

ORIGINAL ARTICLE

Characterization of impurities in commercial spectinomycin by liquid chromatography with electrospray ionization tandem mass spectrometry

Yan Wang, Mingjuan Wang, Jin Li, Shangchen Yao, Jing Xue, Wenbo Zou and Changqin Hu

Analysis of commercial spectinomycin samples with ion-pairing reversed-phase LC coupled with electrospray ionization tandem MS (LC/ESI-MS/MS) indicates that eight additional compounds are present, including actinamine, (4*R*)-dihydrospectinomycin, (4*S*)-dihydrospectinomycin and dihydroxypectinomycin, as well as four new impurities reported, to our knowledge, for the first time. The structures of these compounds were elucidated by comparing their fragmentation patterns with known structures, and NMR was employed to characterize and distinguish (4*R*)-dihydrospectinomycin and (4*S*)-dihydrospectinomycin. Identification of dihydroxypectinomycin isomers is necessary because (4*R*)-dihydrospectinomycin is a minor active pharmaceutical ingredient of spectinomycin, whereas (4*S*)-dihydrospectinomycin is considered to be an impurity (impurity C) by the European Pharmacopoeia (Ph. Eur.).

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INTRODUCTION

Spectinomycin is a broad-spectrum aminoglycoside antibiotic that has been widely used to treat human and animal infections. Spectinomycin was originally developed to treat *Neisseria gonorrhoeae* infections in patients allergic to penicillin, but it is presently used to treat various aerobic Gram-negative and Gram-positive organisms¹ and *Mycoplasma sp.*^{2,3} in various bacterial respiratory and enteric infections.

Spectinomycin is produced by *Streptomyces spectabilis*. Spectinomycin contains several biosynthetically related compounds including (4*S*)-dihydrospectinomycin, (4*R*)-dihydrospectinomycin and dihydroxypectinomycin.^{4,5} Strong acidic hydrolysis can produce actinamine (impurity A), and in basic solutions, actinospectinoic acid (impurity B) is formed.⁶ Other impurities have been described in the European Pharmacopoeia (Ph. Eur.)⁷ (Figure 1).

Pharmaceutical safety is influenced by impurities present in manufactured products,^{8–11} and impurities may depend on the manufacturing process and compromise product quality. Thus, impurity characterization is necessary to ensure the safety of commercial spectinomycin.

Evaporative light scattering detection (ELSD) is a universal approach for analyzing many non-UV-absorbing analytes.^{6,12–19} Frequently, the mobile phase from ELSD is transferred to MS detection because it contains volatile salts, unlike pulsed

electrochemical detection that has no volatile mobile phase.^{20,21} Thus, MS can elucidate structural impurities in various aminoglycosides.^{22–26}

Specifically, spectinomycin analysis by LC/tandem MS (LC-MSⁿ) has been reported by Wang *et al.*²⁷ Impurities—C ((4*S*)-dihydroxypectinomycin) and D (dihydroxypectinomycin)—were identified in commercial spectinomycin. However, dihydroxypectinomycin isomers ((4*R*)- or (4*S*)- dihydroxypectinomycin) could not be distinguished by LC-MSⁿ only and required further analysis including NMR. Meanwhile, several other impurities exceeding the International Conference on Harmonization (ICH) identification threshold¹¹ were also observed in commercial spectinomycin and required comprehensive characterization.

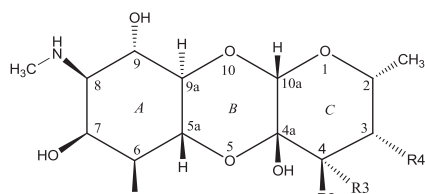
Here we report an improved volatile LC-ELSD method based on the current literature²⁷ that we then improved with MS. Impurities within seven commercial samples from five manufacturers were compared, and eight related substances were fully characterized by the LC system coupled with electrospray ionization MS (LC/ESI-MS/MS) in commercial spectinomycin samples including the minor active pharmaceutical ingredient (API) (4*R*)-dihydroxypectinomycin. With NMR, we characterized the stereo-structure of dihydroxypectinomycin ((4*R*)- or (4*S*)- dihydroxypectinomycin) obtained by reduction of spectinomycin with sodium borohydride (NaBH₄) in methanol. Three known related substances (impurities A,

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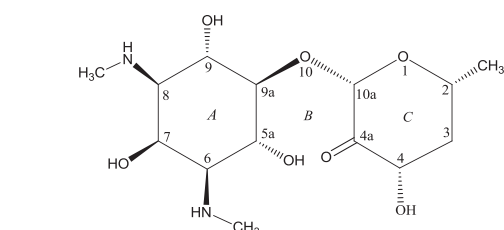
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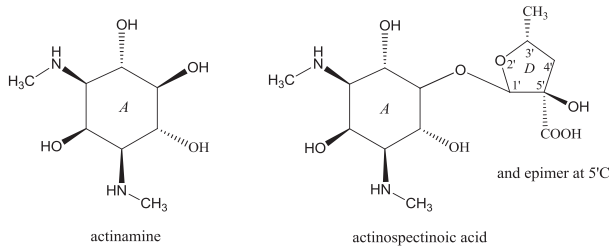
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	[M+H] ⁺	R1	R2	R3	R4	Impurity	Comment ^a
spectinomycin	333.3	CH ₃	R2+R3=O	H	H		Major API
(4R)-dihydrospectinomycin	335.4	CH ₃	OH	H	H		Minor API
(4S)-dihydrospectinomycin	335.4	CH ₃	H	OH	H	C	Known impurity
dihydroxyspectinomycin	351.4	CH ₃	H	OH	OH	D	Known impurity
N-desmethylspectinomycin	319.3	H	R2+R3=O	H	H	E	Known impurity
tetrahydroxyspectinomycin	349.3	CH ₃	R2+R3=O	OH	OH	G	Known impurity

