. Therefore, developing a RP-HPLC method using direct UV detection to estimate low levels of related compounds of netilmicin is not feasible. Other detection technologies, such as pre-column derivatization followed by UV detection, evaporative light scattering detection (ELSD), electrochemical detection (ECD), and refractive index (RI) detection, have major challenges and limitations for use in the analysis of netilmicin. Pre-column derivatization procedures can be difficult to reproduce and cumbersome and are unsuitable for quantitative methods to estimate low levels of compounds in a sample. ELSD is unsuitable for this method because the sensitivity of ELSD is relatively low; the precision and linear dynamic range are also narrow. In general, ECD response is highly sensitive to small changes in temperature, pump pulsations, and any extraneous electrical current. The new generation of ECDs, due to modifications in the design of the detector, have decreased the sensitivity of the ECD response to temperature, pump pulsations, and electrical currents and have also minimized some robustness issues, such as electrode poisoning compared to older generations. However, when ECDs were applied to aminoglycosides analysis, they were cumbersome for routine use and had reproducibility issues (4,5). RI detection is incompatible with gradient methods, which are necessary to separate the related substances of netilmicin. Due to the challenges associated with the previously mentioned conventional HPLC detection systems for use with netilmicin, a charged aerosol detector (CAD) was used as the detector for this method.

CAD is a relatively new universal detection technology, which is sensitive and has a broad dynamic range (6,7). 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 analyte particles are charged by diffusional charge transfer through collision with nitrogen cations, and the charged analyte 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, due to its principle of operation, CAD has a quadratic response versus concentration and has a linear response over only a very short concentration range and at low analyte concentrations. To use the CAD for quantitation of components using a linear fit over the

*Author to whom all correspondences should be addressed: email josepharul@gmail.com.

wide concentration range of 2% (limit of quantitation, LOQ) to 200% of 0.25 mg/mL requires a 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 HPLC–UV methods. A linear fit instead of a quadratic fit was adopted to enable routine use of this HPLC-CAD method in a QC-friendly manner using a single 100% level netilmicin sulfate standard. Retaining netilmicin and its related substances (i.e., *N*-ethyl deoxystreptamine, *N*-ethyl garamine, sisomicin, and other *N*-ethyl derivatives of sisomicin) on typical RP-HPLC columns to obtain the selectivity that is necessary to get baseline separation of all analytes of interest is the second major challenge of this method. In addition, the insolubility of netilmicin 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 netilmicin using both direct and indirect methods have been reported in literature (4,8–17). The indirect methods involve either pre- or post-column derivatization with *o*-phthalaldehyde or dansyl chloride (5-dimethylamino-1-naphthalene sulfonyl chloride) with UV detection. Two direct detection method using electrochemical detection (16) and mass spectrometry (MS) (17) have been reported. The methods reported in the literature lack selectivity to separate and quantitate netilmicin and all of its related compounds (deoxystreptamine, *N*-ethyl garamine, sisomicin, and ethyl sisomicin derivatives) that has been demonstrated using this method. This manuscript describes the development and validation of a sensitive, selective, and robust HPLC method to determine the composition of netilmicin and to estimate its related substances using a pentafluorophenyl stationary phase and CAD. To the best of our knowledge, this is the first report of an analytical method that can quantitate netilmicin and can also estimate its related substances without derivatization using CAD.

Experimental

Materials

All the organic solvents used were HPLC-grade. Acetonitrile, dichloromethane, and pentafluoropropionic acid (PFPA) ≥ 98% were purchased from Sigma (St. Louis, MO) and trifluoroacetic acid (TFA) ≥ 99.5% were purchased from Alfa Aesar (Ward Hill, MA). Water (18.2 MΩcm) was obtained using a Milli-Q system (Millipore, Billerica, MA). Netilmicin sulfate was provided by the Global Quality Services-Analytical Sciences group in Schering-Plough (Union, NJ). Sisomicin sulfate, USP reference standard was purchased from Fisher Scientific (Waltham, MA). *N*-ethyl garamine sulfate was also provided by Global Quality Services-Analytical Sciences group in Schering-Plough. Deoxystrept-amine sulfate was obtained through a contract research organization, Syncom BV (Groningen, the Netherlands).

