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Determination of spectinomycin hydrochloride and its related substances by HPLC–ELSD and HPLC–MS^n

Jian Wang [a,*], Xiaojun Hu [b], Ying Tu [a], Kunyi Ni [a]

[a] *Department of Analytical Chemistry, China Pharmaceutical University, Nanjing 210009, PR China*
[b] *Medical College, Zhejiang University, Hangzhou 310006, PR China*

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Abstract

A new and simple high-performance liquid chromatography–evaporative light scattering detection (HPLC–ELSD) method for the determination of spectinomycin hydrochloride and its related substances was developed. The column was Agilent SB-C_{18} (250 mm × 4.6 mm, 5 μm).The mobile phase was 25 mM trifluoroacetic acid. The drift tube temperature was 40 °C. The pressure of nebulizing gas was 3.5 bar. Good separation of spectinomycin from main related substances could be achieved. The standard curve was rectilinear in the range of 0.07–3.8 mg/ml ($r = 0.9997$). Precision was 1.0% (R.S.D.). The limit of detection was 6 μg/ml. The method is simple and rapid, and the results are accurate and reproducible. The HPLC–MS^n method was used to characterize the structures of impurities contained in the spectinomycin. In positive mode, impurities were elucidated by use of electrospray ion trap mass spectrometry in the multi-stage MS full scan mode. The possible structures of impurities C and D in spectinomycin were deduced based on the HPLC–MS^n data.

Keywords: Spectinomycin hydrochloride; Impurities; HPLC–ELSD; HPLC–MS^n

1. Introduction

Spectinomycin hydrochloride belongs to a class of compounds known as aminoglycoside antibiotics. Like many other aminoglycosides, spectinomycin hydrochloride lacks a suitable chromophore, which is necessary for UV detection. For this reason, the analysis of spectinomycin hydrochloride is performed using pre-column or post-column derivation methods [1,2]. Such methods, which need sample treatments, make the HPLC system more complicated (such as reaction coil, extra pump, etc.) and are time-consuming. Futhermore, the disadvantages of derivation methods in terms of samples include: introduction of non-controlled impurities, degradation products and the most important one, unable to detect impurities of the analyte lacking of the specific functional group required for derivation.

Evaporative light scattering detection (ELSD) is considered as a universal detection mode suitable for non-absorbing analytes [3–5]. 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 can be detected. Currently, the detector response is well described and shows a double logarithmic relationship between the signals and the concentrations of analytes. Such response allows all molecules in a sample to give a proportional signal (which means same sensitivity). This principle is applied in the search of impurities in pharmaceutical products. At the same time, the structural information of impurities in pharmaceutical products can be obtained by HPLC–MS^n [6–10].

The objective of this study was to develop a rapid and simple chromatographic method which allows a direct sample introduction without any derivation treatment. HPLC–ELSD method was first used to determine the content of spectinomycin hydrochloride and its related substances. HPLC–MS^n method was used to characterize the structures of impurities contained in the sample. 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

* Corresponding author. Tel.: +86 571 8645945; fax: +86 571 86459427.
E-mail address: wangjian63@mail.hz.zj.cn (J. Wang).

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detection and characterization of spectinomycin hydrochloride and its related substances.

2. Experimental

2.1. Chemicals and reagents

Spectinomycin hydrochloride reference substance and drug substance (batch numbers: 020126, 020128, 020130, 030810, 030814 and 030818) were provided by Zhejiang Jinhua Kangenbei Pharmaceutical Co. Ltd. (Jinhua, China). Trifluoroacetic acid and pentafluoropropionic acid were analytical grade.

2.2. Instrumentation

2.2.1. LC apparatus

An Agilent 1100 series liquid chromatography (LC) system equipped with a binary pump was connected to an Agilent G1313A autosampler. Chromatographic separation was carried out at room temperature using an Agilent SB-C18 analytical column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of 25 mM trifluoroacetic acid. The flow rate was 1.0 ml/min. Detector used was a Dikma SEDEX 75 ELSD detector. The drift tube temperature was 40 °C. The pressure of nebulizing gas was 3.5 bar. GAIN was set at 4 and 6 for assay of spectinomycin and related substances, respectively. The injection volume was 10 μl.

