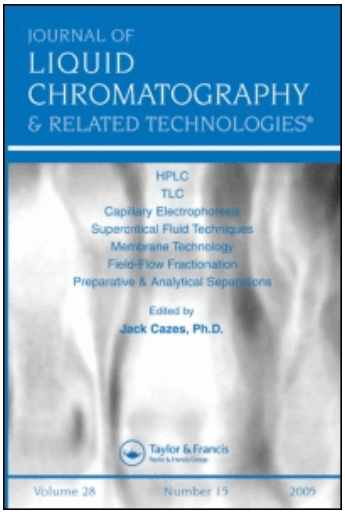

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Determination of Four Aminoglycoside Antibiotics by Liquid Chromatography with Pulsed Electrochemical Detection

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Abstract: A new and simple liquid chromatographic method using a column packed with poly(styrene-divinylbenzene) and pulsed electrochemical detection on a gold electrode for the determination of vertilmicin sulfate (new drug), micronomycin sulfate, etimicin sulfate, and sisomicin sulfate and their related substances was developed. The mobile phase consisted of an aqueous solution of 38 g L^{-1} sodium sulfate, 0.4 g L^{-1} sodium octanesulfonate, 13 mL L^{-1} (10 mL L^{-1} for micronomycin) tetrahydrofuran, and 50 mL L^{-1} 0.2 M phosphate buffer (pH = 3). The effects of the different chromatographic parameters on the separation were investigated. The specificity, assay linearity, low level assay linearity, and precision of assay were also investigated.

Keywords: Vertilmicin sulfate, Micronomycin sulfate, Etimicin sulfate, Sisomicin sulfate, High performance liquid chromatography, Pulsed electrochemical detection

INTRODUCTION

Vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate belong to a class of compounds known as aminoglycoside antibiotics. Vertilmicin sulfate is a novel drug that was found in the synthesis of netilmicin and it is under registration in China. Like many aminoglycosides, the four drugs

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lack a suitable chromophore, which is necessary for UV detection. For this reason, the analysis of the four drugs is performed using precolumn or postcolumn derivatization methods.[1–3] Such methods, which need sample treatment, make the HPLC system more complex (reaction coil, extra pump, etc.) and are very time consuming. In fact, several drawbacks could be listed against a sample derivatization process: introduction of non-controlled impurities, degradation products, and, most important, impurities of the analyte lacking the specific functional group required for derivatization could not be detected.

The principle aim of this work was to develop a sensitive and simple liquid chromatography with pulsed electrochemical detection, which allows a direct sample introduction without any derivatization. Liquid chromatography with pulsed electrochemical detection was used to determine the content of vertilmicin sulfate (new drug), micronomycin sulfate, etimicin sulfate, and sisomicin sulfate and their related substances. The analysis of several aminoglycoside antibiotics by liquid chromatography with pulsed electrochemical detection[4–7] have been described. However, no liquid chromatography with pulsed electrochemical detection has been described to analyze the content of vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate and their related substances.

EXPERIMENTAL

Chemicals and Reagents

Vertilmicin sulfate standard, drug substance (batch numbers: 20040124, 20040218, 20040328), and impurity A were offered by Zhejiang Conler Pharmaceutical Co. Ltd. (Wenzhou, China). Micronomycin sulfate standard, drug substance (batch numbers: 0402093, 0402094, 0402095) and gentamicin C1a were offered by Wuxi First Pharmaceutical factory (Wux, China). Etimicin sulfate standard, drug substance (batch numbers: 20030203, 20030310, 20030804) were offered by Wuxi Shanhe Pharmaceutical Co. Ltd. (Wuxi, China). Sisomicin sulfate standard and drug substance (batch numbers: 040510, 040516, 040526) were offered by Zhejiang Aotuokang Pharmaceutical Co. Ltd. (Jinhua,China).

Water was distilled twice from a glass apparatus. Sodium sulfate anhydrous, sodium 1-octanes-ulfonate, and potassium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany); 0.5 M sodium hydroxide solution from Fluka, tetrahydrofuran from Sigma-Aldrich.

