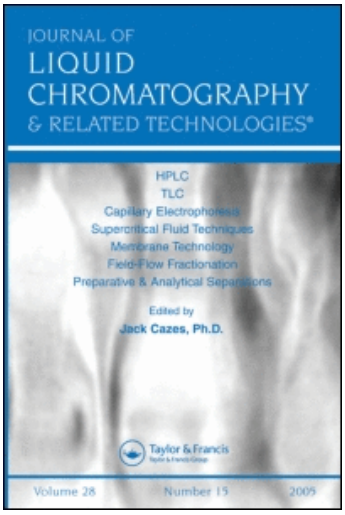

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Determination of Astromycin Sulfate and its Related Substances by HPLC using Evaporative Light Scattering Detection and Electrospray Ion Trap Mass Spectrometry

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Abstract: A new and simple high performance liquid chromatography evaporative light scattering detection for the determination of astromycin sulfate and its related substances was developed. The high performance liquid chromatography electrospray ion trap mass spectrometry was used to identify the structures of a degradation product of astromycin. The column was Agilent SB-C_{18}. The mobile phase was 50 mM trifluoroacetic acid-tetrahydrofuran. The drift tube temperature was 40°C. The pressure of the nebulizing gas was 3.5 bar. The standard curve was rectilinear in the range of 290 ~ 1460 $\mu g\,mL^{-1}$. Precision expressed as the R.S.D was 0.9%. The limit of detection was 6.0 $\mu g\ mL^{-1}$. The possible structure of a degradation product was deduced, based on the HPLC-MS^n data.

Keywords: Astromycin sulfate, High performance liquid chromatography, Evaporative light scattering detection, Electrospray ion trap mass spectrometry, Degradant, Related substance

INTRODUCTION

Astromycin sulfate belongs to a class of compounds known as aminoglycoside antibiotics. Like many aminoglycosides, astromycin sulfate lacks a suitable chromophore, which is necessary for UV detection. For this reason, the

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analysis of astromycin sulfate is performed using precolumn or postcolumn derivatization methods.[1,2] Such methods, which need sample treatment, make the HPLC system more complex (reaction coil, extra pump, etc.) and were very time consuming. In fact, several drawbacks could be listed against a sample derivatization process: introduction of non controlled impurities, degradation products, and the most important, impurities of the analyte lacking the specific functional group required for derivatization could not be detected.

Evaporative light scattering detection (ELSD) is described as a universal detection mode suitable for non-adsorbing 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; any compound less volatile than the mobile phase could be detected. The detector response is now well described and shows a double logarithmic relationship between the signal and the analyte concentration. Such response allows all molecules of the sample to give a proportional signal (same sensitivity). This principle is in good agreement with the search of impurities in pharmaceutical products. The structural information may be obtained by HPLC-MS^n,[6–10] and these characteristics are applicable to the structural elucidation of impurities in pharmaceutical products.

The principle aim of this work was to develop a rapid and simple chromatographic method, which allows a direct sample introduction without any derivatization. HPLC-ELSD was first used to determine the content of astromycin sulfate and its related substances. The high performance liquid chromatography electrospray ion trap mass spectrometry (HPLC-MS^n) was employed to identify the structures of a degradant (impurity A) of astromycin. The method development 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. This permits the use of evaporative light scattering detection and mass spectrometry that enables the identification and detection of astromycin sulfate and its related substances.

EXPERIMENTAL

Chemicals and Reagents

Astromycin sulfate reference substance, drug substance (batch numbers: 020301, 020501, and 021201) were offered by Zhejiang Haizheng Pharmaceutical Co. Ltd. (jiaojiang,China); trifluoroacetic acid and tetrahydrofuran were analytical grade.

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-C_{18} analytical column (250 × 4.6 mm, 5 μm). The mobile phase consisted of 50 mM trifluoroacetic acid-tetrahydrofuran (97 : 3). Detector used was an Dikma SEDEX 75 ELSD detector. The drift tube temperature was 40°C. The pressure of nebulizing gas was 3.5 bar. The flow rate was 1.0 mL min^{-1}.

The 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 p.s.i. (N_2)], dry gas [N_2 (8 L min^{-1})], 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 (3,0000), dwell time (300 ms).