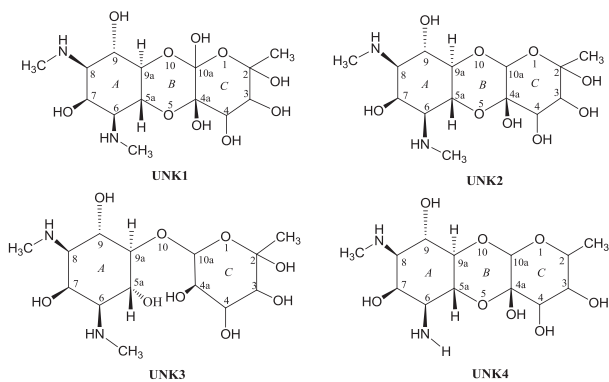


	[M+H] ⁺	Impurity	Comment ^a
triol spectinomycin	335.4	F	Known impurity



	[M+H] ⁺	Impurity	Comment ^a
actinamine	207.3	A	
actinospectinoic acid	351.4	B	Known impurities

^aSymbolizes that the descriptors of compounds are consistent with those presented in Ph. Eur. 7.0.



Peak ^b	[M+H] ⁺	Proposed name	
UNK1	2	383.3	heptahydroxyspectinomycin
UNK2	3	367.3	hexahydroxyspectinomycin
UNK3	4	369.3	triol trihydroxyspectinomycin
UNK4	6	337.3	N-desmethyl dihydroxyspectinomycin

^bSee Figure 2 for the peak numbers.

Figure 1 Spectinomycin structures, known impurities and proposed structures.

C and D) and four new yet undescribed substances were also identified.

RESULTS AND DISCUSSION

Development and optimization of the LC-MS method

First, 0.1 M trifluoroacetic acid (TFA) aqueous solution for isocratic elution was selected as a mobile phase because it offered good separation and a sharp peak with LC-ELSD.²⁷ Optimized LC-ELSD system specificity was confirmed by separation of sample 1 and the impurity mixture solution. This approach was validated with a pulsed electrochemical detection method described in the Ph. Eur.⁷ The final LC-ELSD method is detailed in Methods/Experimental section.

Next, TSKgel C₁₈, Venusil C₁₈ and Phenomenex C₁₈ columns were selected as recommended by the antibiotic-column selection database.²⁸ Considering the total analysis time and peak shape, the TSKgel ODS-100V column was most suitable for MS because of its small particle size and short column length. Data repeatability and robustness offered by the column were validated.

LC conditions were then optimized. Because of ion suppression produced by TFA, a lower concentration of TFA (0.04 M) and postcolumn addition of 70% methanol (0.5 ml min⁻¹) were applied to enhance ionization to stabilize the spray and increase the signals. Concurrently, to increase the amount of impurities injected, ensure detection and minimize possible contamination caused by the principal peak, valve switching technology was employed to remove the concentrated main peak of spectinomycin (from 10.5 to 12 min) during characterization of related substances in commercial spectinomycin. A typical chromatogram of sample 1 obtained by LC/ESI-MS is shown in Figure 2.

Fragmentation pathways of known compounds by LC/ESI-MS/MS

For convenience, the three rings of spectinomycin and its impurities A, B and C, and carbons and oxygen in the structure are also numbered (Figure 1). The precursor ions [M+H]⁺ of spectinomycin (API), actinamine and (4S)-dihydrospectinomycin can be easily observed at *m/z* 333, 207 and 335, respectively, in accordance with their theoretical molecular weights. Their corresponding adduct ions of [M+Na]⁺ further confirmed each [M+H]⁺. Their fragmentation pathways are shown in Figure 3a.