Instrumentation

A Dionex DX-600 series liquid chromatography (LC) system equipped with a GP50 binary pump was connected to an AS50 autosampler with a

fixed loop of 20 μL. The column (250 × 4.6 mm I.D.) was packed with poly(styrene-divinylbenzene) PLRP-S (1000 A, 8 μm, (Polymer Labs, Shropshire, UK). The temperature of the column was maintained at 40°C. The ED50 pulsed electronchemical detector was equipped with a gold working electrode, an Ag/AgCl reference electrode, and a stainless steel counter electrode. The 0.5 M sodium hydroxide solution was added post-column using a PC10 pneumatic device, allowing pulse free addition of the base. The cell of the pulsed electrochemical detector was placed in a temperature oven to keep the temperature at 35°C.

Chromatography

The mobile phase consisted of an aqueous solution containing 38 g L^{-1} of sodium sulfate, 0.4 g L^{-1} of sodium 1-octanesulfonate, 13 mL L^{-1} (10 mL L^{-1} for micronomycin) of tetrahydrofuran, and 50 mL L^{-1} of 0.2 M phosphate buffer (pH 3.0), and was sonicated before use. The flow rate was 1 mL min^{-1}. All substances to be analyzed were dissolved in the mobile phase. To allow pulsed electrochemical detection, 0.5 M NaOH was added post-column (0.3 mL min^{-1}) through a mixing tee from a helium pressurized reservoir (1.6 bar) and mixed in a packed reaction coil (1.2 m, 500 μL), linking to the electrochemical cell. The flow rate for the addition of the base is not critical, but it should be reproducible between runs and must be pulse free. It was necessary to raise the pH of the mobile phase to approximately 13 to improve the sensitivity of the detection. The 0.5 M NaOH solution was made starting from a 50% (m/m) aqueous solution, which was pipetted into helium degassed water to avoid carbonates that foul the electrodes. The time and voltage parameters for the pulsed electrochemical detector were set as follows: E_1, E_2, and E_3 were, respectively, +0.05 V, +0.75 V, and −0.15 V, with the assigned pulse duration t_1: 0–0.40 s, t_2: 0.41–0.60 s, and t_3: 0.61–1.00 s. Integration of the signal was done between 0.20 and 0.4 s.

Preparation of Standard Solution and Sample Solution

Preparation of the standard solution: vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate standards were simply dissolved in the mobile phase to obtain a concentration level within the working range. Concentrations of standard solution used were: 0.1, 0.1, 0.1, 0.05 mg mL^{-1}, respectively, for the analysis of vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, sisomicin sulfate, and 0.01, 0.02, 0.005 mg mL^{-1}, respectively, for the estimation of lower level impurities of vertilmicin sulfate, micronomycin sulfate, and sisomicin sulfate.

Preparation of sample solution: vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate substance was simply dissolved in the mobile phase to obtain a concentration level within the working range. Concentrations of sample solution used were: 0.1, 0.1, 0.1, 0.05 mg mL^{-1}, respectively, for the analysis of vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, sisomicin sulfate, and 1.0, 0.5, 0.5 mg mL^{-1}, respectively, for the estimation of lower level impurities of vertilmicin sulfate, micronomycin sulfate, sisomicin sulfate.

RESULTS AND DISCUSSION

Development of the Chromatography

The influences of the different chromatographic parameters on the separation were investigated. The pH in the range from pH 2.0 to pH 6.0 showed nearly no influence on the retention times of the different drug components, but above pH 4.0, the peak symmetry of the main component was poorer. The influence of the column temperature was examined at 30, 40, and 50°C. As expected, retention times of the components decreased as the column temperature was increased. Sodium octanesulfonate, as an ion-pairing agent, was added to retain the drug molecules which are positively charged at pH 3.0. The concentration of sodium octanesulfonate in the mobile phase was varied in the range from 0.3 to 0.5 g L^{-1}. As expected, retention times decreased by lowering the concentration of sodium octanesulfonate. The influence of the sodium sulfate in the mobile phase on the retention times of the various drug components was examined in the range from 34 to 40 g L^{-1}. An increase of the sodium sulfate resulted in decreases of retention times. Tetrahydrofuran was added to the mobile phase to improve the peak symmetry of the main peak. Increase of tetrahydrofuran concentration in mobile phase resulted in decrease of the retention times. Taking advantage of the conclusion drawn from the previous steps of the study, in order to achieve complete separation of drug from main related substances, the selected mobile phase was an aqueous solution containing 38 g L^{-1} of sodium sulfate, 0.4 g L^{-1} of sodium 1-octanesulfonate, 13 mL L^{-1} (10 mL L^{-1} for micronomycin) of tetrahydrofuran, and 50 mL L^{-1} of 0.2 M phosphate buffer (pH 3.0).