Instrumentation and chromatographic conditions

Waters 2695 Alliance HPLC system (Milford, MA) 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). Data acquisition, analysis, and reporting were performed using Millennium32 and/or Empower chromatography software (Milford, MA). 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, 100 × 4.6 mm i.d., 5-µm particle size) is manufactured by Restek Corporation (State College, PA) and distributed by Fisher Scientific.

Chromatographic conditions of the final method

The solvent used in mobile phase A was pentafluoropropionic acid–water–acetonitrile (0.1:96:4, v/v/v), and the solvent used in mobile phase B was trifluoroacetic acid–water–acetonitrile (1:96:4, v/v/v). The gradient program is listed in Table I. Using highly aqueous mobile phases can lead to loss of mobile phase from stationary phase pores and/or stationary phase collapse, resulting in a reduction in retention of analytes. To prevent loss of mobile phase from the pores of the packing material, the column was washed with mobile phase, water–acetonitrile (20:80, v/v) for 10 min. The column temperature was maintained at 40°C, and a flow rate of 1.5 mL/min was used. The CAD was set at 100 pA gain and a medium noise filter. The sample injection volume was 20 µL.

Synthesis of the ethyl derivatives of Sisomicin by reductive alkylation (18)

As we were unable to find commercial sources for the ethyl derivatives of sisomicin, we synthesized limited amounts of these compounds for the development and pre-validation of the method. Sisomicin sulfate (500 mg) was dissolved in 50 mL of water in a round-bottomed flask. To this solution, 100 µL of acetaldehyde was added with stirring, and the solution was

Table I. 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 (PFPA) elution
3.00	100.0	0.0	0.0	Linear	
3.50	0.0	100.0	0.0	Linear	Second isocratic ion-pair reagent (TFA) elution
30.00	0.0	100.0	0.0	Linear	
Column wash and equilibrate to initial conditions†					
30.50	0.0	0.0	100.0	Linear	Column wash
40.50	0.0	0.0	100.0	Linear	
41.00	100.0	0.0	0.0	Linear	Column equilibration
45.00	100.0	0.0	0.0	Linear	

* Use of highly aqueous mobile phases (96% 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

† The gradient time from 30–45 min is for column wash and equilibration only, and no data acquisition is necessary.

allowed to stir for 10 min. 50 mg of sodium cyanoborohydride was added to the flask, and the reaction mixture was allowed to stir for 15 min. This reaction mixture contains a mixture of ethyl derivatives of sisomicin including netilmicin sulfate.

Sample preparation

Standard solutions of netilmicin sulfate were prepared by dissolving the compound in water to have an analytical concentration of approximately 0.25 mg/mL. As netilmicin is a semi-synthetic aminoglycoside, to obtain all possible related compounds of netilmicin, sisomicin was alkylated to obtain netilmicin and a mixture of ethyl derivatives of sisomicin. A specificity mixture solution was prepared by mixing *N*-ethyl garamine sulfate with the sisomicin sulfate reaction mixture containing the ethyl derivatives of sisomicin (including netilmicin).

To determine the linearity of netilmicin sulfate, triplicate preparations of netilmicin sulfate solutions at each of seven levels of sample concentration were prepared. The seven levels of sample concentration were 1%, 2%, 20%, 40%, 50%, 100%, and 200% of the netilmicin sulfate analytical concentration (0.25 mg/mL). To determine the linearity of netilmicin sulfate-related substance, sisomicin sulfate was prepared at each of three levels of sample concentration. The three levels of sample concentration for sisomicin sulfate were 1%, 2%, and 10.0% compared to the analytical concentration of netilmicin sulfate. The limit of detection (LOD) and LOQ of the tested compounds, netilmicin sulfate and sisomicin sulfate, are 0.0025 mg/mL and 0.005 mg/mL, which correspond to 1% and 2% of the netilmicin analytical concentration of 0.25 mg/mL, respectively.

Calculation

The composition of netilmicin sulfate is calculated as a percent area by area normalization. The percent area of netilmicin sulfate can be calculated using the equation shown below.