Sample Preparation

Drug substances were simply dissolved in water to obtain a concentration level within the working range. Concentrations of astromycin solution used for the assay and related substances, were 0.6 and 3.0 mg mL^{-1}, respectively.

Quantitation

An external standard calibration curve with three calibration points ranging from 80 to 120% of the analytical assay concentration (0.6 mg mL^{-1}) was used for the astromycin assay. A separate external standard calibration curve was used for the assay of low level related substances, with three calibration points ranging from 1.0 to 2.0% of the sample concentration (3.0 mg mL^{-1}). Calculations were based on peak areas.

RESULTS AND DISCUSSION

Method Development

Selection of the Mobile Phase

It is difficult to retain the AGs 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. In order to permit the use of evaporative light scattering detection, the mobile phases can not contain any non-volatile reagents. Perfluoranated carboxylic acids, such as

heptafluorobutyric acid,[4,5] were reported for use as ion pair reagents to facilitate the retention of AGs in the reversed phase mode. An Agilent Extend-C_{18} (250×4.6 mm, 5 μm) basic column with basic mobile phase[3] consisting of water-ammonium hydroxide-acetic acid (96 : 3.6 : 0.4), was tested to separate astromycin from its impurities without adding ion pair reagents, but the HPLC chromatograms failed to demonstrate sharp symmetrical peaks and good separation. An Agilent SB-C_{18} (250×4.6 mm, 5 μm) column with acidic mobile phase consisting of 50 mM trifluoroacetic acid and tetrahydrofuran (97 : 3) or 50 mM pentafluoropropionic acid and methanol (50 : 50) was tested to separate astromycin from its impurities, and the HPLC chromatograms showed to have sharp symmetrical peaks and good separation.

The mobile phases consisting of various concentrations of trifluoroacetic acid (25, 50, 100 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 astromycin increased and the chromatograms showed sharp symmetrical peaks and good separation. If concentrations of trifluoroacetic acid was lower than 50 mM, astromycin could not be completely separated from impurity A. However, high concentration (100 mM) with pH below 2.0 would do harm to the chromatographic column. Therefore, 50 mM trifluoroacetic acid was selected as the ion pair reagent.

The mobile phases with the presence of methanol, acetonitrile, tetrahydrofuran, acetone, and dioxane had some influence on the resolution and peak shape; mobile phase with the presence of tetrahydrofuran was the best to separate astromycin from impurity A. Therefore, tetrahydrofuran was selected as the organic solvent. The results are shown in Table 1.

Peak shape is strongly dependant on retention time and the increase of retention time resulted in poor peak shape. The experiment showed that the proportion of tetrahydrofuran to water containing 50 mM trifluoroacetic acid was selected to be 3 : 97, in order to achieve a retention time of astromycin close to 6 min (at smaller retention time, astromycin may overlap with impurities, while at greater retention time, peak broadening and asymmetry would increase, resulting in a decrease of ELSD response factor).

Table 1. Influence of organic solvent on resolution and peak shape

Organic solvent	Plates, astromycin	Resolution between astromycin and impurity A
Methanol	3951	1.40
Acetonitrile	4726	1.56
Tetrahydrofuran	4693	1.82
Acetone	4631	1.38
Dioxane	6793	0.98

Peak shape is strongly dependant on sample concentration and injection volume, and the increase of sample concentration and injection volume resulted in poor peak shape. It was satisfactory, that for assay of astromycin and related substances, the concentrations were 0.6 and 3.0 mg mL^{-1}, respectively, and the injection volume was 10 μL.

Optimization of ELSD Conditions

The drift tube temperature was tested at 40, 50, 60°C 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 satisfactory. The pressure of nebulizing gas (2.5, 3.5, 4.0 bar) was tested to study the influence on ELSD response and signal-to-noise ratio, and the results showed that 3.5 bar was satisfactory. The gain, ranging from 4 to 6, was tested and the results showed that ELSD response increased with higher gain value 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^{-1}, respectively. Therefore, gain was set at 4 and 6 for assay of astromycin and related substances, respectively.

Method Validation

A 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.