Peak shape is strongly dependent upon sample concentration. The increase of sample concentration caused electrodes to overload, and resulted in poor peak shape. There is no good linearity in the range from 0.20 to 1.2 mg mL^{-1} for the determination of the contents of the four drugs. The experimental results showed, in a satisfactory manner, that for assay of the four drugs and their related substances, the concentrations were 0.05–0.1 mg mL^{-1} and 0.5–1.0 mg mL^{-1}, 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 to determine the limit of detection.

Specificity

The ability of the chromatographic system to resolve the drugs from its possible impurities was investigated in order to assure that they do not interfere (peak overlapping) with the drugs. Vertilmicin from impurity A (a main impurity in vertilmicin) could be completely separated. Micromycin from gentamicin C_{1a} (a main impurity in micromycin) could be completely separated. Etimicin from gentamicin C_{1a} (a main impurity in etimicin) could be completely separated. Sisomicin from impurity B (a heat degradation impurity in sisomicin) could be completely separated. The chromatograms were shown in Figures 1–11.

Forced degradation studies: Test samples were stored under relevant stress conditions (light, heat, acid/base hydrolysis, and oxidation, respectively). Vertilmicin sulfate and micronomycin sulfate showed light and heat stability, while degradation compounds were produced under acid/base hydrolysis and oxidation conditions. Etimicin sulfate and sisomicin sulfate

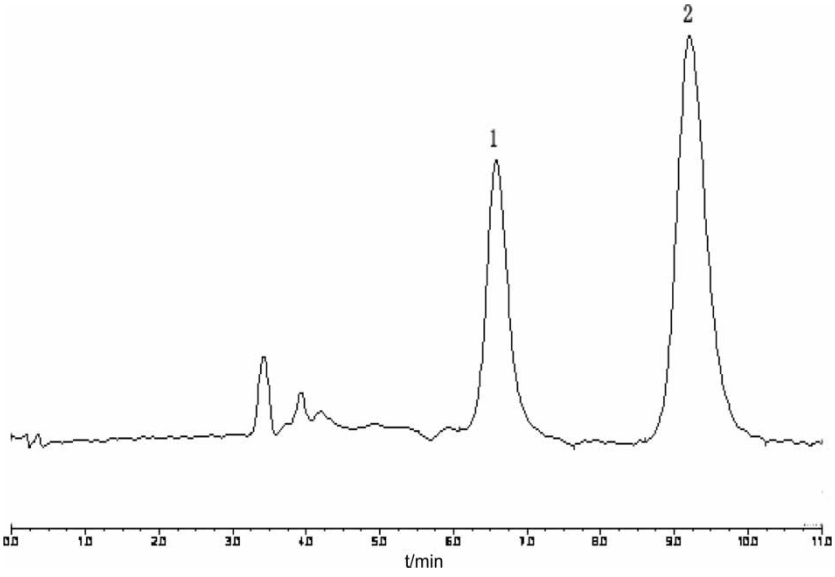

Figure 1. Chromatogram of vertilmicin spiking with impurity A. 1. Impurity A; 2. Vertilmicin.

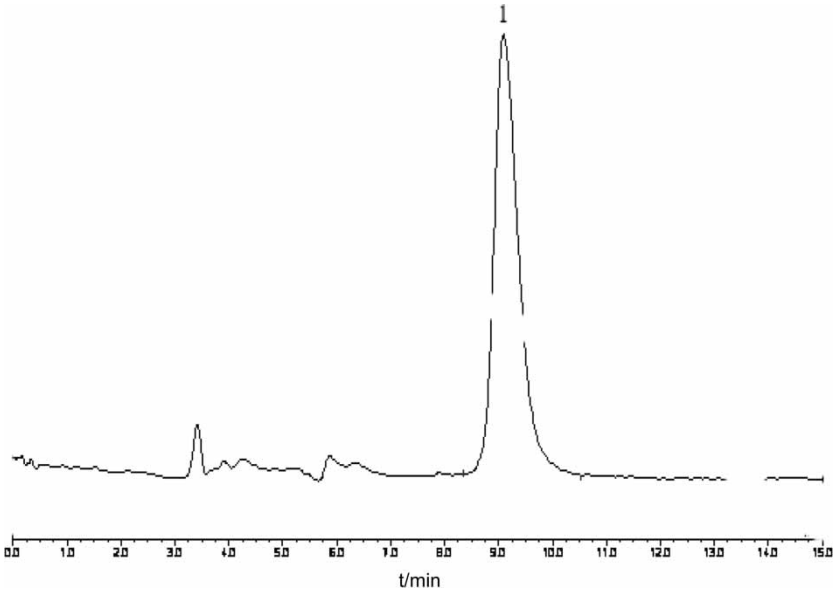

Figure 2. Chromatogram of vertilmicin sulfate assay. 1. Vertilmicin.