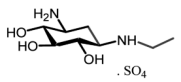
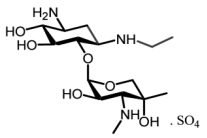
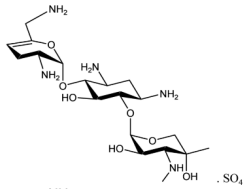
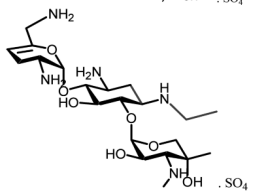
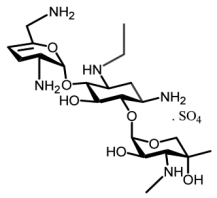
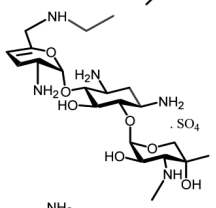
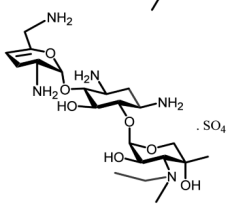
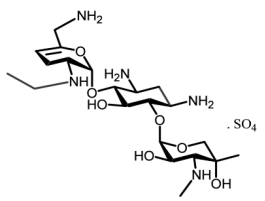
Results and Discussion

HPLC method development

The main objective was to develop a sensitive and rugged RP-HPLC method that can separate netilmicin from its known (*N*-ethyl garamine and sisomicin) and unknown related substances (ethyl sisomicin derivatives prepared by the alkylation of sisomicin and impurities formed from stress studies). Chemical structures of netilmicin and its related substances are shown in Table II. Netilmicin and ethyl-sisomicin derivatives (such as 3-*N*-ethyl sisomicin, 2'-*N*-ethyl sisomicin, 6'-*N*-ethyl sisomicin, and 3''-*N*-ethyl sisomicin) differ from one another only in the position of substitution of the ethyl group on the amine functional group on one of the three rings of sisomicin.

As netilmicin is a semi-synthetic derivative of sisomicin, it can be expected that the source of the related substances of netilmicin would be the by-products of the synthesis of

Table II. Compound Name, Chemical Structure, Identity, and Physical Properties of Netilmicin and its Related Substances

Compound Name	Structure	Identity	Properties
N-ethyl deoxystreptamine Sulfate		Related substance	UV inactive, water soluble
N-ethyl garamine Sulfate		Related substance	UV inactive, water soluble
Sisomicin Sulfate		Related substance	Weak UV absorbance, water soluble
Netilmicin Sulfate		API	Weak UV absorbance, water soluble
3-*N*-ethyl Sisomicin Sulfate		Related substance	Weak UV absorbance, water soluble
6′-*N*-ethyl Sisomicin Sulfate		Related substance	Weak UV absorbance, water soluble
3″-*N*-ethyl Sisomicin Sulfate		Related substance	Weak UV absorbance, water soluble
2′-*N*-ethyl Sisomicin Sulfate		Related substance	Weak UV absorbance, water soluble

netilmicin from sisomicin. Therefore, a reductive alkylation of sisomicin (18) was performed using acetaldehyde and sodium cyanoborohydride to synthesize netilmicin and a mixture of ethyl derivatives of sisomicin (Figure 1). Ion-pairing agents can form an ion-pair with the amine groups of netilmicin and its related substances to enable retention of these compounds on RP-HPLC columns, which is otherwise unfeasible due to their polar nature. In addition, ion-pairing agents can provide the additional selectivity in resolving these compounds on RP-HPLC columns. As non-volatile additives are incompatible with the CAD, it was necessary to explore volatile ion-pairing agents (e.g., carboxylic acid and perfluorocarboxylic acids) during method development activities.

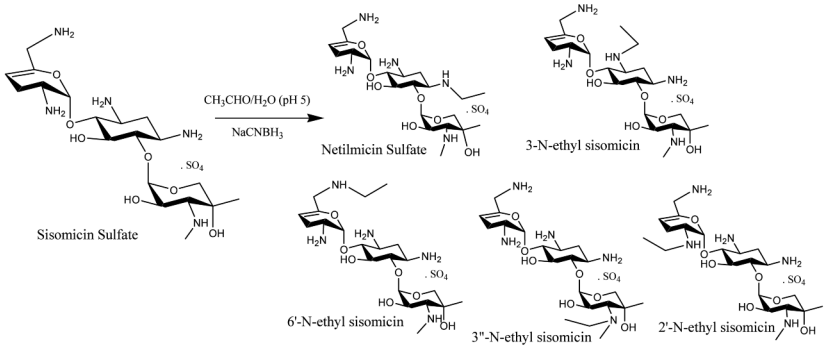


Figure 1. Reductive alkylation of sisomicin using acetaldehyde and sodium cyanoborohydride.