Specificity

The ability of the chromatographic system to resolve astromycin sulfate from its possible impurities was investigated. The impurity A was examined in order to assure that it does not interfere (peak overlapping) with astromycin. For the related substances test, samples were stored under relevant stress conditions (light, heat, acid/base hydrolysis, and oxidation, respectively). Samples showed light and heat stability, while degradation compounds were produced under acid/base hydrolysis and oxidation conditions. Astromycin could be completely separated from impurity A and degradants. The chromatograms for the determination of astromycin 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.

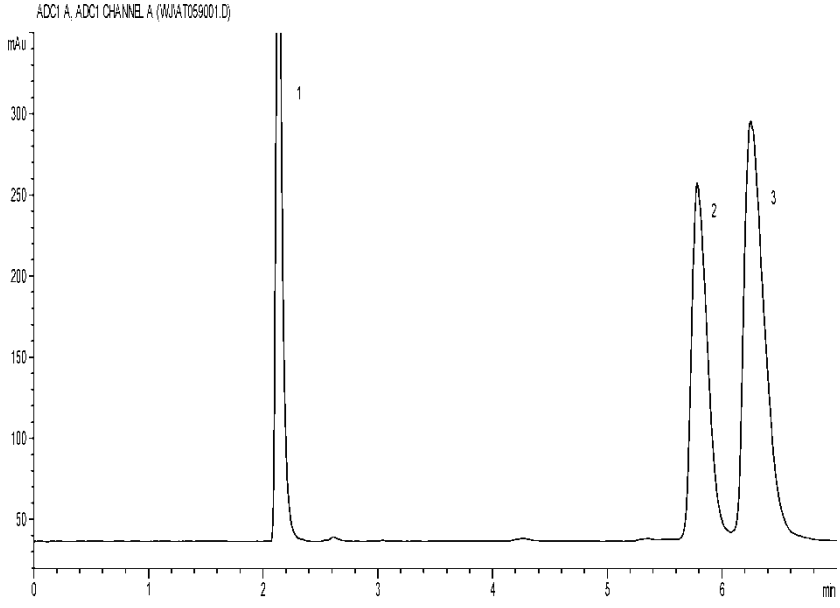

Figure 1. Chromatogram of astromycin spiked with impurity A. 1. sulfate; 2. astromycin; 3. impurity A.

For the assay of astromycin sulfate, the linearity of response was determined by preparing, in duplicate, five astromycin sulfate solutions ranging from 50 to 250% of the assay concentration ($0.6\,mg\,mL^{-1}$). Each solution was analyzed using the recommended HPLC system. The regression curves were obtained by plotting log (concentration) versus log (peak area). The regression equation was $\log A = 1.220 \log C + 0.072$. The corresponding coefficient r was 0.9999. The result indicated good linearity whatever the astromycin studied.

For the assay of related substances (low level linearity), five astromycin sulfate solutions were prepared with concentrations ranging from 0.5 to 2.5% of the sample assay concentration ($3.0\,mg\,mL^{-1}$). The solutions were injected into the HPLC system. The regression curves were obtained by plotting log (concentration) versus log (peak area). The regression equation was $\log A = 1.095 \log C + 0.288$. The corresponding coefficient r was 0.9977. The result indicated good linearity whatever the astromycin studied.

Precision of the Assay

Six replicate sample solutions at 100% of the test concentration ($0.6\,mg\,mL^{-1}$) were prepared and then assayed for astromycin using the recommended HPLC system and sample preparation. The relative standard deviation (R.S.D) value was 0.9% ($n = 6$). The result was satisfactory.

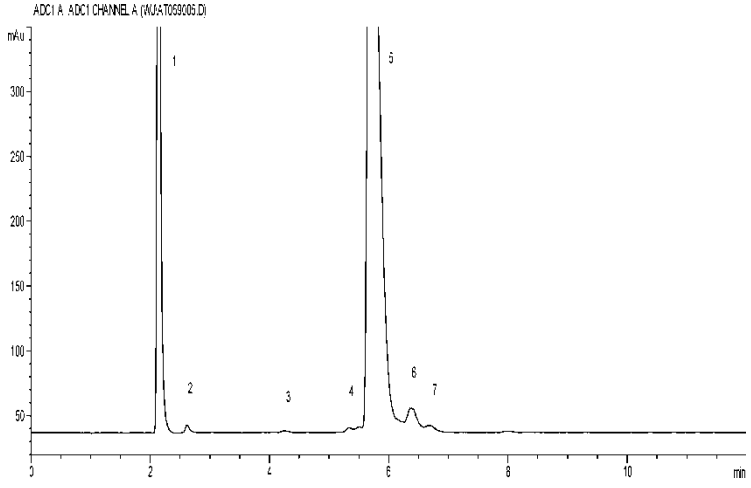

Figure 2. Chromatogram of related substances in astromycin sulfate. 1. sulfate; 5. astromycin; 6. impurity A; 2, 3, 4, 7, other impurities.