showed light and oxidation stability, while degradation compounds were produced under acid/base hydrolysis and heat conditions. Vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate could be completely separated from their degradation compounds.

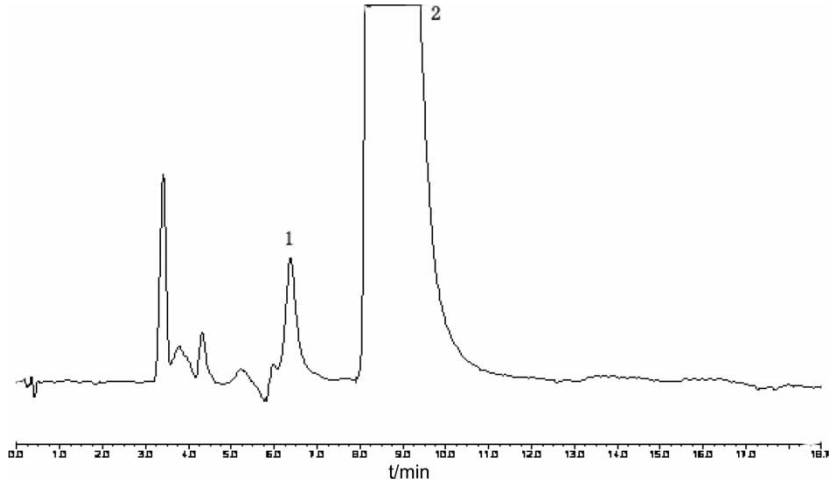

Figure 3. Chromatogram of related substances in vertilmicin sulfate. 1. Impurity A; 2. Vertilmicin.

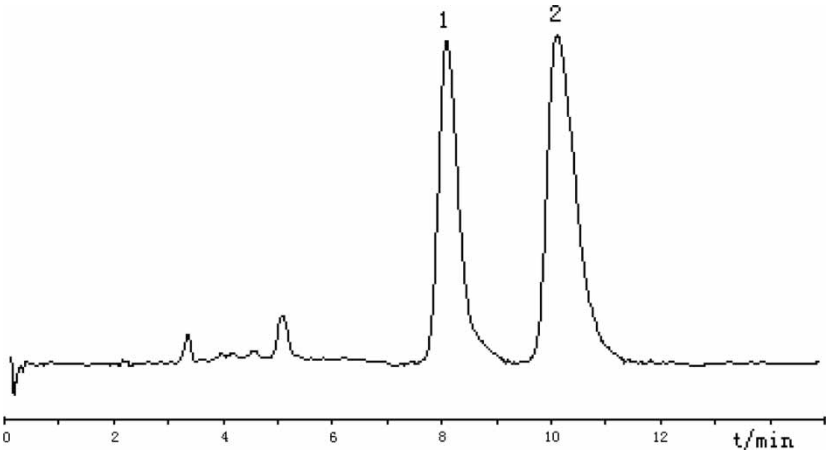

Figure 4. Chromatogram of micronomycin sulfate spiking gentamycin C_{1a}. 1. Gentamycin; 2. Micronomycin.

Acid degradation: About 10 mg of drug was weighed and transferred to a 10 mL volumetric flask. 1 M HCl, 0.5 mL, was added and allowed to stand for 8 hrs at room temperature. After 8 hrs, the drug treated with 1 M HCl was neutralized with 1 M NaOH and diluted with mobile phase to get a concentration of about 1 mg mL^{-1}. The solution was injected into the HPLC system.

