Determination of Vertilmicin Sulfate and Its Related Substances by HPLC–ELSD and HPLC–MS^2

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Abstract

A new and simple high-performance liquid chromatography (HPLC)–evaporative light scattering detection (ELSD) method for the determination of vertilmicin sulfate and its related substances is developed. The column is an Agilent SB-C_{18} (250 × 4.6 mm, 5 µm). The mobile phase is 0.05 mol/L trifluoroacetic acid–methanol (85:15). Good separation of vertilmicin from the main related substances is achieved. The standard curve is rectilinear in the range of 270–1350 µg/mL (r = 0.9998). The average recovery is 99.8%. The limit of detection is 10 µg/mL. The HPLC–mass spectrometry–mass spectrometry (MS^2) method is used to characterize the structures of vertilmicin sulfate and its related substances. In positive mode, vertilmicin sulfate and its related substances are elucidated by use of electrospray ion trap MS in the multi-stage MS full scan mode. The possible structure of an unknown impurity in vertilmicin is deduced based on the HPLC–MS^2 data.

Introduction

Vertilmicin sulfate belongs to a class of compounds known as aminoglycoside antibiotics. It is a novel drug that is found during the synthesis of netilmicin, and it is under registration in China. Like many other aminoglycosides, vertilmicin sulfate lacks a suitable chromophore, which is necessary for UV detection. For this reason, the analysis of vertilmicin sulfate is performed using precolumn or post-column derivation methods (1,2,3). Such methods, which need sample treatments, make the high-performance liquid chromatography (HPLC) system more complicated (such as reaction coil, extra pump, etc.) and are time-consuming. Furthermore, the disadvantages of derivation methods in terms of samples include: introduction of non-controlled impurities, degradation products, and, the most important one, the inability to detect impurities of the analyte that are not of the specific functional group required for derivation.

Evaporative light scattering detection (ELSD) is described as a universal detection mode suitable for non-absorbing analytes (4–6). The chromatographic mobile phase is nebulized with an inert gas and evaporated in a drift tube. The response does not depend on the solute-optical properties, and any compound less volatile than the mobile phase could be detected. Currently, the detector response is well described and shows a double logarithmic relationship between the signals and the concentrations of analytes. Such a response allows all molecules in a sample to give a proportional signal (which means they have the same sensitivity). This principle is applied in the search for impurities in pharmaceutical products. At the same time, the structural information of impurities in pharmaceutical products can be obtained by HPLC–mass spectrometry–mass spectrometry (MS^2) (3,7–10).

The objective of this study was to develop a rapid and simple chromatographic method, which allows a direct sample introduction without any derivation treatment. The HPLC–ELSD method was first used to determine the content of vertilmicin sulfate and its related substances. The HPLC–MS^2 method was used to characterize the structures of vertilmicin sulfate and its related substances. The HPLC system demonstrates a unique synergistic approach that uses a novel low-pH stable reversed-phase silica column and a simple mobile phase that was designed not to contain any non-volatile reagents, which permits the use of ELSD and MS for the detection and characterization of vertilmicin sulfate and its related substances.

Experimental

Chemicals and reagents

Vertilmicin sulfate standard and drug substance (batch numbers: 20040124, 20040218, and 20040328) and its injection (100 mg vertilmicin/2 mL, batch numbers: 20050530) were

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provided by Zhejiang Conler Pharmaceutical Co. (Wenzhou, China). Trifluoroacetic acid was analytical grade, and methanol was chromatographic grade.

Instrumentation

LC apparatus

An Agilent 1100 series liquid chromatography (LC) system equipped with a binary pump was connected to an Agilent G1313A autosampler (Agilent, Palo Alto, CA). Chromatographic separation was carried out at room temperature using an Agilent SB-C_{18} analytical column (250 × 4.6 mm, 5 µm). The mobile phase consisted of 0.05 mol/L trifluoroacetic acid–methanol (85:15). The flow rate was 1.0 mL/min. The detector used was a Dikma SEDEX 75 ELSD detector (Alfortville, France). The drift tube temperature was 40°C. The pressure of the nebulizing gas was 3.5 bar. GAIN was set at 4 and 6 for the assay of vertilmicin and related substances, respectively. The injection volume was 10 µL.

