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Determination of vertilmicin in rat serum by high-performance liquid chromatography using 1-fluoro-2,4-dinitrobenzene derivatization

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Abstract

A procedure for the high-performance liquid chromatographic determination of vertilmicin in rat serum was described using pre-column derivatization. The serum proteins were precipitated with acetonitrile and vertilmicin in the supernatant was derivatized with 1-fluoro-2,4-dinitrobenzene. Etimicin was selected as the internal standard. The mobile phase consisted of methanol−20 mM ammonium acetate (80:20, v/v), and flow-rate was 0.9 ml/min. Ultraviolet detection was set at 365 nm. The reaction products were chromatographed on a C_{18} column kept at 40 °C. A good linearity was found in the range of 0.5–250 μg/ml. Both intra- and inter-day precisions of vertilmicin, expressed as the relative standard deviation, were less than 7.4%. Accuracy, expressed as the relative error, ranged from −0.1 to 3.6%. The mean absolute recovery of vertilmicin at three different concentrations was 92.5%. Serum volumes of 50 μl were sufficient for the determination of vertilmicin. The method was proved suitable for the pharmacokinetic study of vertilmicin in rats.

Keywords: Derivatization, LC; Vertilmicin; 1-Fluoro-2,4-dinitrobenzene

1. Introduction

Vertilmicin {1-*N*-ethyl-verdamicin, *O*-3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1 → 6)-*O*-[2,6-diamino-2,3,4,6,7-pentadeoxy-β-L-threo-hept-4-enopyranosyl-(1 → 4)]-2-deoxy-N^1-ethyl-D-streptamine, CAS 59711-97-6, Fig. 1}, a new semisynthetic aminoglycoside derived from verdamicin (CAS 49863-48-1), was in the stage of preclinical development. In order to investigate the pharmacokinetics of vertilmicin in animals, it was essential to develop a sensitive method for the analysis of vertilmicin in body fluids. Up to now, the determination of vertilmicin has not been reported, but a wide variety of methods for the bioanalysis of aminoglycoside antibiotics in biological samples have been published over the years, including microbiological assay [1], immunoassays [2,3], capillary electrophoresis [4], thin layer chromatography [5], gas-liquid chromatography [6], and high-performance liquid chromatography with detection of UV [7–14], fluorescene [15], electrochemical [16] and mass spectrometry [17,18]. Vertilmicin has a structure of aminoglycoside and the methods mentioned above should also be applicable to it. HPLC assays using 1-fluoro-2,4-dinitrobenzene (FDNB) derivatization had been developed for neomycin, gentamicin, sisomicin, fortimicin C, amikacin, tobramycin, paromomycin and netilmicin [7–14], and HPLC with UV detection was a widely used equipment. Therefore, we developed an analytical method of pre-column derivatization with FDNB, based on the method reported for gentamicin and tobramycin by Barends et al. [8,9], to determine vertilmicin in rats.

2. Experimental

2.1. Chemicals

Vertilmicin sulfate (lot nr: 20020220, stated potency 544.48 μg/mg, one molecule vertilmicin is bound to 2.5

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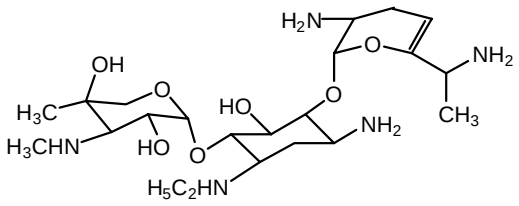
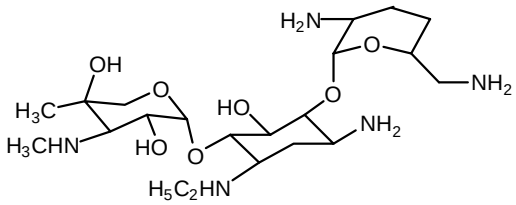


Vertilmicin



Etimicin

Fig. 1. Chemical structures of vertilmicin and etimicin (internal standard).

molecules sulfate), was obtained by courtesy of Kangle Pharmaceutical Co. Ltd (Zhejiang, China). Etimicin sulfate was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 1-Fluoro-2,4-dinitrobenzene (FDNB) was analytical grade from E. Merck (Darmstadt, Germany). Methanol and acetonitrile were of HPLC-grade. Tris(hydroxymethyl)aminomethane (Tris) and ammonium acetate were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation and chromatographic conditions

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of a pump (LC-6AT), an autosampler (SIL-10A_{XL}), a system controller (SCL-10A), a UV detector (Spectra 100), a column oven (TC-1) and a LC-Workstation (Ckchrom). Chromatogaphy was performed on a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., particle 10 μm, Dikma, Beijing, China). The mobile phase consisted of methanol−20 mM ammonium acetate (80:20, v/v). The flow-rate was 0.9 ml/min. Ultraviolet detection was performed at wavelength of 365 nm and the column temperature was kept at 40 °C.