2.2.2. Mass spectrometry

LC–MS experiment was carried out on an Agilent1100 ion trap mass spectrometer. The column effluent was split using a zero-dead-volume "T" connector, with approximately one quarter of the flow being fed to the mass spectrometer. The MSD was equipped with an ESI 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 (8 l/min)], dry gas temperature (325 °C), spray capillary voltage (3500 V), skimmer voltage (40 V), ion transfer capillary exit (94 V), scan range (100–1200 *m/z*), spectra average (5), ion current control (on), target (30,000), dwell time (300 ms).

2.2.3. Software

All data acquired were processed by Agilent Chemstation Rev. A. 09.01 software (Agilent, Palo Alto, CA).

2.3. Procedures

2.3.1. Sample preparation

Drug substances were dissolved in water to obtain a concentration level within the working range. Concentrations of spectinomycin solution were 1.5 and 6.0 mg/ml for the assay of spectinomycin and related substances, respectively.

2.3.2. Quantitation

An external standard calibration curve with calibration points ranging from 80 to 120% of the assay concentration (1.5 mg/ml) was used for the spectinomycin assay. A separate external standard calibration curve was used for the assay of low level related substances with calibration points ranging from 1.0 to 3.0% of the sample concentration (6.0 mg/ml). Calculations of the contents were based on peak areas and external standard calibration curve. The best linear fit of the calibration curve data points was obtained using a logarithmic type curve.

3. Results and discussion

3.1. Development of the chromatography

3.1.1. 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 forms of ion-pair modes or basic mobile phases. Perfluoranated carboxylic acids such as heptafluorobutyric acid [4,5] were used as ion-pair reagents to facilitate the retention of aminoglycosides in the reversed-phase mode.

In order to permit the use of evaporative light scattering detection, the mobile phases can not contain any non-volatile reagents. The acidic mobile phase consisting of 50 mM pentafluoropropionic acid–methanol (60:40) was tested to separate spectinomycin from its impurities, but the chromatogram failed to demonstrate sharp symmetrical peaks and good separation. The acidic mobile phase consisting of 25 mM trifluoroacetic acid was tested to separate spectinomycin from its impurities and the chromatogram showed sharp symmetrical peaks and good separation. The mobile phases consisting of various concentrations of trifluoroacetic acid (12.5, 25 and 50 mM) were tested to investigate the influence on peak shape, resolution and retention time. The results showed that as the concentrations increased, the retention time of spectinomycin increased and the chromatograms showed sharp symmetrical peaks and good separation. If concentration of trifluoroacetic acid was lower than 25 mM, spectinomycin could not be completely separated from impurities. However, high concentration (50 mM) with pH below 2.0 would do harm to the chromatographic column. Therefore, 25 mM trifluoroacetic acid was selected as the ion-pair reagent. The results were shown in Table 1. It was shown that the mobile phases containing methanol, acetonitrile, tetrahydrofuran, acetone, and dioxane had adverse effect on the resolution and peak shape. Therefore, the organic solvent was not added in the mobile phase.

Table 1
Influence of concentrations of trifluoroacetic acid on resolution and peak shape

Mobile phase (mM)	pH	Plate (spectinomycin)	Resolution	
			With impurity D	With impurity C
12.5	2.16	1231	1.41	3.83
25	2.05	1621	3.66	7.80
50	1.82	2047	5.19	10.28

3.1.2. Influence of retention time on peak shape of spectinomycin

It was shown that peak shape was strongly dependent on retention time and the increase of retention time resulted in poor peak shape. Trifluoroacetic acid (25 mM) was selected in order to achieve a retention time of spectinomycin close to 8 min. It was shown that at smaller retention time spectinomycin may overlap with impurities, while at greater retention time, peak broadening and asymmetry would increase resulting in decrease of ELSD response factor.