MS

LC–MS experimentation was carried out on an Agilent 1100 ion trap MS. The column effluent was split using a zero-dead-volume "T" connector, with approximately one quarter of the flow being fed into the MS. The mass selective detector (MSD) was equipped with an electrospray ionization source. The ionization mode was positive. The interface and MSD parameters were as the follows: nebulizer pressure, 25 psi (N_2); dry gas, N_2 (8L/min); dry gas temperature, 325°C; spray capillary voltage, 3500 V; skimmer voltage, 40 V; ion transfer capillary exit, 94 V; scan range, 100 to 1200 *m/z*; spectra average, 5; ion current control, on; target, 30000; and dwell time, 300 ms.

Software

All data acquired were processed by Agilent Chemstation Rev. A. 09.01 software.

Procedures

Sample preparation

Drug substance and its injection were simply dissolved in water to obtain a concentration level within the working range. Concentrations of vertilmicin solution were 0.6 and 5.0 mg/mL for the assay of vertilmicin and related substances, respectively.

Quantitation

An external standard calibration curve with three calibration points, ranging from 80% to 120% of the assay concentration (0.6 mg/mL), was used for the vertilmicin assay. A separate external standard calibration curve was used for the assay of low-level related substances with three calibration points ranging from 0.5% to 1.5% of the sample concentration (5.0 mg/mL). Calculations of the contents were based on peak areas and the external standard calibration curve. The best linear fit of the calibration curve data points was obtained using a logarithmic-type curve.

Results and Discussion

Development of the chromatography

Selection of the mobile phase

It is difficult to retain the aminoglycosides in the reversed-phase mode, even with purely aqueous eluents. Therefore, most chromatographic methods were based on some form of ion-pair modes or basic mobile phases. In order to permit the use of ELSD, the mobile phases cannot contain any non-volatile reagents. Perfluoranated carboxylic acids, such as heptafluorobutyric acid (5,6), were used as ion-pair reagents to facilitate the retention of aminoglycosides in the reversed-phase mode.

An Agilent Extend-C_{18} (250 × 4.6 mm, 5 µm) basic column with a basic mobile phase consisting of water–ammonium hydroxide–acetic acid (96:3.6:0.4) (4) was tested to separate vertilmicin and its impurities without adding ion-pair reagents, but the chromatogram failed to demonstrate sharp symmetrical peaks and good separation. An Agilent SB-C_{18} (250 × 4.6 mm, 5 µm) column with an acidic mobile phase consisting of 0.05 mol/L trifluoroacetic acid–methanol (85:15) or 0.05 mol/L pentafluoropropionic acid–methanol (50:50) was tested to separate vertilmicin from its impurities, and the chromatograms showed sharp symmetrical peaks and good separation.

Selection of the concentration of trifluoroacetic acid

Mobile phases consisting of various concentrations of trifluoroacetic acid (0.025, 0.05, and 0.10 mol/L) were tested in order to investigate the influence on peak shape, resolution, and retention time. The results showed that as the concentrations increased, the retention time of vertilmicin increased, and the chromatograms showed sharp symmetrical peaks and good separation. However, a high concentration (0.10 mol/L) with a pH below 2.0 would do harm to the chromatographic column. Therefore, 0.05 mol/L trifluoroacetic acid was selected as the ion-pair reagent.

Influence of organic solvent on resolution and response

The mobile phases with the presence of methanol, tetrahydrofuran, acetone, and dioxane had some influence on the resolution and response. The mobile phase with the presence of methanol was optimal to separate vertilmicin from impurity A

Table I. Influence of Organic Solvent on Resolution and ELSD Response of Vertilmicin Sulfate

Mobile phase	Response (peak area)	Resolution between vertilmicin and impurity A
0.05 mol/L Trifluoroacetic acid–methanol (85:15)	630	4.56
0.05 mol/L Trifluoroacetic acid–tetrahydrofuran (96:4)	413	3.51
0.05 mol/L Trifluoroacetic acid–acetone (92:8)	500	3.77
0.05 mol/L Trifluoroacetic	480	3.49

(the characterization of the unknown impurity is described later) and to achieve high sensitivity. Therefore, methanol was selected as the organic solvent. The results are shown in Table I. Resolutions were calculated from the expression:

$$2(t_{R2} - t_{R1}) / (W_1 + W_2)$$

where t_R is retention time and W is peak width.

