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Determination of isepamicin sulfate and related compounds by high performance liquid chromatography using evaporative light scattering detection

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Abstract

A simple reversed phase high performance liquid chromatographic method was developed for the analysis of isepamicin sulfate. The use of evaporative light scattering detection eliminates the need for sample derivatization. Separation of the isepamicin aminoglycoside from structurally similar related compounds was achieved using two Waters X-Terra RP18 columns connected in tandem at 10°C. The assay of isepamicin sulfate and the estimation of its impurities was accomplished using external standard calibration curves at two sample concentrations: 1.6 mg ml^{-1} for the analysis of isepamicin sulfate and 8.0 mg ml^{-1} for the estimation of lower level impurities. The limit of detection was 0.1%. The specificity, assay linearity, low level assay linearity and assay repeatability were also investigated.

Keywords: Isepamicin sulfate; Aminoglycoside

1. Introduction

Isepamicin sulfate belongs to a class of compounds known as aminoglycoside antibiotics. Like many aminoglycosides, isepamicin sulfate lacks a suitable chromophore, which is necessary for UV detection. For this reason the analysis of isepamicin sulfate is usually performed using pre-column or post-column derivatization methods [1–6]. Derivatization may also be used to increase the specificity of isepamicin and it's related compounds. These methods usually require labor intensive sample preparation and typically contain salt laden mobile phases, which are abusive to HPLC pumps and injectors.

In comparison, a method based on the direct analysis of analyte saves valuable time with respect to sample preparation and system set up. Sample derivatization also increases the complexity of an HPLC method by requiring additional system components such as an extra reagent pump, a reaction coil and a mixing T. Other disadvantages of sample derivatization include the possible creation of degradation products and the introduction of impurities as a result of the derivatization procedure.

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In addition, certain impurities may not be detected if the compound lacks the specific functional group required for derivatization. Other non-derivatizing methods using perfluorinated ion pairing reagents with refractive index detection have been reported for the analysis of various aminoglycoside antibiotics [7,8]. Detection using refractive index prohibited the effective use of gradient elution due to significant baseline drift, which could interfere with the accuracy and detectability of the analysis. The method used a polymeric reversed phase column with acidic mobile phases containing trifluoroacetic acid and other perfluoranated carboxylic acids, but the HPLC chromatograms failed to demonstrate sharp symmetrical peaks that would typically be seen with a silica based column [8].

The main objective of this paper was to develop a quick and simple HPLC method capable of resolving isepamicin sulfate from it's geometric isomer, D-isepamicin, and other related compounds (Fig. 1) without the use of derivatization. The HPLC system demonstrates a unique synergistic approach that uses a new novel 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 (ELSD) that enables