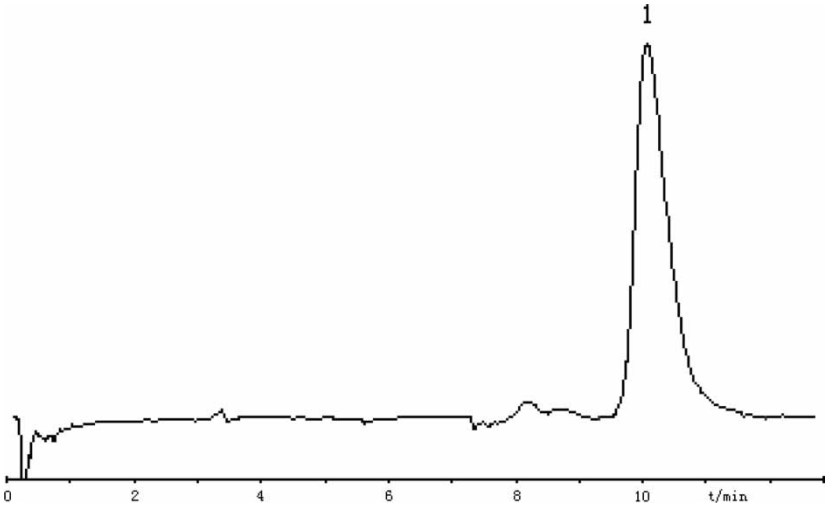

Figure 5. Chromatogram of micronomycin sulfate assay. 1. Micronomycin.

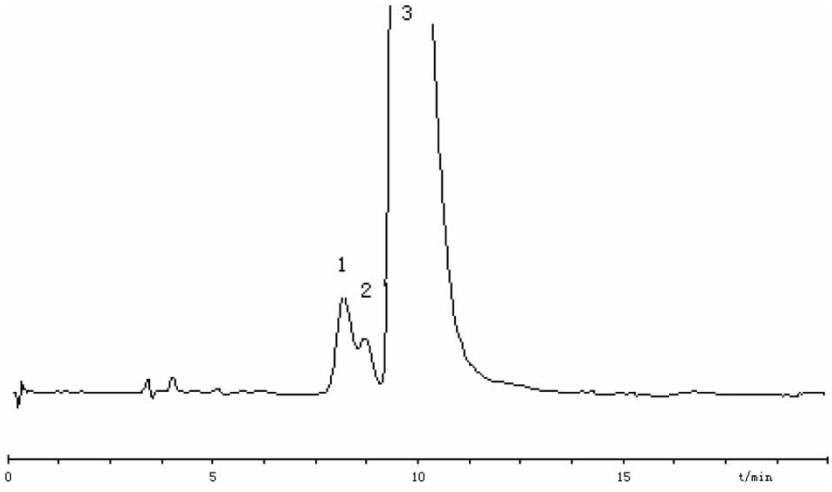

Figure 6. Chromatogram of related substances in micronomycin sulfate. 1. Gentamycin; 2. Unknown impurity; 3. Micronomycin.

Basic degradation: About 10 mg of the drug was weighed and transferred to a 10 mL volumetric flask. 1 M NaOH, 0.5 mL, was added and allowed to stand for 8 hrs at room temperature. After 8 hrs, the drug treated with 1 M NaOH was neutralized with 1 M HCl and diluted with mobile phase to get

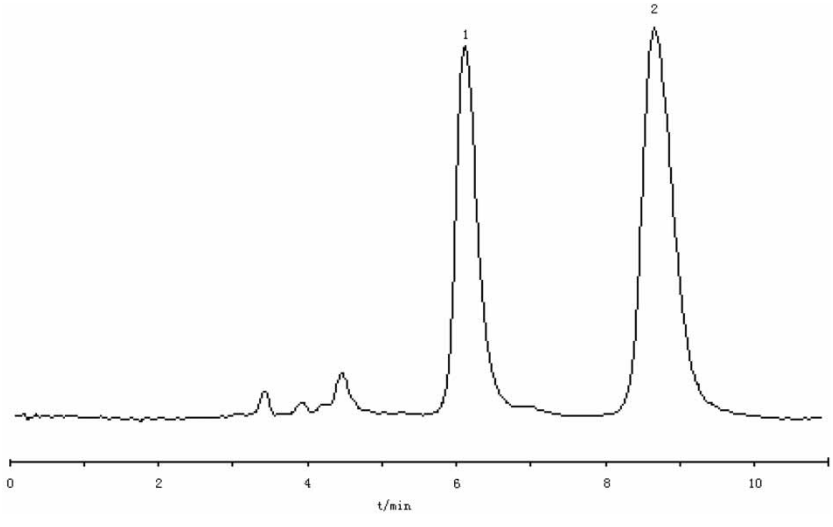

Figure 7. Chromatogram of etimycin sulfate spiking gentamycin C_{1a}. 1. Gentamycin; 2. Etimycin.