Influence of retention time on the peak shape of vertilmicin

It was shown that peak shape was strongly dependent on retention time, and the increase of retention time resulted in poor peak shape. The proportion of methanol-to-water containing 0.05 mol/L trifluoroacetic acid was selected to be 15:85 in order to achieve a retention time of vertilmicin close to 7 min. It was shown that at a shorter retention time vertilmicin may overlap with impurities, but when a longer retention time was used, peak broadening and asymmetry would increase, resulting in a decrease of the ELSD response factor.

Influence of sample concentration and injection volume on peak shape

The study showed that peak shape was strongly dependent on sample concentration and injection volume. Increasing the sample concentration and injection volume resulted in poor peak shape. It was optimal that for the assay of vertilmicin and related substances, the concentrations were 0.6 and 5.0 mg/mL, respectively, and that the injection volume was 10 µL.

Optimization of ELSD conditions

In this study, a Dikma Technologies SEDEX 75 ELSD was used. Various temperatures at 40°C, 50°C, and 60°C were tested to study the influence on ELSD response and on the signal-to-noise ratio (s/n), and the results showed that there was a higher ELSD response at a lower temperature, and 40°C was optimal. Various pressures (2.5, 3.5, and 4.0 bar) were tested to study the influence on ELSD response and s/n, and the results showed that 3.5 bar was optimal. GAIN, ranging from 4 to 6, was tested, and the results showed that ELSD response increased with higher GAIN values, but resulted in increased baseline noise. When GAIN was set at 4 and 6, the limit of detection (LOD) (s/n = 3) was 15 and 10 µg/mL, respectively. Therefore, GAIN was set at 4 and 6 for the assay of vertilmicin and related substances, respectively.

Method validation

Preliminary method validation was performed to determine if the HPLC system was acceptable with respect to the specificity, linearity of response, precision, and accuracy and to determine the LOD.

Specificity

The ability of the chromatographic system to resolve vertilmicin sulfate from its possible impurities was investigated. Excipient was examined in order to assure that they did not interfere (peak overlaping) with vertilmicin. For the related substances test, samples were stored under relevant stress conditions (light, heat, acid–base hydrolysis, and oxidation, respectively).

The samples showed light and heat stability, but degradation products were produced under acid–base hydrolysis and oxidation conditions. Vertilmicin could be completely separated from impurity A, excipient, and degradation products. The chromatograms for the determination of vertilmicin sulfate and its related substances are shown in Figures 1–4.

Linearity of response

It is now well known that ELSD gives non-direct linear response. A plot of log *I* versus log *m* provides a linear response as a plot of the peak area versus the sample concentration in double logarithmic coordinates.

For the assay of vertilmicin sulfate, the linearity of response was determined by preparing, in duplicate, five vertilmicin sulfate solutions ranging from 50% to 250% of the assay concentration (0.6 mg/mL). Each solution was analyzed using the recommended HPLC system. The regression curve was obtained by plotting log(concentration) versus log(peak area). The regression equation was log A = 1.263 log C – 0.6685. The corresponding coefficient (*r*) was 0.9998. The result indicated good linearity.

For the assay of related substances (low-level linearity), five

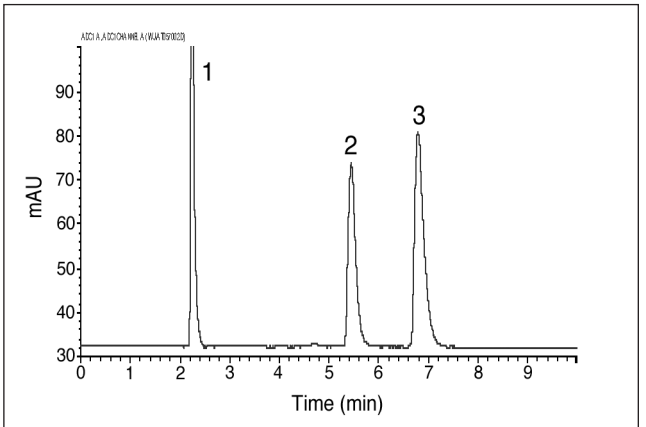

Figure 1. Chromatogram of vertilmicin spiked with impurity A. Peak numbers are: sulfate, 1; impurity A, 2; and vertilmicin, 3.