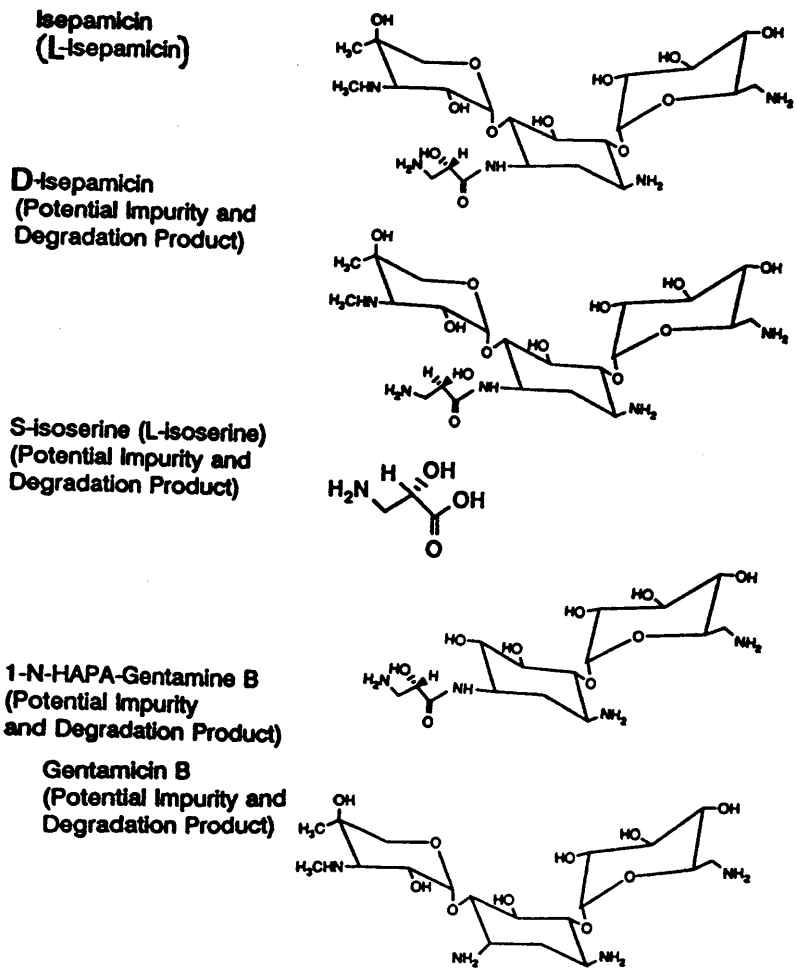

Fig. 1. Structure of isepamicin sulfate and related compounds.

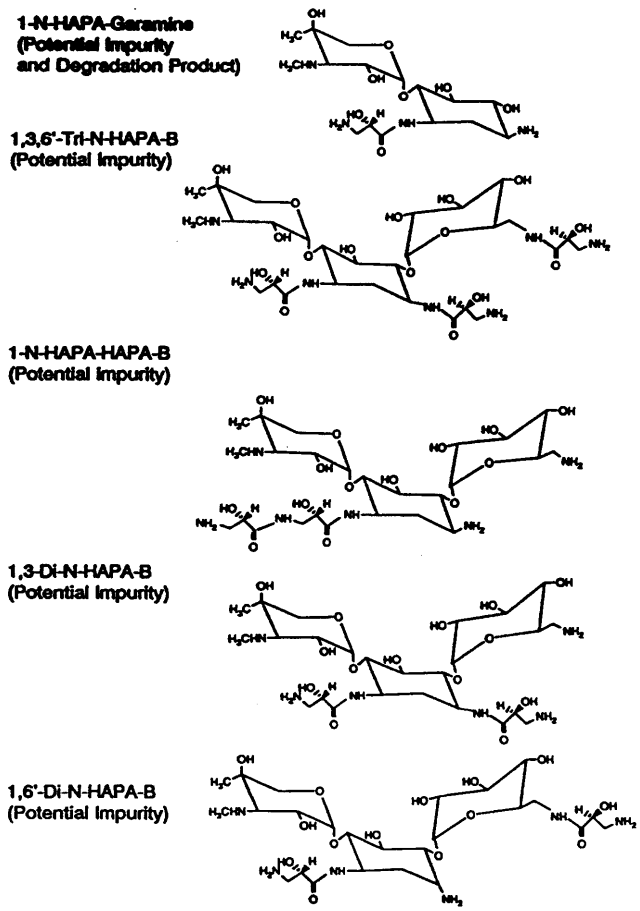

Fig. 1. (*Continued*)

the detection of compounds lacking a UV chromophore, and eliminates the need for sample derivatization.

2. Experimental

2.1. Chemicals

Isepamicin sulfate and related compounds were prepared at Schering Plough Corp., Kenilworth NJ USA.

Water: distilled and then purified through Milli Q Reagent Water system. Millipore Corp.

Ammonium hydroxide: Fisher Certified ACS. Plus approx. 14.8 N.

Glacial acetic acid: Fisher Certified ACS. Plus approx. 17.4 N.

2.2. Instrumentation

The liquid chromatograph was a Hewlett Packard 1100 series containing a quaternary pump, vacuum degasser, thermostatted column compartment and autoinjector. The data system used for the acquisition and quantification of the chromatographic data was a PE Nelson 900 series interface with Turbochrome software.

The analytical columns consist of two Waters X-Terra RP 18, 3.5 μm columns, 50×4.6 and 150×4.6 mm ID, connected in tandem using a double slipfree column connector, 6 cm $\times$ 0.005″

ID (available from Keystone Scientific, Inc.). The columns are thermostatted at 10 ± 2°C. The mobile phase consists of water:ammonium hydroxide:acetic acid (96.0:3.6:0.4). The mobile phase flow rate is 0.4 ml min^{-1} for 15 min followed by a linear increase to 1.0 ml min^{-1} at 20 min. During the course of the flow rate program the pressure typically increases from 130 to 330 bar. The detector used was a SEDERE SEDEX 55 Evaporative light scattering detector, which was upgraded to be equivalent to the SEDEX 75 model via installation of an improved nebulizer and drift tube. The drift tube temperature was set at 51°C with the gain set at 8. The nebulizer gas was nitrogen at 3.5 bar. The injection volume used was 20 μl.