3.1.3. 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, and the increase of sample concentration and injection volume resulted in poor peak shape. It was optimal that for assay of spectinomycin and related substances, the concentrations were 1.5 and 6.0 mg/ml, respectively, and the injection volume was 10 μl.

3.2. Optimization of ELSD conditions

In this study, Dikma Technologies SEDEX 75 evaporative light scattering detector was used and drift tube temperature recommended was 40 °C. Based on this, various temperatures at 40, 50 and 60 °C were tested to study the influence on ELSD response and signal-to-noise ratio. The results showed that there was higher ELSD response at lower temperature and 40 °C was optimal.

The pressure of nebulizing gas recommended was 3.5 bar. Based on this, various pressures (2.5, 3.5 and 4.0 bar) were tested to study the influence on ELSD response and signal-to-noise ratio, and the results showed that 3.5 bar was optimal.

The used and recommended GAIN were 1–12 and 4–6, respectively. Based on this, GAIN ranging from 4 to 6 was tested, and the results showed that ELSD response increased with higher GAIN values but resulted in increase of baseline noise. When GAIN was set at 4 and 6, LOD (S/N = 3) was 9.0 and 6.0 μg/ml, respectively. Therefore, GAIN was set at 4 and 6 for assay of spectinomycin and related substances, respectively.

3.3. 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 to determine the limit of detection.

3.3.1. Specificity

The ability of the chromatographic system to resolve spectinomycin hydrochloride from its possible impurities was investigated. Impurities C and D were examined in order to assure that they do not interfere (peak overlapping) with spectinomycin. 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 while degradation products were produced under acid/base hydrolysis and oxidation conditions (retention time 2.6, 4.3 and 2.9 min, respectively). Spectinomycin could be completely separated from impurities C, D and degradation products. The chromatographs for the determination of spectinomycin hydrochloride and its related substances were shown in Figs. 1 and 2.

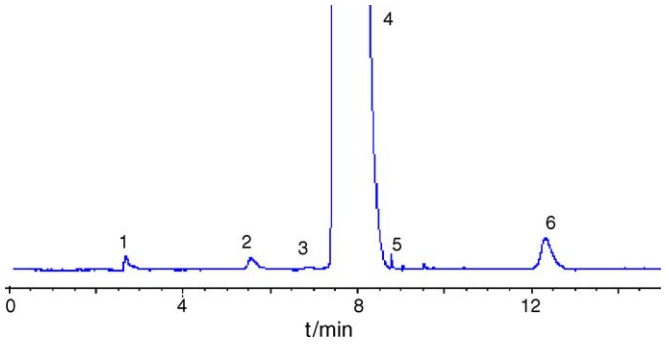

Fig. 1. Chromatogram of related substances in spectinomycin hydrochloride. 2: Impurity D, 4: spectinomycin, 6: impurity C.

3.3.2. 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 spectinomycin hydrochloride, the linearity of response was determined by preparing in duplicate five spectinomycin hydrochloride solutions ranging from 50 to 250% of the assay concentration (1.5 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 $\log A = 1.195 \log C + 2.767$. The corresponding coefficient r was 0.9997. The result indicated good linearity.

For the assay of related substances (low level linearity), five spectinomycin hydrochloride solutions were prepared with concentration ranging from 0.5 to 5.0% of the sample concentration (6.0 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 equa-

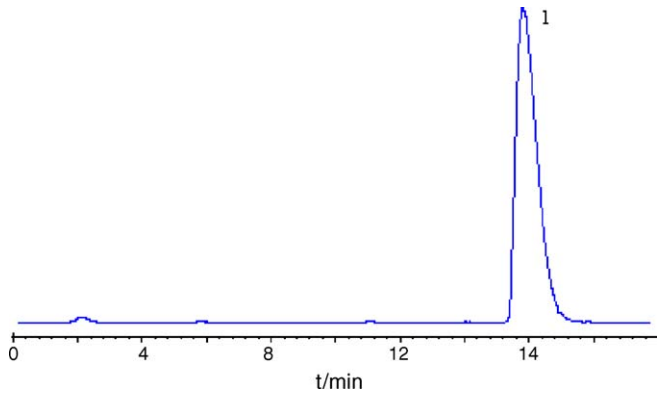

Fig. 2. Chromatogram of spectinomycin hydrochloride assay. 1: Spectinomycin.

tion was $\log A = 1.438 \log C - 1.572$. The corresponding coefficient r was 0.9995. The result indicated good linearity.