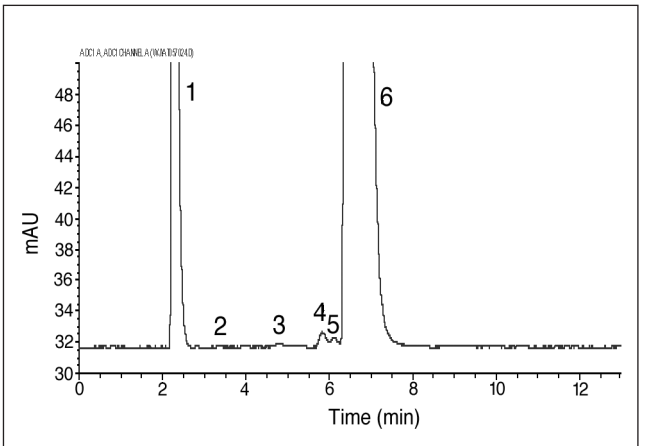

Figure 2. Chromatogram of related substances in vertilmicin sulfate. Peak numbers are: sulfate, 1; impurity A, 4; vertilmicin, 6; and other impurities, 2, 3, and 5.

vertilmicin sulfate solutions were prepared with concentration ranging from 0.5% to 2.5% of the sample concentration (5.0 mg/mL). The solutions were injected into the HPLC system. The regression curve was obtained by plotting log(concentration) versus log(peak area). The regression equation was log A = 1.332 log C – 0.8743. The corresponding coefficient (*r*) was 0.9997. The result indicated good linearity.

Precision of the assay

Sample solutions at 100% of the test concentration (0.6 mg/mL) were prepared and then assayed for vertilmicin using the recommended HPLC system. Within-day precision, expressed as the relative standard deviation (RSD), was 1.0% (*n* = 6), and between-day precision, expressed as the RSD, was 1.2% (*n* = 3).

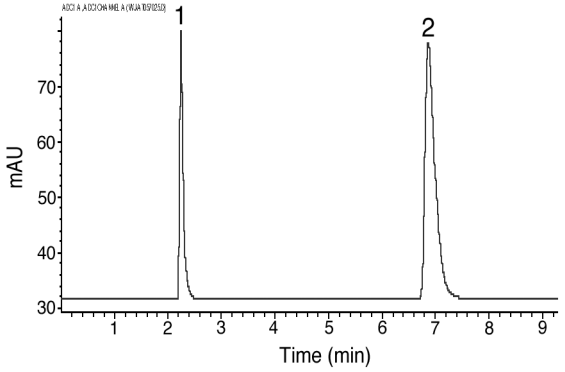

Figure 3. Chromatogram of vertilmicin sulfate assay. Peak numbers are: sulfate, 1; and sertilmicin, 2.

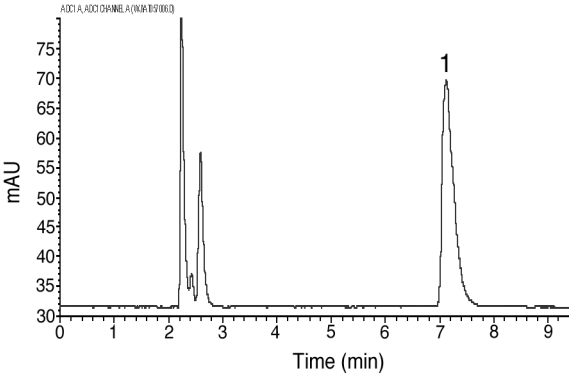

Figure 4. Chromatogram of vertilmicin sulfate injection assay. The peak number is: vertilmicin, 1.

Accuracy of the assay

The accuracy of the new method was evaluated by performing a recovery experiment. The recovery was obtained by injecting blank samples containing a known amount of vertilmicin sulfate. The average recovery was 99.8% (RSD = 1.0%) and revealed sufficient accuracy. The results of the recovery of the HPLC method are presented in Table II.