2.3. Sample preparation

Isepamicin sulfate is a disulfate salt that is very soluble in water. The sulfate will combine with the ammonium ion in the mobile phase to produce ammonium sulfate. The ammonium sulfate is detected by ELSD and co-elutes with a potential degradation product, *S*-isoserine, which masks the detection of *S*-isoserine. This co-elution is prevented if the isepamicin sulfate sample solution (8.0 mg ml^{-1}) is prepared in a 19 mM barium acetate solution. The sulfate from the isepamicin sulfate sample is precipitated as barium sulfate. The barium sulfate precipitate is filtered through a 0.45 μ acrodisc CR syringe filter (Gelman Laboratory). If the sample diluent contains too much barium acetate, the barium acetate will also be detected as a chromatographic peak and may interfere with a different impurity (Fig. 2). For this reason, the sample diluent must contain the proper stoichiometric amount of barium acetate to isepamicin sulfate (a 2:1 molar ratio).

2.3.1. Assay for isepamicin sulfate

An 8.0 mg ml^{-1} sample solution in 19 mM barium acetate is filtered and diluted 5 ml into 25 ml (1.6 mg ml^{-1}) using water as the final diluent (Fig. 3).

2.3.2. Assay for related compounds

A filtered 8.0 mg ml^{-1} isepamicin sulfate sample solution is directly injected into the liquid chromatograph. A high concentration of isepam-

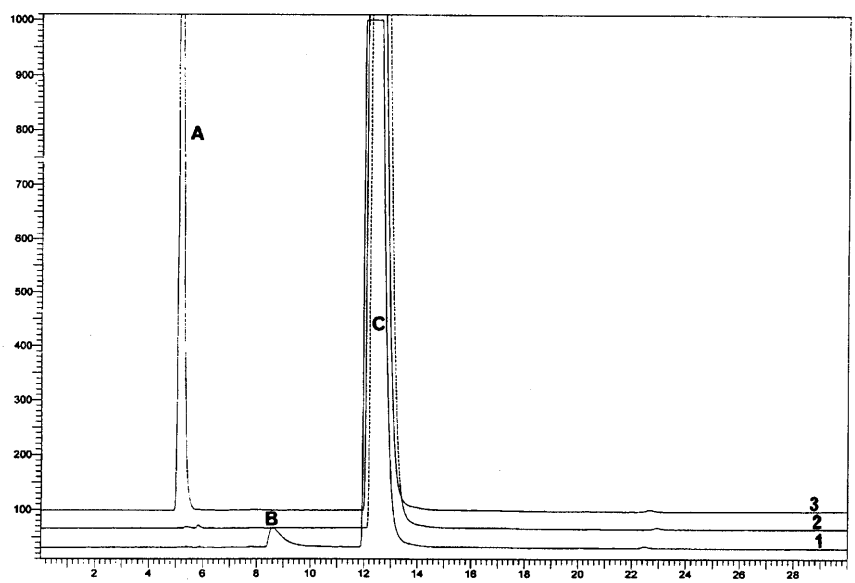

Fig. 2. 1, 8.0 mg ml^{-1} isepamicin sulfate sample solution using 20 mM barium acetate diluent; 2, 8.0 mg ml^{-1} isepamicin sulfate sample solution using 19 mM barium acetate; 3, 8.0 mg ml^{-1} isepamicin sulfate in water. A, ammonium sulfate; B, barium acetate; C, isepamicin.

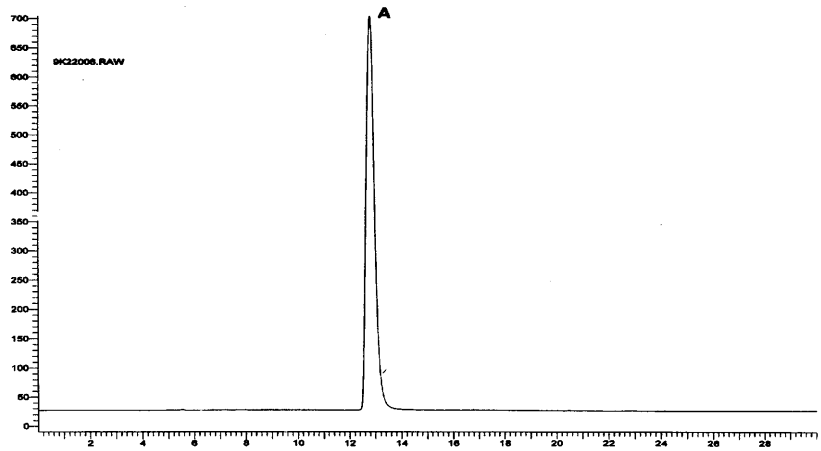

Fig. 3. Isepamicin sulfate, 1.6 mg ml^{-1}; A, isepamicin.