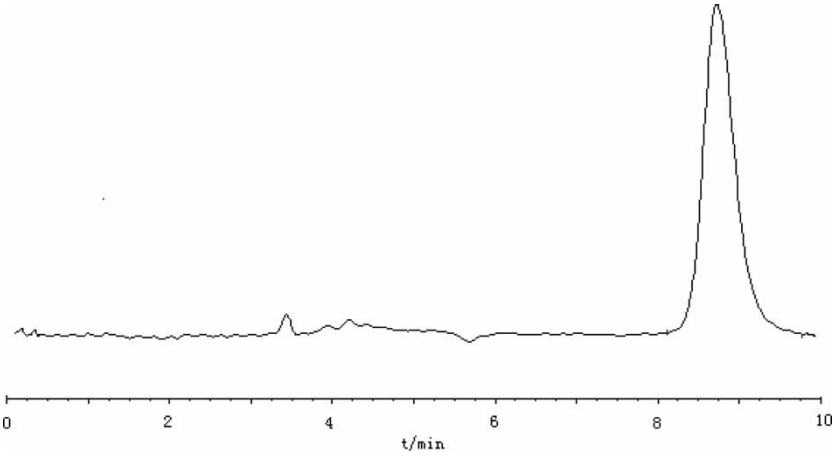

Figure 8. Chromatogram of etimycin sulfate assay. 1. Etimycin.

a concentration of about 1 mg mL^{-1}. The solution was injected into the HPLC system.

Oxidative degradation: About 10 mg of drug was weighed and transferred to a 10 mL volumetric flask. Thirty percent of H_2O_2, 0.5 mL,

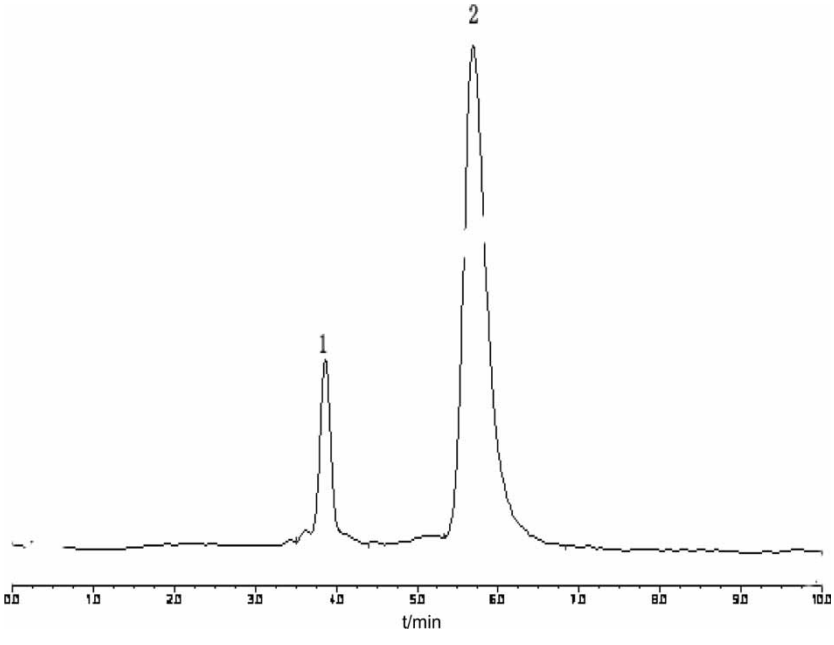

Figure 9. Chromatogram of sisomicin sulfate by high temperature damage. 1. Impuirity B; 2. Sisomycin.