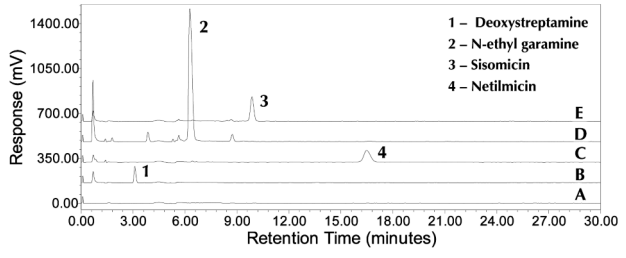


Figure 2. Representative chromatograms of diluent (A), deoxystreptamine (B), netilmicin (C), N-ethyl garamine (D), and sisomicin (E).

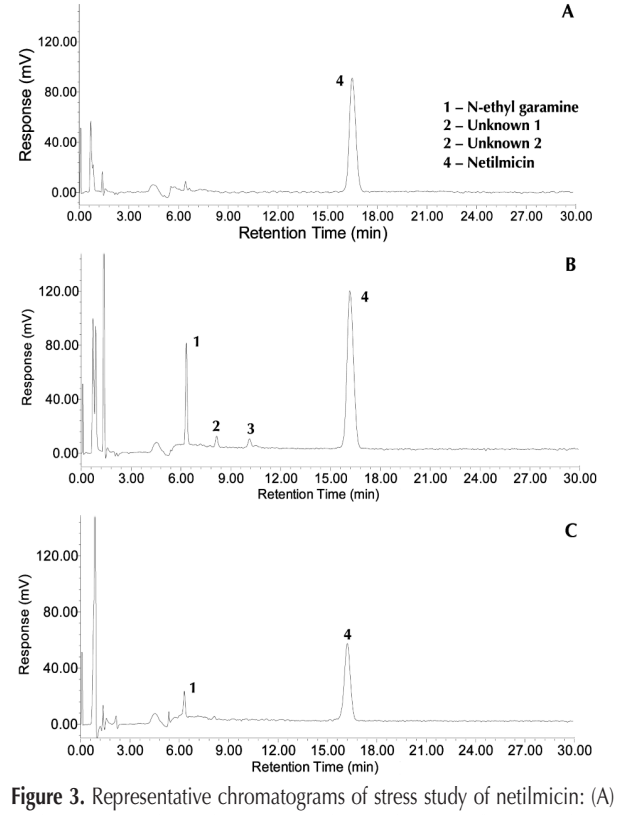


Figure 3. Representative chromatograms of stress study of netilmicin: (A) netilmicin, (B) netilmicin heat stress, and (C) netilmicin acid stress (HCl).

Method development activities were initiated using a pentafluorophenyl column with mobile phases containing ion-pairing agents. A pentafluorophenyl stationary phase was selected because in addition to the typical hydrophobic interaction between analyte and stationary phase, it can provide a dipole-dipole interaction and Pi-Pi 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 netilmicin 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 retain sisomicin and netilmicin. Hence, pentafluorophenyl stationary phase was selected. The Restek Allure PFP column (100 × 4.6 mm, 5-µm particles) was selected for this method because this column provided the best separation of all the ethyl derivatives of sisomicin from netilmicin. The composition of the mobile phase was modified to investigate different ion-pairing agents (e.g., TFA, PFPA, and heptafluorobutyric acid). In addition, various organic modifiers (e.g., methanol, isopropanol, etc.) in combination with water at different proportions were also investigated. Netilmicin was successfully separated from *N*-ethyl garamine and sisomicin using a mobile phase of trifluoroacetic acid–water–acetonitrile (1:96:4, v/v/v). However, deoxystreptamine was found to elute at the solvent front under these conditions. Modifying the concentration of TFA and acetonitrile did not provide the desired improvement in retention of deoxystreptamine. Using even low concentrations of the higher fluorocarboxylic acid homologs, PFPA (0.1%) and heptafluorobutyric acid (0.05%), increased the retention of netilmicin considerably. The first compound, deoxystreptamine, is eluted at the solvent front on all columns including pentafluorophenyl columns under isocratic conditions. To achieve the goal of retaining deoxystreptamine and eluting netilmicin and related substances within a reasonable time period, a shallow ion-pair gradient was employed. The ion-pair gradient uses 0.1% PFPA from time-zero to 3 min to retain deoxystreptamine. Then 1.0% TFA is introduced to elute ethylgaramine, sisomicin, and netilmicin (Table I). The PFPA concentration (0.1%) used in the ion-pair gradient was selected to elute the deoxystreptamine close to 3 min and prevents its overlap with solvent peaks near the void volume. The TFA concentration of 1% was ideal to resolve the sisomicin alkyl groups within a reasonable run time.