Chemical structures of spectinomycin and its known impurities listed in the Ph. Eur.⁷ indicate that fragile positions are ring C groups (that is, hydroxyls) and not ring A groups during fermentation. The abundance of the product ion at *m/z* 207 indicates that hydroxylation sites mostly likely occur in ring C, partly because of the strong steric effect from the two methylamino groups in ring A. The protonated molecules at *m/z* 333 and 335 all yield the characteristic product ions at *m/z* 207 (F1), 189 (F2) and 158 (F3) by the consecutive loss of ring C (-126u (spectinomycin)/-128u ((4S)-dihydrospectinomycin)), water (-18u) and a methylamino group (-31u), respectively. This also indicates the presence of a nonmodified ring A that was confirmed by actinamine MS² spectra. The loss of water at the C-4 position (-18u) was observed (product ion F4 at *m/z* 317) in the (4S)-dihydrospectinomycin MS² spectrum. A loss of spectinomycin was 28u. This is a product ion F4 at *m/z* 305, corresponding to a loss of carbon monoxide. The product ion F5 corresponding to the loss of the acetaldehyde group (CH₃CHO, -44u) from ring C confirms the substituent group(s) at C-4 and C-3. For example, the product ions F5 of spectinomycin at *m/z* 289 and (4S)-dihydrospectinomycin at *m/z* 291 verify that their C-4 substituents are carbonyl (=O) and hydroxyl (-OH), respectively.

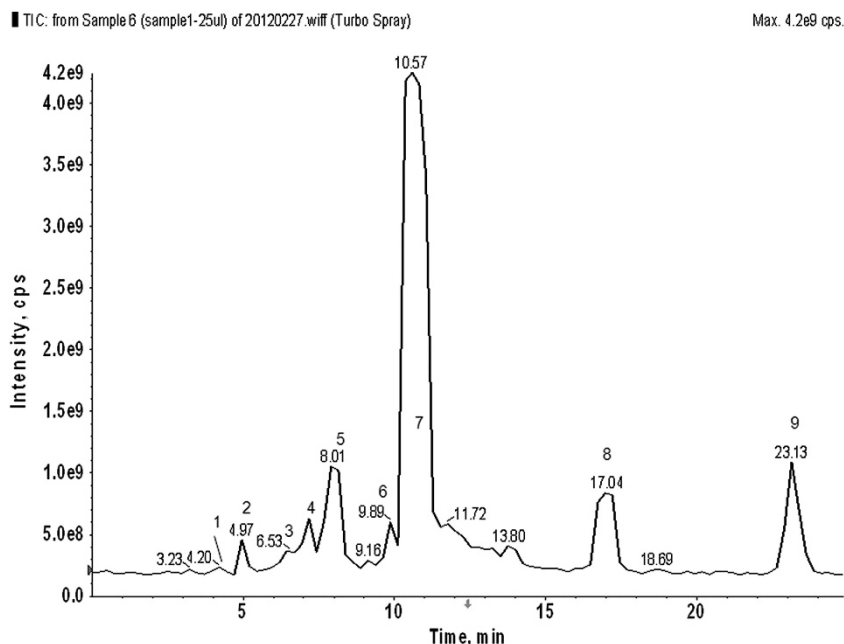


Figure 2 A typical total ion current (TIC) chromatogram of sample 1. 1, actinamine (impurity A, m/z 207); 2, UNK1^a (m/z 383); 3, UNK2^a (m/z 367); 4, UNK3^a (m/z 369); 5, dihydroxypectinomycin (impurity D, m/z 351); 6, UNK4^a (m/z 337); 7, spectinomycin (major active pharmaceutical ingredient (API), m/z 333); 8, (4*R*)-dihydroxypectinomycin (minor API, m/z 335); 9, (4*S*)-dihydroxypectinomycin (impurity C, m/z 335). UNK (unknown): new impurities described in this paper. ^aSee Figure 1 for their structures. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Characterization of the stereo-structure of dihydroxypectinomycin obtained by reduction of spectinomycin with NaBH₄ in methanol by NMR

Identification of dihydroxypectinomycin isomers is critical: (4*R*)-dihydroxypectinomycin is a minor API component of spectinomycin, whereas (4*S*)-dihydroxypectinomycin is an impurity (impurity C, see Figure 1).⁷ Dihydroxypectinomycin is produced by reduction of spectinomycin with NaBH₄ in methanol (see Methods/Experimental section) and NMR spectroscopy confirmed its configuration, including the C-4 in ring C. The assignments of C and H atoms of dihydroxypectinomycin were verified based on its ¹H- and ¹³C- NMR data (see Table 1). Two-dimensional experiments such as heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation were employed to assign spectra, especially for two methylamino groups. More importantly, the absolute configuration of the stereocenter of C-4 was identified with ¹H–¹H rotating-frame Overhauser effect spectroscopy (ROESY) data (Table 1). The ROESY spectrum clearly shows a correlation between H-10a and H-2, both of which correlate to H-4. This suggests that the three protons are adjacent to each other and on the same side of ring C. Because the absolute configurations of C-10a and C-2 were known as 10a*S* and 2*R*, respectively, the absolute configuration of C-4 in the structure of dihydroxypectinomycin was thus characterized as 4*S*.

Investigation of compounds present in commercial bulk samples of spectinomycin

Seven commercial samples from different origins were screened for impurities using LC-ELSD and LC-MS (Table 2). A double logarithmic curve obtained with reference solutions was used to quantify these impurities.

Based on the fragmentation behavior of known components (Figure 3a) and knowledge of known fragmentation mechanisms,²⁹ the impurities in different samples were investigated by LC-MSⁿ.