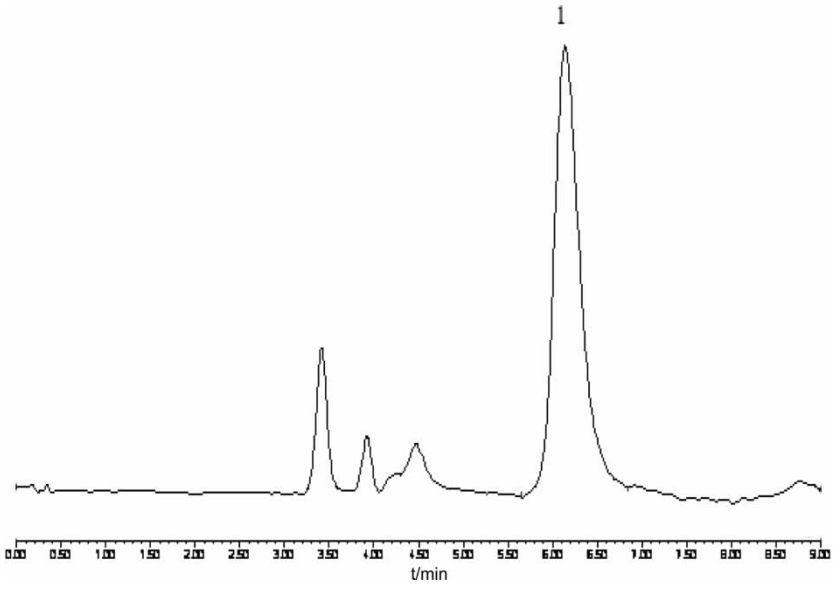

Figure 10. Chromatogram of sisomicin assay. 1. Sisomycin.

was added and allowed to stand for 8 hrs at room temperature. After 8 hrs, the drug treated with 30% H_2O_2 was diluted with mobile phase to get a concentration of about 1 mg mL^{-1}. The solution was injected into the HPLC system.

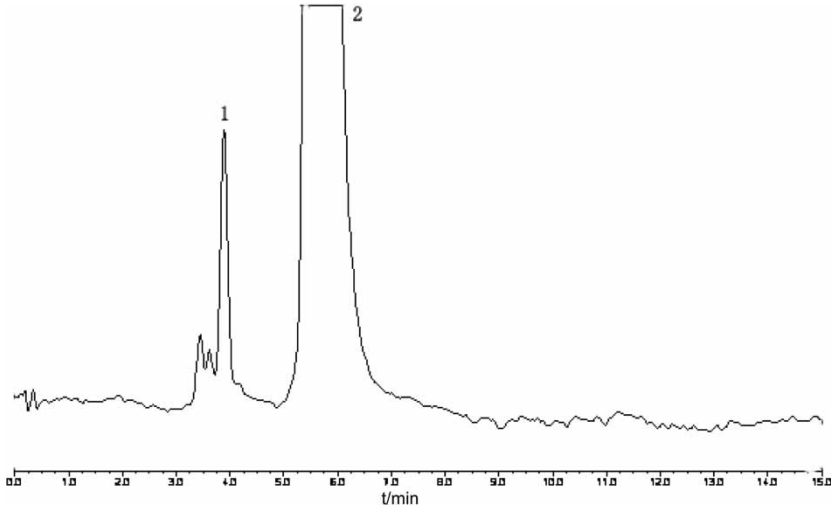

Figure 11. Chromatogram of related substances in sisomicin sulfate. 1. Impurity; 2. Sisomycin.

Heat degradation: About 10 mg of drug was weighed and transferred to a glass bottle and allowed to stand for 3 hrs in a oven maintained at 120°C. After 8 hrs, the drug was diluted with mobile phase to get a concentration of about 1 mg mL^{-1}. The solution was injected into the HPLC system.

Photolytic degradation: About 10 mg of the drug was weighed and transferred into a Petri dish, which is exposed to light at 4500 Lx for 48 hrs. After 48 hrs, the drug was diluted with mobile phase to get a concentration of about 1 mg mL^{-1}. The solution was injected into the HPLC system.

Linearity of Response

For the assay of vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate, the linearity of response was determined by preparing, in duplicate, five standard solutions ranging about from 40 to 200% of the assay concentration (0.1 mg mL^{-1}). For the assay of related substances (low level linearity), five standard solutions were prepared with concentrations ranging from 0.5 to 2.5% of the sample concentration (1.0 mg mL^{-1}). The solutions were injected into the HPLC system. The regression curve was obtained by plotting concentration versus peak area. The result indicated good linearity. The results are shown in Table 1.

Precision of the Assay

Six replicate sample solutions at 100% of the test concentration (0.1 mg mL^{-1}) were prepared and then assayed for vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate using the recommended HPLC system and sample preparation. The relative standard deviation (R.S.D.) value was 1.4%, 1.5%, 1.5%, 1.4%, respectively, ($n = 6$).