LOD

The LOD was defined as the lowest concentration of an analyte that could be accurately detected. Its determination could be made by the calculation of the s/n. A ratio of 3 was selected, and successive dilutions of the test solution gave an LOD relative to the vertilmicin peak of 0.2% (m/m). The LOD was 10 µg/mL. Such a limit was in good agreement with what is required by the Food and Drug Administration for the assay of related substances.

Analysis of vertilmicin sulfate drug substance and its injection

Three batches of drug substances and one batch of its injection were analyzed using the recommended HPLC system. The results of the analysis of vetilmicin sulfate and related substances for drug substances and its injection are shown in Table III.

The characterization of vertilmicin and impurity by HPLC–MS^2

The HPLC–MS^2 method was used to characterize the structures of vertilmicin and impurity A, contained in the vertilmicin. The mobile phase used for these experiments was described in the LC apparatus section. The eluent was the same as that described earlier for the HPLC–ELSD method.

The characterization of vertilmicin by HPLC–MS^2

In MS full scan mode, the major ion detected was a protonated molecular ion, $[M+H]^+$ *m/z* 490. It was demonstrated that vertilmicin gave abundant product ion at *m/z* 350 by loss of the C-ring (amino-α-D-glucopyranose) in MS^2 full scan mode. Furthermore, the prominent fragmentation ion at

Table II. The Recoveries of Vertilmicin Sulfate

	1	2	3	4	5	6	7	8	9
Amount spiked (mg)	9.38	9.45	9.31	11.72	11.65	11.78	14.08	14.15	14.02
Amount found (mg)	9.28	9.31	9.30	11.86	11.55	11.80	13.98	14.15	14.20
Relative Recovery (%)	98.95	98.51	99.90	101.20	99.15	100.15	99.31	99.98	101.30

m/z 191 was formed from the fragmentation ion at *m/z* 350 by loss of the A-ring (amino-α-D-glucopyranose), though the characteristic fragmentation ion at *m/z* 160 was formed from the fragmentation ion at *m/z* 350 by loss of the B-ring (2-deoxy-D-streptamine). Figure 5 shows the mass spectrum of vertilmicin. The proposed scheme for the fragmentation of vertilmicin in the multi-stage MS full scan mode is shown in Figure 6.

Table III. The Results of Analysis of Vertilmicin Sulfate and Its Related Substances (*n* = 2)

Batches	Content of vertilmicin (%)	Content of impurity A (%)	Content of total impurity (%)
20040124	55.7	0.4	0.9
20040218	54.1	0.5	0.9
20040328	56.8	0.4	0.8
20040530	102.5	0.3	1.0

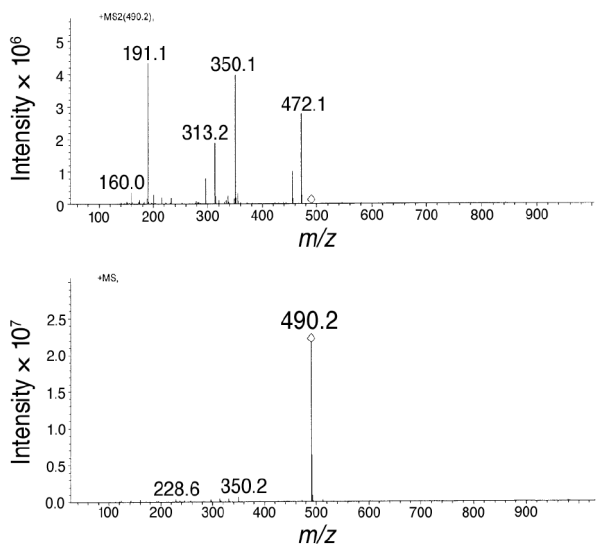

Figure 5. (+)-electrospray ionization–MS^2 spectra of vertilmicin.