Using a highly aqueous mobile phase (96% water) on a typical RP-HPLC column causes the loss of ability by the stationary phase to maintain hydrophobic interaction with the analytes, possibly due to desolvation (19). 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 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. The reduction of retention time was minimized after the addition of this extra step at the end of each gradient run. Representative chromatograms of diluent, deoxystreptamine sulfate, *N*-ethyl garamine sulfate, sisomicin sulfate, and netilmicin sulfate are shown in Figure 2. The chromatograms were obtained using the method conditions described earlier.

Preliminary validation

Preliminary validation of this method was conducted to determine and demonstrate that the response linearity, specificity, robustness, LOQ, and LOD of this method using CAD detection is comparable with the UV detection, which is the most widely used detection system for chromatographic methods. Unlike the linear response of UV detectors, CAD detectors have quadratic response, and therefore, the criteria for the coefficient of determination r^2 of the linear regression analysis of CAD peak area versus concentration plots were set at a values of > 0.99 compared to the typical r^2 values of > 0.999 used with UV detectors. The results of the linearity, specificity, robustness, LOQ, and LOD experiments are described below.

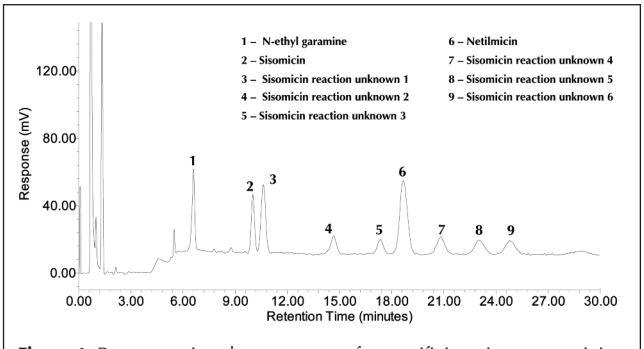


Figure 4. Representative chromatogram of a specificity mixture containing the ethyl derivatives of sisomicin (reaction mixture of sisomicin reductive alkylation) and *N*-ethyl garamine sulfate.

Table IV. The Resolution of Netilmicin and Related Substances in the Specificity Mixture Under Selected HPLC Parameter Robustness Conditions

Compound Name	Method condition	Column temp.		Flow rate (mL/min)		Injection volume	
		35°C	45°C	1.3	1.7	18 µL	22 µL
N-ethyl garamine	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Sisomicin	10.4	10.1	12.0	11.1	11.0	10.7	10.6
Sisomicin Uk* 1	1.5	1.7	1.4	1.7	1.6	1.5	1.5
Sisomicin Uk* 2	7.0	7.2	6.2	7.5	7.4	6.9	7.2
Sisomicin Uk* 3	4.0	3.6	3.4	4.0	4.2	4.1	4.2
Netilmicin	1.7	1.6	1.6	1.7	1.7	1.9	1.7
Sisomicin Uk* 4	2.2	2.0	2.6	2.5	2.6	2.3	2.3
Sisomicin Uk* 5	2.0	2.4	2.1	4.4	2.4	2.1	2.1
Sisomicin Uk* 6	1.7	1.3	1.7	2.4	1.6	1.6	1.4

* Uk = unknown.

Method specificity

The method specificity was demonstrated by successfully resolving netilmicin from its related substances in samples that were subjected to stress studies (Figure 3) and by successfully resolving netilmicin from sisomicin and ethyl derivatives of sisomicin in a specificity test mixture consisting of the sisomicin sulfate reductive alkylation reaction mixture spiked with ethyl garamine sulfate (Figure 4).