Eight related substances were identified in the commercial samples including (4*R*)-dihydroxypectinomycin (minor API), actinamine (impurity A), (4*S*)-dihydroxypectinomycin (impurity C) and dihydroxypectinomycin (impurity D). In addition, four new related substances were identified that, to the best of our knowledge, have not been reported. The proposed structures of these compounds are shown in Figure 1.

Interpretation of the compounds present in sample 1. The [M + H]⁺ collision-induced dissociation (CID) spectra acquired for the five related substances observed in sample 1 are shown in Figure 4. The compound in peak 1 ([M + H]⁺ m/z 207) was identified as actinamine (impurity A)⁷ because the [M + H]⁺ CID spectrum and the retention time (RT) are the same as those of the actinamine reference substance. The characteristic product ions of ring A at m/z 207 (F1), 189 (F2) and 158 (F3) were also observed in the [M + H]⁺ CID spectra of other compounds, except UNK4.

The loss of ring C (–176u) with the cleavage at ring B observed in the [M + H]⁺ CID spectrum of compound UNK1 ([M + H]⁺ m/z 383) in peak 2 (Figure 4a) is 48u more than the corresponding loss (–128u, that is, 335–207 = 128u) obtained with (4*S*)-dihydroxypectinomycin. Its much shorter RT and abundant losses of water molecules (–18u) in its [M + H]⁺ CID spectrum revealed that the 48u difference may be because of the presence of three additional polar hydroxyl groups in ring C. No product ion corresponding to the loss of methanol ([M + H–CH₃OH]⁺, m/z 351) was observed, indicating its C2–CH₃ was not replaced by –CH₂OH. Thus, the possible positions of the additional hydroxyl groups were identified as C3, C2 and C-10a, respectively, in comparison with the structure of (4*S*)-dihydroxypectinomycin.

The proposed structure of UNK1 (see Figure 1) can be further demonstrated by the following observations: (1) after consecutive losses of two water molecules, keto–enol tautomerism of the product

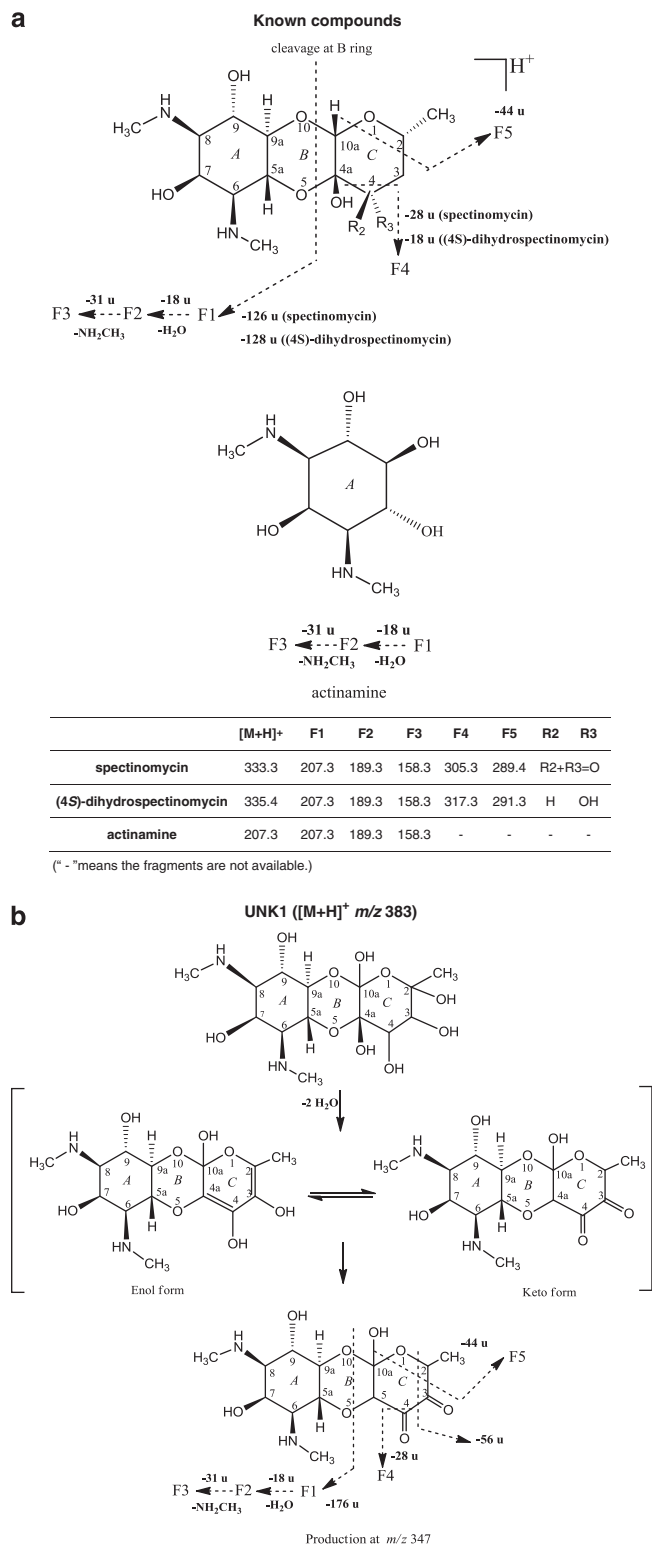


Figure 3 Plausible fragmentation pathways of known compounds (a) and that of UNK1 ($[M+H]^+$ m/z 383) (b).