Table 1. Regression equation, correlation coefficient and detection limit

Drug substance	Linear range (mg/mL)	Regression equation	Correlation coefficient	Detection limit (μg/mL)
Vertilmicin	0.03–0.15	$A = 529.8\ C + 1.8$	0.9997	
Vertilmicin	0.005–0.025	$A = 0.777\ C - 0.376$	0.9987	1.0
Impurity A	0.005–0.020	$A = 1.196\ C - 0.159$	0.9991	0.5
Micronomycin	0.03–0.20	$A = 557.2\ C + 4.1$	0.9991	
Micronomycin	0.005–0.020	$A = 0.819\ C - 0.242$	0.9990	1.1
Gentamicin C_{1a}	0.003–0.013	$A = 0.810\ C - 0.134$	0.9997	1.1
Etilmicin	0.02–0.15	$A = 218.0\ C + 4.1$	0.9990	1.0
Sisomicin	0.02–0.10	$A = 157.3\ C + 7.6$	0.9990	
Sisomicin	0.002–0.012	$A = 0.589\ C - 0.096$	0.9971	0.5

Table 2. The results of assay and related substances determination for vertilmicin sulfate

Batches	Content of vertilmicin (%)	Content of impurity A (%)	Content of total impurity (%)
20040124	56.7	0.6	1.2
20040218	55.1	0.7	1.4
20040328	56.0	0.6	1.3

Table 3. The results of assay and related substances determination for micronomycin sulfate

Batches	Content of micronomycin (%)	Content of gentamicin C_{1a} (%)	Content of total impurity (%)
0402093	57.98	1.77	2.62
0402094	57.80	1.72	2.58
0402095	57.82	1.75	2.60

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 vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate peak of 0.05–0.1% (m/m). The limits of detection were shown in Table 1. Such limits were in good agreement with that required for the assay of related substances.

Analysis of Vertilmicin Sulfate, Micronomycin Sulfate, Etimicin Sulfate, and Sisomicin Sulfate Substance

The four drug substances were analyzed using the described HPLC system and sample preparation. The results of determination of assay and related

Table 4. The results of assay determination for etimicin sulfate

Batches	Content of etimicin (%)
20030203	58.6
20030310	58.2
20030804	59.0

Table 5. The results of assay and related substances determination for sisomicin sulfate

Batches	Content of sisomicin (%)	Content of impurity B (%)	Content of total impurity (%)
040510	54.2	0.8	1.5
040516	55.4	1.2	2.1
040526	54.3	1.0	1.8

substances for vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, and sisomicin sulfate are shown in Tables 2–5.

CONCLUSION

The described liquid chromatography with pulsed electrochemical detection provides a rapid and simple analysis for vertilmicin sulfate, micronomycin sulfate, etimicin sulfate, sisomicin sulfate, and their related substances without derivatization. The method is sensitive and accurate.

REFERENCES

1. Stead, D.A. Current methodologies for the analysis of aminoglycosides. J. Chromatogr. B **2000**, *74*, 69–93.
2. Zhou, M.; Wei, G. Determination of vertilmicin in rat serum by high-performance liquid chromatography using 1-fluoro-2,4-dinitrobenzene derivatization. J. Chromatogr. B **2003**, *798*, 43–48.
3. Edder, P.; Cominoli, A.; Corvi, C. Determination of streptomycin residues in food by solid-phase extraction and liquid chromatography with post-column derivatization fluorometric detection. J. Chromatogr. A **1999**, *830*, 345–351.
4. Adams, E.; Van Vaerenbergh, G.; Roets, E. Anlysis of amikacin by liquid chromatography with pulsed electrochemical detection. J. Chromatogr. A **1998**, *819*, 93–97.
5. Adams, E.; Dalle, J.; De Bie, E. Analysis of kanamycin by liquid chromatography with pulsed electrochemical detection. J. Chromatogr. A **1997**, *766*, 133–139.
6. Adams, E.; Schepers, R.; Roets, E. Determination of neomycin sulfate by liquid chromatography with pulsed electrochemical detection. J. Chromatogr. A **1996**, *741*, 233–240.
7. Graham, A.E.; Speicher, E.; Williamson, B. Analysis of gentamicin sulfate and a study of its degradation in dextrose solution. J. Pharm. Biomed. Anal. **1997**, *15*, 537–543.

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