Stress studies were conducted using heat, acid, and base. When netilmicin is subjected to heat, it undergoes degradation to form *N*-ethyl garamine and two unknown degradants. The RP-HPLC method described in this paper was able to successfully resolve netilmicin from its three degradants, as shown in Figure 3B. Acid treatment primarily leads to the formation of *N*-ethyl garamine due to hydrolysis, the resulting chromatogram is shown in Figure 3C. Base-treated netilmicin solutions did not reveal any detectable peaks due to the high background noise from the base. The reductive alkylation of sisomicin using acetaldehyde and sodium borohydride leads to the formation of about six unknown compounds besides netilmicin. This analytical method is capable of successfully resolving netilmicin, sisomicin, and *N*-ethyl garamine from all six unknown compounds, as shown in Figure 4, demonstrating that the method described in this report is a stability-indicating method.

Linearity

For netilmicin sulfate, the linearity range investigated covered the concentration range from 0.0025 mg/mL to 0.5 mg/mL, which corresponds to 1% to 200% of the analytical concentration of netilmicin sulfate of 0.25 mg/mL. For sisomicin sulfate, the linearity range investigated covered the concentration range from 0.0025 mg/mL to 0.025 mg/mL, which corresponds to 1% to 10% of netilmicin sulfate analytical strength. 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 = 21) for netilmicin sulfate and greater than 0.99 (n = 9) for the related substance sisomicin sulfate.

LOD and LOQ

The LOD and LOQ of netilmicin sulfate and sisomicin sulfate are 0.0025 mg/mL and 0.005 mg/mL, 1% and 2%, respectively, of the netilmicin sulfate analytical concentration of 0.25 mg/mL. Signal to noise (S/N) values of greater than 10 for the LOQ and greater than 3 for LOD were routinely observed.

Method robustness

Deliberate variations in HPLC parameters were made to demonstrate the robustness of the

Table III. Variations in HPLC Parameters to Test Method Robustness

Testing conditions	Procedural conditions	Procedural Variations												
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Flow rate (mL/min)	1.5	1.3	1.7	–*	–	–	–	–	–	–	–	–	–	–	–
Column temp. (°C)	35	–	–	35	45	–	–	–	–	–	–	–	–	–	–
Acetonitrile % in mobile phase	H_2O–ACN (96:4)	–	–	–	–	95:5	97:3	–	–	–	–	–	–	–	–
PFPA proportion in mobile phase A	0.1% PFPA	–	–	–	–	–	–	0.09%	0.11%	–	–	–	–	–	–
TFA proportion in mobile phase B	1% TFA	–	–	–	–	–	–	–	–	1.1%	0.9%	–	–	–	–
Injection vol. (µL)	20	–	–	–	–	–	–	–	–	–	18	22			

* Blank cell (–) indicates that the parameter is the same as the procedural parameter.

method. The parameters tested include injection volume, flow rate, temperature, and proportion of acetonitrile and ion-pairing agents in mobile phase (Table III). We evaluated the method robustness based on the changes in the resolution between the peaks in the unknown netilmicin sulfate and sisomicin sulfate and the tailing factor of netilmicin sulfate under the tested conditions. The resolution between sisomicin and sisomicin reaction unknown 1 was found to be ≥ 1.4, and the tailing factor was found to be ≤ 2.0 under the various chromatographic conditions tested. The resolution of netilmicin and its related substances in the specificity mixture obtained under a few representative HPLC conditions are summarized in Table IV. It can be seen that netilmicin and all its related substances under various chromatographic conditions are well-resolved.

Conclusion

The HPLC method described in this report is the first known method, which can separate and accurately estimate all the individual analogues of netilmicin, including all the related compounds by direct detection (i.e., no derivatization) using a CAD. A preliminary validation of the method demonstrated linearity, specificity, and robustness of the CAD detection that is comparable with UV detection. The analytical method described in this paper has been successfully used to determine the composition of netilmicin sulfate and also to estimate its related substances. This method is also a stability-indicating method for netilmicin because it can separate all the known and unknown degradation products of netilmicin and can accurately quantitate the content of netilmicin in any sample. Thus, this method can be used for routine analysis of netilmicin, including the analysis of stability samples as this is also a stability-indicating method.

Acknowledgments

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.