Limit of Detection (LOD)

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 astromycin peak of 0.2% (m/m). The limit of detection

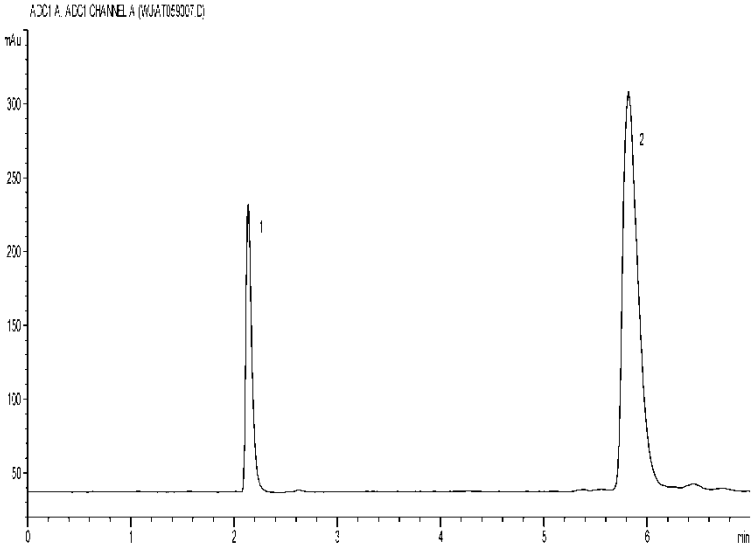

Figure 3. Chromatogram of astromycin sulfate assay. 1. sulfate; 2. astromycin.

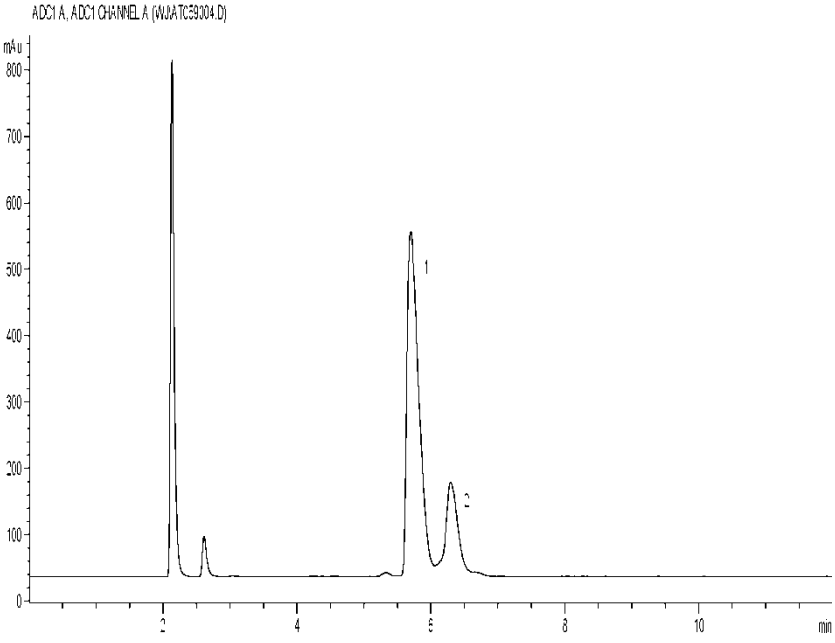

Figure 4. Chromatogram of astromycin sulfate by hydrolytic damage. 1. astromycin; 2. impurity A.

was 6.0 $\mu g\,mL^{-1}$. Such limit was in good agreement with that required for assay of related substances.

Analysis of Astromycin Sulfate Drug Substance

Three batch drug substances were analyzed using the recommended HPLC system and sample preparation. The results of determination of assay and related substances for astromycin are shown in Table 2.