icin is injected onto the HPLC column to improve sensitivity for detection of low levels of related compounds, while still maintaining adequate resolution between isepamicin and its nearest eluting known related compound, the geometric isomer, D-isepamicin (Fig. 4).

3. Quantitation

An external standard calibration curve with calibration points ranging from 80 to 120% of the analytical assay concentration (1.6 mg ml^{-1}) was used for the isepamicin sulfate assay. A separate external standard calibration curve was used for the assay of low level related compounds with calibration points ranging from 0.1 to 1.0% of the sample concentration (8.0 mg ml^{-1}). Calculations were based on peak areas. The best linear fit of the calibration curve data points was obtained using a polynomial type curve.

4. Validation

Preliminary method validation was performed to determine if the HPLC system was acceptable with respect to the specificity, repeatability and detector linearity and to determine the limit of detection.

4.1. Specificity

The ability of the system to resolve isepamicin sulfate from structurally related compounds was investigated. A qualitative specificity solution was prepared in water containing isepamicin sulfate spiked with nine related compounds (Fig. 5).

4.2. Linearity of response

4.2.1. Assay for isepamicin sulfate

The linearity of response was determined by preparing in duplicate five isepamicin sulfate solutions ranging from 50 to 150% of the assay concentration (1.6 mg ml^{-1}). Each solution was

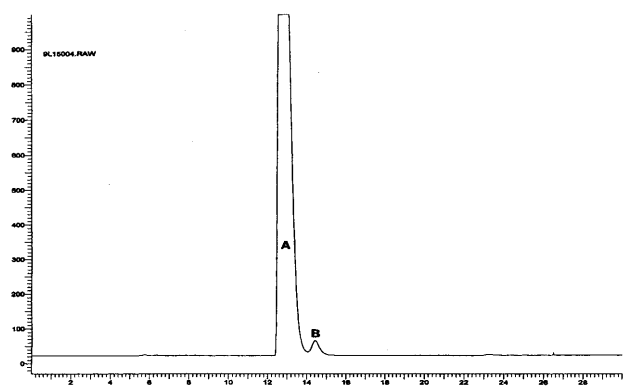

Fig. 4. Isepamicin sulfate spiked with 1.7% D-isepamicin; A, isepamicin; B, D-isepamicin.

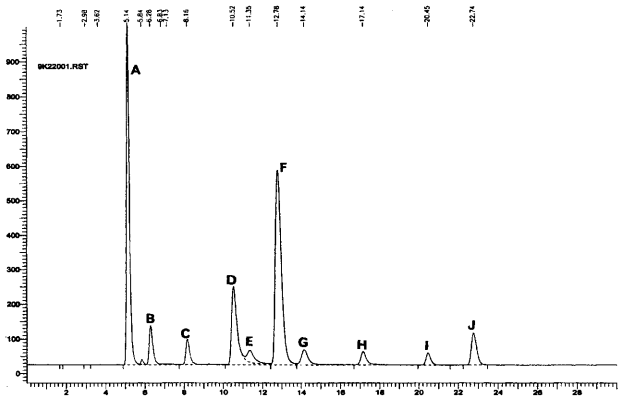

Fig. 5. Specificity solution: A, *S*-isoserine; B, 1-*N*-HAPA-gentamine-B; C, 1-*N*-HAPA-garamine; D, 1,3-di-*N*-HAPA-B; E, 1,3,6′-tri-*N*-HAPA-B; F, isepamicin; G, D-isepamicin; H, 1,6′-di-*N*-HAPA-B; I, 1-*N*-HAPA-HAPA-B; J, gentamicin-B.