ion at m/z 347 may occur, as shown in Figure 3b. The subsequent neutral loss of 28u was because of the loss of carbon monoxide at C-4 in accordance with that of spectinomycin (F4). (2) Next, the subsequent loss of 44u was in accordance with that of spectinomycin

or (4S)-dihydrospectinomycin (product ion: F5) as shown in Figure 3a, confirming its C2-CH₃ was normal. (3) The loss of 56u may be because of the combined loss of ethylene (CH₂=CH₂, -28u) and carbon monoxide (-28u). The plausible fragmentation pathway of UNK1 is shown in Figure 3b.

The $[M+H]^+$ CID spectrum of UNK2 ($[M+H]^+$ m/z 367) in peak 3 is shown in Figure 4b. Similar to UNK1, its loss of ring C (-160u) is 32u higher than that of (4S)-dihydrospectinomycin (-128u). The shorter RT and abundant losses of water molecules in its $[M+H]^+$ CID spectrum indicate that two additional hydroxyl groups are located in ring C. The subsequent losses of 28u, 44u and 56u are similar to those of UNK1 (Figure 3b). Based on these results, the proposed structure of UNK2 is shown in Figure 1.

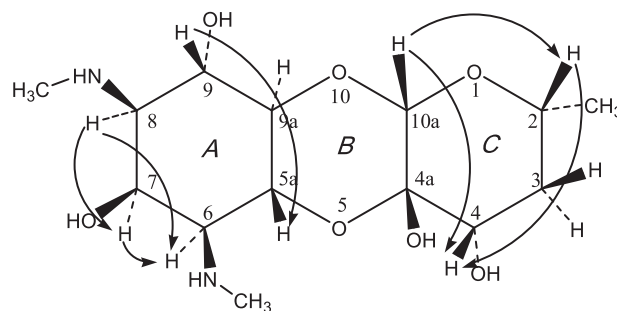
The RTs of UNK3 and UNK2 are close to each other, indicating similar polarities. The protonated molecule of compound UNK3 ($[M+H]^+$ m/z 369) in peak 4 and its loss of ring C (-162u) (see Figure 4c) are both 2u higher than those of UNK2. This suggests UNK3 may be the ring-opened derivative of UNK2. The abundant losses of water molecules (3 × 18u) in its $[M+H]^+$ CID spectrum confirmed the presence of the additional hydroxyl groups in ring C. The loss of 28u (carbon monoxide) was because of keto-enol tautomerism after water molecule loss, similar to that of UNK1. The possible structure of UNK3 is shown in Figure 1.

The compound in peak 5 ($[M+H]^+$ m/z 351) was identified as dihydroxyspectinomycin (impurity D).⁷ As shown in Figure 4d, the characteristic ions at m/z 207 (F1), 189 (F2) and 158 (F3) revealed the presence of a normal ring A. The loss of ring C (-144u) is 16u higher than the corresponding loss (-128u) obtained with (4S)-dihydroxyspectinomycin and this indicates one additional hydroxyl group is located in ring C. Its shorter RT versus (4S)-dihydroxyspectinomycin confirmed that peak 5 has one additional hydroxyl group. Its fragmentation behavior after loss of one water (-18u) is similar to that of (4S)-dihydroxyspectinomycin (that is, the subsequent loss of one water (-18u) or acetaldehyde (-44u)) that further confirmed the compound in peak 5 was dihydroxyspectinomycin. Its fragmentation pathway agreed with reports in the literature.²⁷

The $[M+H]^+$ CID spectrum acquired for the compound (UNK4) in peak 6 ($[M+H]^+$ m/z 337) is shown in Figure 4e. Unlike other compounds, its product ions F1, F2 and F3 were at m/z 193, 175 and 144—all 14u less than the normal ones (207 [F1], 189 [F2] and 158 [F3]). This suggests its ring A was aberrant with 6-*N*-desmethyl or 8-*N*-desmethyl. Two hydroxyl groups adjacent to methylamino group at C8 could produce a stronger steric effect on the methylamino group at C8 than that at C6. Thus, the compound with 6-*N*-desmethyl is more likely to be present in the fermentation process of spectinomycin. An aberrant ring A was proposed based on the structure of *N*-desmethyl-spectinomycin (impurity E, see Figure 1). The other fragmentation behaviors were similar to those of dihydroxyspectinomycin. Thus, peak 6 in UNK4 ($[M+H]^+$ m/z 337) was identified as *N*-desmethyldihydroxyspectinomycin (see Figure 1).

The protonated molecules ($[M+H]^+$ m/z 335) and the $[M+H]^+$ CID spectra of the compounds in peaks 8 and 9 are the same as those of (4S)-dihydroxyspectinomycin and this indicates that they are isomers. The RT of the compound in peak 9 was the same as that of (4S)-dihydroxyspectinomycin prepared in this study. Thus, the compounds corresponding to peaks 8 and 9 were identified as (4*R*)-dihydroxyspectinomycin and (4*S*)-dihydroxyspectinomycin, respectively.