Table 2. The results of assay and related substances determination for astromycin (n = 2)

Batches	Astromycin (%)	Impurity A (%)	Total impurity (%)
020301	58.0	1.1	2.0
020501	58.5	1.5	2.3
021201	58.3	1.3	2.2

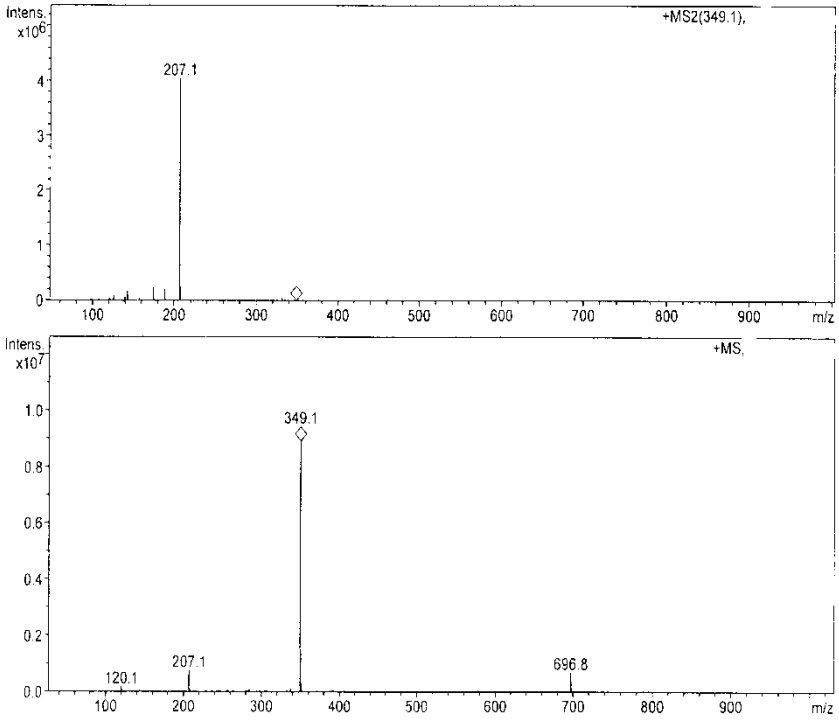

Figure 5. (+)-ESI-MS spectra.

Characterization of Degradant by HPLC-MS^n

The HPLC-MS^n method was used to characterize the structures of a degradant of astromycin. The mobile phase used for these experiments was described in 2.2.1. The eluent is the same as the one described above for the HPLC-ELSD method.

From Figure 2, we can see one impurity was repeatedly found above an apparent level. In MS full scan mode, the major ion detected was protonated

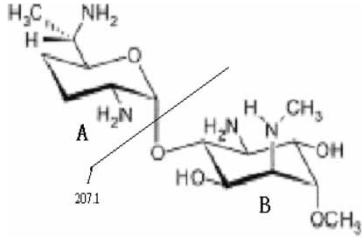

Figure 6. Structure and ESI-MS fragmentation pathway of imputity A.

molecular ion, $[M + H]^+$ m/z 349. It was demonstrated that the astromycin gave abundant product ion at m/z 207 by loss of the A-ring in MS^2 full scan mode. The possible structure of the unknown impurity in astromycin was deduced based on the HPLC-MS^n data (the structure is shown in Figure 6). We isolated this impurity from crude astromycin and proceeded to confirm its structure by NMR, which was called impurity A. Figure 5 shows the mass spectrum of impurity A. Figure 6 shows a proposed scheme for fragmentation of impurity A in the multi-stage MS full scan mode.

The experiment showed that the astromycin solution was dissolved in water and stored for 1 month at room temperature, and the content of impurity A increased largely. The chromatograms are shown in Figure 4. This indicated that impurity A was a degradant of astromycin. The antimicrobial activity of astromycin and the degradant was determined. The antimicrobial potency of astromycin was 580 IU mg^{-1}, and the antimicrobial potency of the degradant was only 18 IU mg^{-1}.

CONCLUSION

The described HPLC-ELSD method provides a rapid and simple analysis for astromycin sulfate and its related substances without derivatization. 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.

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