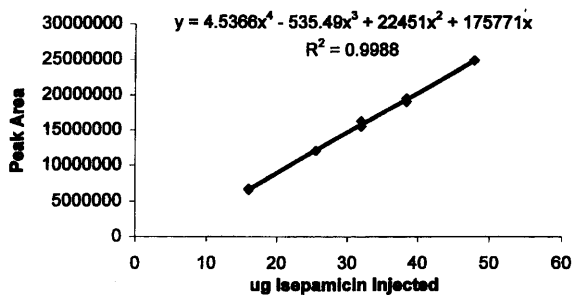

Fig. 6. Isepamicin sulfate response curve (50–150%).

analyzed using the recommended HPLC system. The isepamicin peak area response was plotted against the amount of isepamicin sulfate injected in micrograms (Fig. 6).

4.2.2. Assay for related compounds (low level linearity)

Five isepamicin sulfate solutions were prepared with concentrations ranging from 0.1 to 1.0% of the sample assay concentration (8.0 mg ml^{-1}). The solutions were injected into the HPLC system and the isepamicin peak area response was plotted against the amount injected in micrograms (Fig. 7).

4.3. Assay repeatability

Six replicate sample solutions were prepared and then assayed for isepamicin and related compounds using the recommended HPLC system and sample preparation.

4.4. Limit of detection

Two requirements were used for establishing the limit of detection of a specific compound. The first requirement was to visually confirm the presence of a chromatographic peak in an expanded scale chromatogram. The second requirement was that the peak must have a signal to noise ratio of at least 2.0.

The limit of detection was determined for isepamicin sulfate (Fig. 8). The limit of detection was also determined for *S*-isoserine and D-isepamicin in the presence of isepamicin sulfate at the specified procedural concentration, 8.0 mg ml^{-1} (Fig. 9).

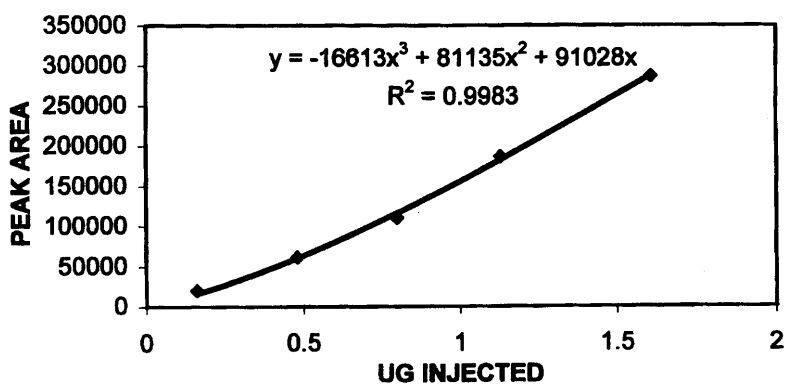

Fig. 7. Low level response curve (0.1–1.0%).

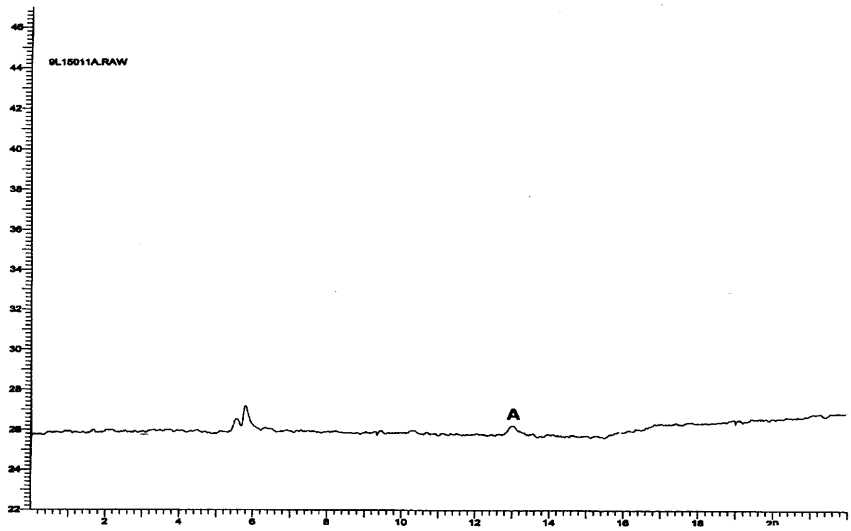

Fig. 8. Limit of detection: A, 0.1% isepamicin sulfate.