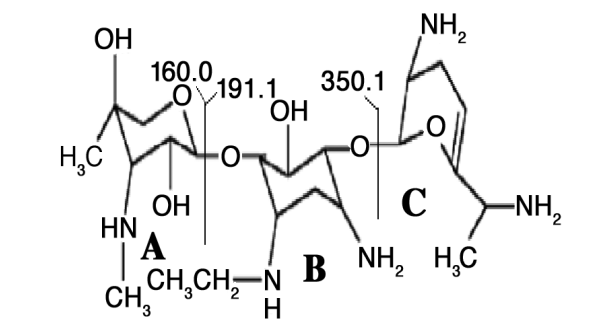

Figure 6. Chemical structure and the ESI–MS^2 fragmentation pathways of vertilmicin.

The characterization of unknown impurity by HPLC–MS^2

Figure 2 shows that an impurity appeared repeatedly above a 0.3% level. In MS full scan mode, the major ion detected was a protonated molecular ion, $[M+H]^+$ *m/z* 462. It was demonstrated that vertilmicin gave an abundant product ion at *m/z* 322 by the loss of the C-ring (amino-α-D-glucopyranose) in MS^2 full scan mode. Furthermore, the prominent fragmentation ion at *m/z* 163 was formed from the fragmentation ion at *m/z* 322 by the loss of the A-ring (amino-α-D-glucopyranose), though the characteristic fragmentation ion at *m/z* 160 was formed from the fragmentation ion at *m/z* 322 by the loss of the B-ring (2-deoxy-D-streptamine). The possible structure of the unknown impurity in vertilmicin was deduced based on the HPLC–MS^2 data (the structure is shown in Figure 7). The impurity was purified, and NMR (^1H NMR, ^{13}C NMR, DEPT, HMQC, and HMBC) experiments were conductted to confirm its structure. The impurity was named as impurity A. Figure 8 shows the mass spectrum of impurity A. The proposed scheme for fragmentation of impurity A in the multi-stage MS full scan mode is shown in Figure 7.

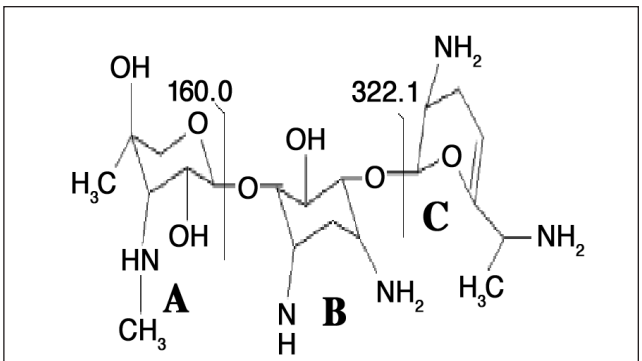

Figure 7. Chemical structure and the ESI–MS^2 fragmentation pathways of impurity A.

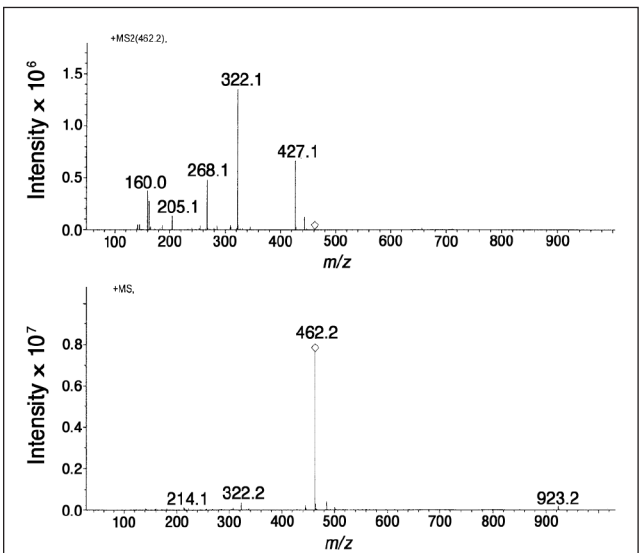

Figure 8. (+)-Electrospray ionization–MS^2 spectra of impurity A.

Conclusion

The described HPLC–ELSD method provides a rapid and simple analysis for vertilmicin sulfate and its related substances without derivation. The method is accurate and reproducible. The structural information was obtained via collision-activated dissociation and these characteristics are applicable to the structural elucidation of vertilmicin sulfate and its related substances.

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