Comparison of different bulk samples. The impurity profiles of seven commercial samples from five different manufacturers were investigated by both LC-MS and LC-ELSD (Table 2). The samples had

Table 1 Key ^1H - ^1H ROESY correlations of dihydrospectinomycin obtained by reduction of spectinomycin with NaBH_4 in methanol


Carbon no.	^{13}C	^1H	^1H - ^1H ROESY
2	67.4	3.58 (1H, m)	4.47 (1H, s); 3.46 (1H, d, $J=6.5$ Hz)
2- CH_3	20.9	1.13 (1H, d, $J=6.5$ Hz)	—
3	38.8	1.45 (1H, m), 1.66 (1H, m)	—
4	71.8	3.46 (1H, d, $J=6.5$ Hz)	3.58 (1H, m); 4.47 (1H, s)
4a	90.7	—	—
5a	66.3	3.60–3.66 (1H, m)	4.15 (1H, t, $J=10.5$ Hz)
6	61.3	3.10 (1H, m)	4.63 (1H, s); 3.25 (1H, m)
6-NH CH_3	30.3	2.65 (3H, s)	—
7	60.0	4.63 (1H, s)	3.25 (1H, m); 3.10 (1H, m)
8	57.7	3.25 (1H, m)	3.10 (1H, m); 4.63 (1H, s)
8-NH CH_3	31.1	2.65 (3H, s)	—
9	64.6	4.15 (1H, t, $J=10.5$ Hz)	3.60–3.66 (1H, m)
9a	70.9	3.60–3.66 (1H, m)	—
10a	95.0	4.47 (1H, s)	3.58 (1H, m); 3.46 (1H, d, $J=6.5$ Hz)

Abbreviations: d, doublet; m, multiplet; ROESY, rotating-frame Overhauser effect spectroscopy; s, singlet; t, triplet. The table shows 500 MHz, $\text{DMSO}-d_6$, δ scale in p.p.m and J in Hz. The symbol '—' indicates that no signals were detected.

Table 2 Comparison of impurities of seven spectinomycin bulk samples by LC-MS and LC-ELSD

Samples		Contents of impurities present in bulk samples (%)						
No.	Company	UNK1 ^a	UNK2 ^a	UNK3 ^a	Dihydroxyspectinomycin	UNK4 ^a	(4 <i>R</i>)-dihydrospectinomycin	(4 <i>S</i>)-dihydrospectinomycin
1	A	0.4	0.3	1.3	3.8	0.4	1.1	1.2
2		—	—	0.3	2.5	0.6	3.9	0.8
3	B	—	0.2	0.4	4.2	0.5	1.5	—
4	C	—	0.2	0.4	4.2	0.6	0.4	—
5	D	—	—	0.4	0.8	0.7	1.7	—
6		—	—	0.2	1.0	0.5	1.7	—
7	E	—	0.1	0.2	1.5	0.4	—	—

Abbreviation: ELSD, evaporative light scattering detection. The symbol '—' indicates that impurity was not detected in the sample. ^aTheir structures are shown in Figure 1.

unique impurities that are not uncommon because different strains can be used for the fermentation processes. The largest impurity was dihydroxyspectinomycin (impurity D). (4*R*)-dihydrospectinomycin (minor API) was present in almost all samples, whereas its stereo-isomer of (4*S*)-dihydrospectinomycin (impurity) was not. The newly identified impurities, such as UNKs 3 and 4, were also found in all samples. In contrast, impurities E, F and G were not detected in all samples. There is a significant difference between the impurity profiles of samples 1 and 2, although both were from company A, suggesting that production or the fermentation process might be inconsistent.

METHODS/EXPERIMENTAL SECTION

Reagents and samples

HPLC-grade methanol was obtained from Fisher Scientific (Fairlawn, NJ, USA). HPLC-grade TFA was supplied by Merk-Schuchardt (Hohenbrunn, Germany). A Milli-Q water purification system (Millipore, Billerica, MA, USA) was used to further purify glass-distilled water.

Reference samples of spectinomycin and impurities A (actinamine) and B (actinospectinoic acid) were provided by National Institutes for Food and Drug Control, Beijing, People's Republic of China. The descriptors of impurities A–G are consistent with those presented in Ph. Eur. 7.0.⁷ Seven batches of samples 1–7 of spectinomycin were obtained from five different manufacturers (companies A–E).