3.3.3. Precision of the assay

Six replicate sample solutions at 100% of the test concentration (1.5 mg/ml) were prepared and then assayed for spectinomycin using the recommended HPLC system and sample preparation. The relative standard deviation (R.S.D.) value was 1.0% ($n = 6$).

3.3.4. Limit of detection (LOD)

The limit of detection is defined as the lowest concentration of analyte that can be accurately detected. Its determination could be made by the calculation of the signal-to-noise ratio. A ratio of 3 was selected and successive dilutions of the test solution gave a LOD relative to the spectinomycin peak of 0.1% (m/m). The limit of detection was 6.0 μg/ml. Such limit was in good agreement with what required for assay of related substances.

3.4. Analysis of spectinomycin hydrochloride drug substance

Six batches of drug substances were analyzed using the recommended HPLC system. The results of determination of spectinomycin and related substances were shown in Tables 2 and 3. The results were in agreement with the results of determination by the microbiological assays of antibiotics in Chinese Pharmacopoeia.

3.5. The characterization of impurities by HPLC–MS^n

The HPLC–MS^n method was used to characterize the structures of impurities contained in the spectinomycin. The mobile phase used for these experiments was described in Section 2.2.1.

Table 2
The results of assay determination for spectinomycin hydrochloride ($n = 2$)

Batches	ELSD method (%)	Microbiological method (%)
020126	77.8	80.5
020128	77.3	80.9
020130	77.1	80.0
030810	77.4	
030814	77.7	
030818	77.2	

Table 3
The results of related substances determination in spectinomycin hydrochloride

Batches	Content of impurity D (%)	Content of impurity C (%)	Content of total impurity (%)
020126	0.9	2.3	3.1
020128	0.8	2.1	3.3
020130	0.8	2.0	3.2
030810	0.8	1.9	2.8
030814	0.8	2.4	3.3
030818	0.5	2.5	2.6

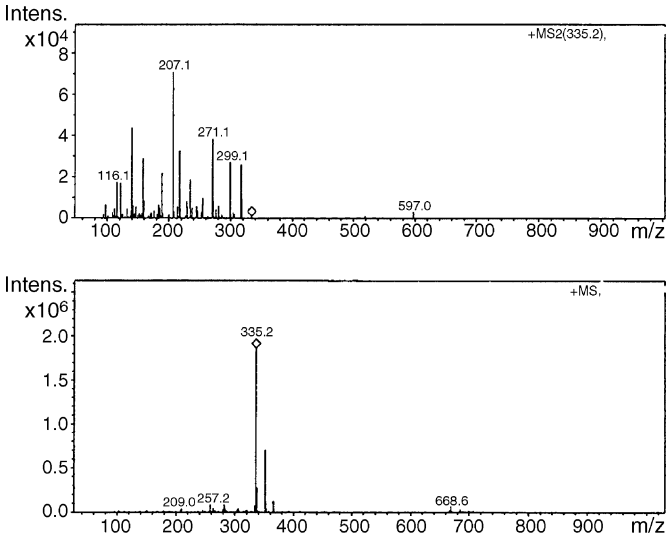

Fig. 3. (+)-ESI-MS^2 spectra of impurity C.

The eluent is the same as the one described above for the HPLC–ELSD method.