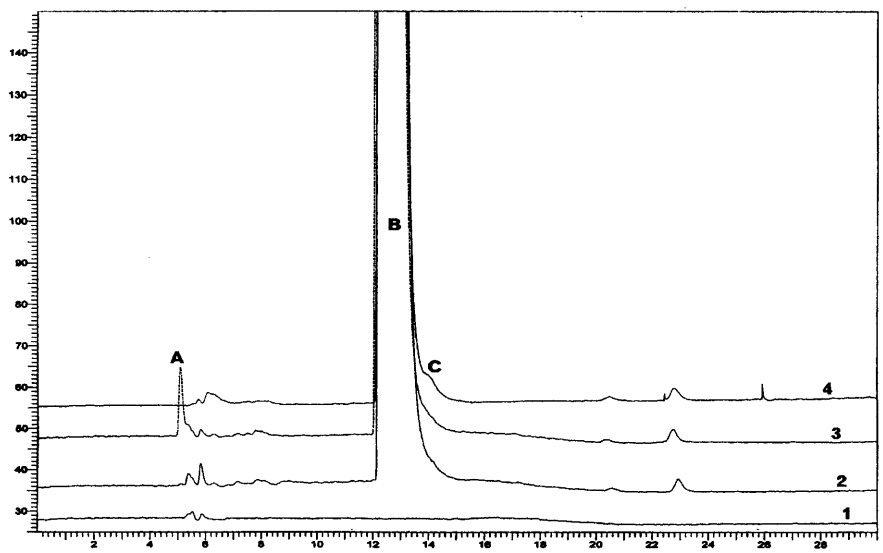

Fig. 9. 1, water blank; 2, 8.0 mg ml^{-1} isepamicin sulfate; 3, 8.0 mg ml^{-1} isepamicin sulfate spiked with *S*-isoserine (0.1%); 4, 8.0 mg ml^{-1} isepamicin sulfate spiked with D-isepamicin (0.2%); A, *S*-isoserine; B, isepamicin; C, D-isepamicin.

5. Results and discussion

The HPLC system demonstrates good specificity. All nine spiked related compounds are resolved from isepamicin sulfate as well as from each other.

Satisfactory detector response was obtained using a polynomial curve fit of the calibration points ranging from 50 to 150% of the isepamicin sulfate assay concentration. The correlation coefficient (R^2) was 0.9988. The lower level response curve of isepamicin sulfate, from 0.1 to 1.0% of the sample

concentration in the analysis of related compounds, was also satisfactory with an R^2 equal to 0.9983. The tendency of evaporative light scattering detection to demonstrate a slightly non-linear response has been previously documented [9,10]. This intrinsic behavior seems to be a detector characteristic and is probably a direct result of the ELSD mechanism of operation.

An inherent advantage of ELSD is its non-discriminating response of isepamicin sulfate and its related compounds; all related compounds will give a similar response per unit concentration.

The analysis of six replicate isepamicin sulfate samples demonstrated acceptable assay repeatability. An average assay value of 96.0% was obtained with a 1.6% R.S.D. Each sample solution contained the same six related compounds. The total average percent related compounds obtained was 1.4%, with a 6.2% R.S.D. The isepamicin sulfate samples were analyzed 'as-is' and the assay results were not corrected for moisture content. This may help explain the discrepancy seen with the mass balance.

A 0.1% limit of detection was obtained for isepamicin sulfate. In the presence of isepamicin sulfate (at 8.0 mg ml^{-1}) a 0.2% limit of detection was obtained for D-isepamicin, primarily due to a decrease in resolution, which was created when the low level D-isepamicin was partially obscured by the tail end of the much greater adjacent main peak. A 0.1% isoserine peak is easily detected in the presence of isepamicin sulfate. The short retention time and narrow bandwidth of isoserine yields a lower limit of detection, which is about 0.05%.

6. Conclusions

The described HPLC method provides a quick and simple assay of isepamicin sulfate and its related compounds without the use of derivatization. The system demonstrates excellent specificity and eliminates the potential problems that are encountered with a derivatization method. The direct analysis of isepamicin sulfate using ELSD also reduces analysis time with respect to sample preparation and system set up, which increases laboratory efficiency and productivity. In addition, the HPLC pump and injector will experience less wear and tear due to the absence of abusive mobile phase additives or ion pairing reagents.

Further optimization of ELSD parameters such as the drift tube temperature or the nebulizer gas pressure may reduce baseline noise and thereby decrease the limit of detection. This method can also be modified for the analysis of other aminoglycosides with the proper mobile phase modifications, or by using gradient elution. The HPLC system also has potential utility as an LC-MS method.

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