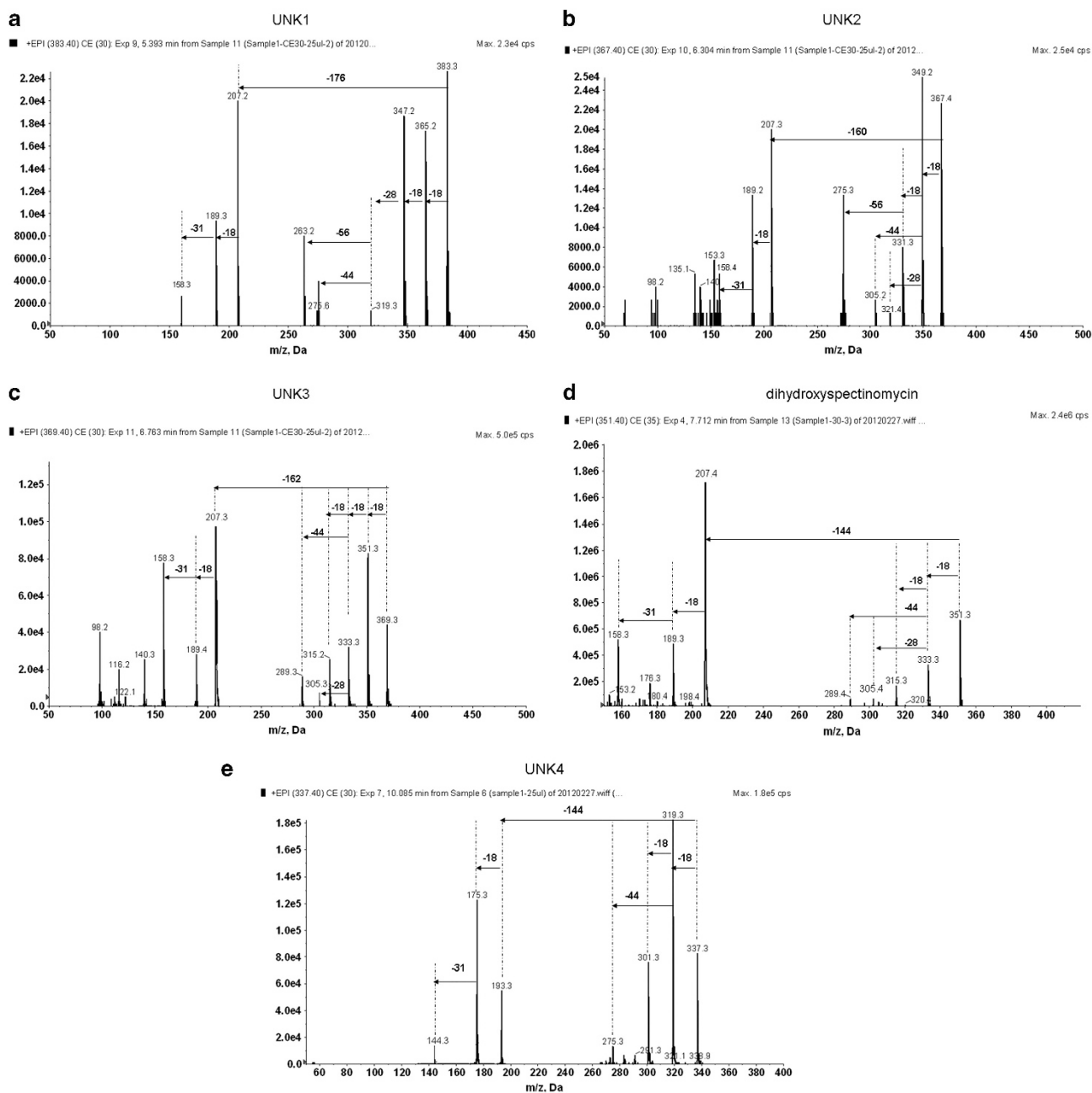


Figure 4 $[M+H]^+$ collision-induced dissociation (CID) spectra acquired for the compounds in peaks 2, 3, 4, 5 and 6. These are the result of isolation and collisional activation of the precursor ions at m/z 383 (a), 367 (b), 369 (c), 351 (d) and 337 (e) (from top to bottom), respectively.

Dihydroxypectinomycin (sodium salt) was prepared as follows: 18.9 g of spectinomycin hydrochloride was added to 625 ml of methanol, and the solution was stirred for 30 min until opacification. Gaseous ammonia was then introduced into the solution to adjust the pH to 10.5. Then, 6.4 g of NaBH_4 was added gradually while cooling on an ice bath. After standing for ~ 2.5 h at ambient temperature, the solution was filtered and adjusted to pH 3.0 with 6 M hydrochloric acid. The residue obtained by vacuum evaporation at room temperature was then added to 188 ml of methanol and evaporated again. The operation was repeated in another 63 ml of methanol and filtered. To this second solution, 250 ml of ether was added and the precipitate was removed by filtration/evaporation at room temperature (purity by ELSD $\geq 95\%$).

Solution preparation

A mixture of actinamine, actinospectinoic acid, spectinomycin and dihydroxypectinomycin in distilled water was prepared at 0.1 mg ml^{-1} each as the

impurity mixture solution. Sample solutions in distilled water were prepared at 6 mg ml^{-1} . Reference solutions were 0.03 , 0.12 and 0.3 mg ml^{-1} (corresponding to 0.5 – 5.0% of the sample concentration). Sample solutions were prepared immediately before use to avoid anomer formation. Dihydroxypectinomycin was freshly prepared in deuterated DMSO at $\sim 20 \text{ mg ml}^{-1}$ for the NMR experiment.