3.5.1. The characterization of impurity C by HPLC–MS^n

In Fig. 1, we could find that an impurity appeared repeatedly above an apparent level. In MS full scan mode, the major ion detected was protonated molecular ion, $[M+H]^+$ m/z 335. It was demonstrated that the spectinomycin gave abundant product ion at m/z 207 by loss of the C-ring and B-ring in MS^2 full scan mode. The possible structure of the impurity in spectinomycin was deduced based on the HPLC–MS^n data (the structure is shown in Fig. 5). Its structure is in agreement with the impurity C listed in British Pharmacopoeia, which was named as (2*R*,4*RS*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2H-pyrano[2,3-b][1,4]benzodioxine-4,4a,7,9-tetrol(dihydrospectinomycins). Fig. 3 shows the mass spectrum of impurity C. The proposed scheme for fragmentation of impurity C in the multi-stage MS full scan mode was shown in Fig. 5.

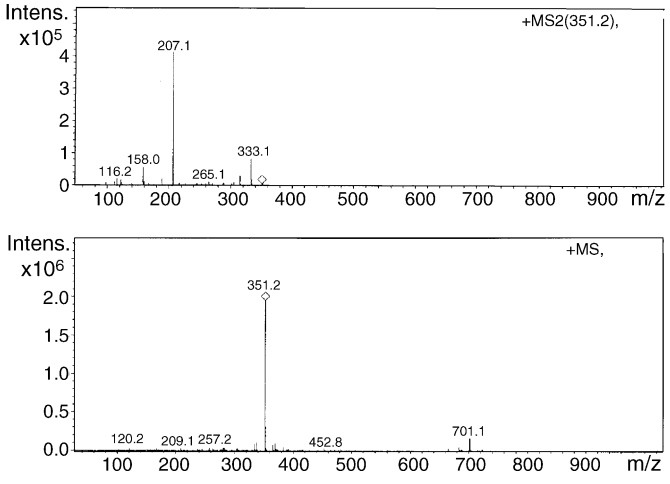

Fig. 4. (+)-ESI-MS^2 spectra of impurity D.

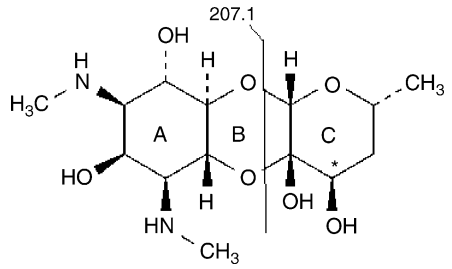

Fig. 5. Chemical structure and the ESI-MS^2 fragmentation pathways of impurity C.

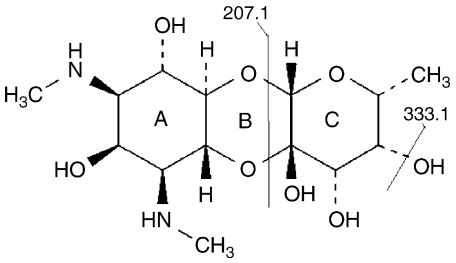

Fig. 6. Chemical structure and the ESI-MS^2 fragmentation pathways of impurity D.

3.5.2. *The characterization of impurity D by HPLC–MS^n*

In Fig. 1, we could find that an impurity appeared repeatedly above an apparent level. In MS full scan mode, the major ion detected was protonated molecular ion, $[M+H]^+$ *m/z* 351. It was demonstrated that the spectinomycin gave abundant product ion at *m/z* 333 by loss of the H_2O, and at *m/z* 207 by loss of the C-ring and B-ring in MS^2 full scan mode. The possible structure of the impurity in spectinomycin was deduced based on the HPLC–MS^n data (the structure is shown in Fig. 6). Its structure is in agreement with the impurity D listed in British Pharmacopoeia, which was named as (2*R*,3*R*,4*S*,4a*S*,5a*R*,6*S*,7*S*, 8*R*, 9*S*, 9a*R*, 10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2H-pyrano[2,3-b][1,4]benzodioxine-3,4,4a,7,9-pentol(dihydroxyspectinomycin). Fig. 4 shows the mass spectrum of impurity D. The proposed scheme for fragmentation of impurity D in the multi-stage MS full scan mode was shown in Fig. 6.

4. Conclusions

The described HPLC–ELSD method provides a rapid and simple analysis for spectinomycin hydrochloride and its related substances without derivations. 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 impurities contained in the spectinomycin.

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