References

1. J.J. Wright. Synthesis of 1-N-ethylsisomicin: a broad-spectrum semisynthetic aminoglycoside antibiotic. *J.C.S. Chem. Commun.* **1976:** 206–208 (1976).
2. F.C. Luft. Netilmicin: a review of toxicity in laboratory animals. *J. Int. Med. Res.* **6:** 286–299 (1978).
3. P. Noone. Sisomicin, netilmicin and dibekacin: A review of their antibacterial activity and therapeutic use. *Drugs* **27:** 548–578 (1984).
4. E. Adams, D. Puelings, M. Rafiee, E. Roets, and J. Hoogmartens. Determination of netilmicin sulfate by liquid chromatography with pulsed electrochemical detection. *J. Chromatogr. A* **812:** 151–157 (1998).
5. C. Ghinami, V. Giuliani, A. Menarini, F. Abballe, S. Travaini, and T. Ladisa. Electrochemical detection of tobramycin or gentamicin according to the Eurpoean Pharmacopeia analytical method. *J. Chromatogr.* **1139:** 53–56 (2007).
6. R.W. Dixon and D.S. Peterson. Development and testing of a detection method for liquid chromatography based on aerosol charging. *Anal. Chem.* **74:** 2930–2937 (2002).
7. P.H. Gamache, R.S. McCarthy, S.M. Freeto, D.J. Asa, M.J. Woodcock, K. Laws, and R.O. Cole. HPLC analysis of non-volatile analytes using charged aerosol detection. *LC-GC Eur.* **18:** 345–354, (2005).
8. G.W. Peng, G.G. Jackson, and W.L. Chiou. High-pressure liquid chromatographic assay of netilmicin in plasma. *Antimicrob. Agents Chemother.* **12:** 707–709 (1977).
9. S.E. Back, I. Nilsson-Ehle, and P. Nilsson-Ehle. Chemical assay, involving liquid chromatography, for aminoglycoside antibiotics in serum. *Clin. Chem.* **25:** 1222–1225 (1979).
10. J. Marples and M.D.G. Oates. Serum gentamicin, netilmicin and tobramycin assays by high performance liquid chromatography. *J. Antimicrob. Chemother.* **10:** 311–318 (1982).
11. L. Essers. An automated high-performance liquid chromatographic method for the determination of aminoglycosides in serum using pre-column sample clean-up and derivatization. *J. Chromatogr.* **305:** 345–352 (1984).
12. M. Santos, E. Garcia, F.G. Lopez, J.M. Lanao, and A. Dominguez-Gil. Determination of netilmicin in plasma by HPLC. *J. Pharm. Biomed. Anal.* **13:** 1059–1062 (1995).
13. S. Dionisotti, F. Bamonte, M. Gamba, and E. Ongini. High-performance liquid chromatographic determination of netilmicin in guinea-pig and human serum by fluorodinitrobenzene derivatization with spectrophotometric detection. *J. Chromatogr.* **434:** 169–176 (1988).
14. H. Kubo, T. Kinoshita, Y. Kobayashi, and K. Tokunaga. Micro-Scale Method for Determination of Tobramycin in Serum Using High-Performance Liquid Chromatography. *J. Liq. Chromatogr.* **7:** 2219–2228 (1984).
15. H. Fabre, M. Sekkat, M.D. Blanchin, and B. Mandrou. Determination of aminoglycosides in pharmaceutical formulations—II. High-performance liquid chromatography. *J. Pharm. Biomed. Anal.* **7:** 1711–1718 (1989).
16. D.C. Rigge and M. F. Jones. Shelf lives of aseptically prepared medicines—stability of netilmicin injection in polypropylene syringes. *J. Pharm. Biomed. Anal.* **35:** 1251–1256 (2004).
17. B. Li, A.V. Schepdael, J. Hoogmartens, and E. Adams. Characterization of impurities in sisomicin and netilmicin by liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **22:** 3455–3471 (2008).
18. J.J. Wright, P.J.L. Daniels, A.K. Mallams, and T.L. Nagabhushan, U.S. Patent 4002742 (1977).
19. N. Nagae, T. Enami, and S. Doshi. The retention behavior of reversed-phase HPLC columns with 100% aqueous mobile phase. *LC/GC* **20:** 964–972 (2002).

Manuscript received August 5, 2009;
revision received October 15, 2009.