Instrumentation

The LC-ELSD was performed on a Shimadzu LC-20A HPLC (Kyoto, Japan) system equipped with LC-20AT binary pumps, a DGU-20A₃ vacuum degasser, a SIL-20AC autosampler, a CTO-10AS VP column oven, an Alltech 2000ES ELSD (Deerfield, IL, USA), an automatic air source generator (Type GCK3308, BCHP, Beijing, China) and Shimadzu LC-solution software was used for data processing.

LC/ESI-MS/MS consisted of a 3201 S1-2 binary pump, a 3202 S1-2 vacuum degasser, a 3014 S1-2 column heater, a 3012 S1-2 column switch system, a 3133

S1-2 sampler from SHISEIDO (Tokyo, Japan), a post-column pump of SHIMADZU LC-10AT and a 3200Q TRAP mass detector (Applied Biosystems, Foster city, CA, USA) controlled by Analyst software (version 1.4.2, MDS/Sciex, Concord, ON, Canada). NMR experiments were performed on an AV500-III high-resolution NMR spectrometer (500 MHz) (Bruker, Bio Spin Corporation, Billerica, MA, USA).

Chromatographic conditions

The mobile phase for LC-ELSD contained 0.1 M of TFA in water for isocratic elution without organic solvent (0.5 ml min⁻¹). Compounds were separated on a TSKgel C₁₈ column (part number: ODS-100V, size: 150 mm × 4.6 mm i.d., 3 μm; TOSOH, Tokyo, Japan). Other columns, including Venusil C₁₈ column (type: ASB, size: 150 mm × 4.6 mm i.d., 5 μm; Agela, Tianjin, China) and Phenomenex C₁₈ column (type: Gemini, size: 250 mm × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA), were also used. Column temperature was maintained at 30 °C and the injection volume was 20 μl. ELSD parameters were as follows: temperature of the drift tube = 110 °C, nitrogen was the carrier gas (2.61 min⁻¹), and gain was 1; the impactor was off.

Chromatographic conditions for LC/ESI-MS/MS were the same as that of LC-ELSD except that TFA was decreased to 0.04 M. A mixture of methanol and water (70:30, v/v) was added postcolumn in a 1:1 proportion with the LC mobile phase at a flow rate of 0.5 ml min⁻¹.

MS

Optimization and MS/MS analysis of spectinomycin samples were carried out according to the instrument manufacturer's instructions. ESI was operated in positive ion mode with an enhanced MS scanning mode for MS1 and enhanced product ion scanning mode for MS2. The optimized MS conditions were ESI-positive ionization mode, declustering potential of 60 V, entrance potential of 10 V, collision energy of 30 V, curtain gas: 20.0 l min⁻¹, ion source gas 1: 65.0 l min⁻¹, ion source gas 2: 60.0 l min⁻¹, ion spray: 5500.0 V, temperature: 500.0 °C and interface heater: on. The acquisition of the first-order mass spectra (Q1 scan) was from 100 to 500 u. The ions of interest were isolated in the ion trap and activated at collision energy of 30 V to get enhanced product ion spectra in the range of 50–400 u. The 'Precursor Ion (Prec)' and 'Neutral Loss (NL)' methods of Analyst software were also used to confirm the individual protonated molecules and proposed fragmentation pathways. Valve switching cut off the major spectinomycin peak during impurity characterization.

NMR spectroscopy

The ¹H, ¹³C and ¹H–¹H ROESY as well as heteronuclear multiple-bond correlation and heteronuclear single quantum coherence NMR experiments were employed to elucidate the stereo-structure of dihydrospectinomycin. The two possible structures—(4R)- or (4S)-dihydrospectinomycin—were obtained by reduction of spectinomycin with NaBH₄ in methanol. NMR experiments were performed in DMSO at 25 °C. The ¹H, ¹³C and ¹H–¹H ROESY chemical shifts were reported on the δ scale in p.p.m. relative DMSO-*d*₆, respectively. A relaxation delay of 1 s and a mixing time of 300 ms were used in the ROESY experiment.

Experimental details on NMR data are available as Supplementary Information.

CONCLUSION

Using the LC-MSⁿ capability of the 3200Q TRAP mass spectrometer, several impurities in commercial spectinomycin samples were characterized: four known compounds and four new substances. The stereo-structure of dihydrospectinomycin obtained by reduction of spectinomycin with NaBH₄ in methanol was identified as (4S)-dihydrospectinomycin according to ¹H–¹H ROESY NMR. Spectinomycin sample impurities from the five manufacturers were different with respect to type and amount according to LC-MSⁿ and LC-ELSD. The major impurities observed were dihydroxyspectinomycin (impurity D) and the minor API (4R)-dihydrospectinomycin, both of which arise from fermentation.

Meanwhile, because isomers could not be distinguished by only LC-MS, the spatial configuration of the chiral carbons in ring C of the four new related substances require confirmation by other techniques, such as NMR. However, because of their low abundance and the difficulty in purification of each, these experiments may be challenging.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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