Finnigan LCQ system (San Jose, CA, USA) was equipped with an atmospheric pressure ionization interface. The electrospray ionization was performed applying parameters: spray voltage 4.25 kV, capillary temperature 180 °C, capillary voltage 6.5 V, a sheath gas (N_2) flow-rate 0.75 ml/min, an auxiliary gas (N_2) flow-rate 0.15 ml/min, and positive ion mode monitoring. The collision gas was He and the collision energy was set at 25 eV.

2.3. Preparation of standard solutions and quality control samples

The stock standard solution of vertilmicin was prepared by dissolving the accurately weighed vertilmicin sulfate in water to give a final concentration of 5.0 mg/ml (calculated as free base). The solution was then successively diluted with water to achieve standard working solutions at concentrations of 0.005, 0.01, 0.1, 0.2, 0.5, 1.0 and 2.5 mg/ml for vertilmicin. A 20.0 μg/ml internal standard working solution was prepared by diluting the 1.0 mg/ml (calculated as free base) stock standard solution of etimicin with Tris 20 mg/ml in water (pH 10.5).

The standard working solutions (100 μl) were used to spike blank rats serum (0.9 ml) to give final concentrations in the range of 0.5–250.0 μg/ml. The serum samples at concentrations of 1.0, 50.0 and 200.0 μg/ml were used as quality control samples in the developing analytical method and during the pharmacokinetic study. All the solutions were stored at 4 °C and were brought to room temperature before use.

2.4. Sample preparation

To a 50 μl aliquot of serum was added 50 μl of the internal standard and 200 μl of acetonitrile. The samples were vortexed for 20 s, and then centrifuged at 4000 × g for 5 min. Transferred 200 μl of the supernatant into an ampoule, added 20 μl of FDNB 250 mg/ml in acetonitrile. Closed the ampoules and placed in a water-bath at 80 °C for 45 min. A 50 μl aliquot of the solution was injected onto the HPLC system.

2.5. Performance characteristics

2.5.1. Assay specificity

The specificity of the method was demonstrated by comparing chromatograms of six independent serum samples from rats, each as a blank and a spiked sample.

2.5.2. Calibration and calculation procedures

Standard curves were constructed using weighted ($w = 1/c^2$) linear least-squares regression analysis of the observed peak area ratios of the derivatized products of vertilmicin and the internal standard [19]. The unknown samples concentrations were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curve.

2.5.3. Precision and accuracy of the assay

In order to evaluate the intra-day precision and accuracy, six replicate samples of each concentration of QC samples were analyzed on the same day. The inter-day validation was evaluated on the QC samples of different days. Intra- and inter-day assays were assessed using six spiked serum samples at each concentrations of 1.0, 50.0 and

200.0 μg/ml. The accuracy was evaluated as the relative error, and the precision was evaluated by the relative standard deviation.

2.5.4. Absolute recovery and analyte stability

The absolute recoveries of vertilmicin and etimicin from serum were determined as follows: Drug-free serum with added vertilmicin (1.0, 50.0 and 200.0 μg/ml) and etimicin (20.0 μg/ml) was processed by the method described above. Absolute recovery was calculated by comparing the peak areas so obtained with those obtained by derivation of aqueous solutions.

The stability of vertilmicin in rat serum was investigated under a variety of storage conditions: performing three cycles of freeze (−20 °C)–thaw (room temperature), under −20 °C freezer and 24 h storage of the derivatized products of vertilmicin and the internal standard at room temperature.

2.6. Application of the analytical method

The established analytical method was used to investigate the profile of vertilmicin, after intravenous injection of 40 mg/kg vertilmicin to six Wistar rats via the tail vein. Blood samples were drawn from each rat by puncture of the retro-orbital sinus. This was performed at 0 min (predose), and 2, 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, and 6 h after intravenous administration. The serum concentration-time curves of vertilmicin in rats were plotted.

3. Results and discussion

3.1. The optimal derivatization conditions

The optimal derivatization conditions were determined by investigation of the effects of pH, FDNB concentration, reaction time and temperature. Dispensed into 50 μl of 200 μg/ml aqueous vertilmicin solution, 50 μl of Tris 20 mg/ml solution (pH ranging from 5 to 10.5), 200 μl of acetonitrile and 30 μl of FDNB 250 mg/ml in acetonitrile (adding 30 μl of FDNB resulted in the same FDNB concentration in the derivatization reaction as that in the sample preparation procedure). Closed the ampoules and placed in a water-bath at 80 °C for 45 min. A 50 μl aliquot of the solutions was injected onto the HPLC system. The pH of Tris 20 mg/ml in water, which was approximately 10.5, was adjusted to 10.0, 9.5, 9.0, 8.5, 8.0, 7.5, 7.0, 6.0 and 5.0 by adding different quantities of 2 M HCl solution, respectively. By comparing peak areas of the derivatized product of vertilmicin under different pH values, the effect of pH on derivatization reaction was observed. According to method described above, FDNB concentration, reaction time and temperature were varied, respectively, to observe the effects of these factors on derivatization reaction. The results were depicted in Figs. 2 and 3.

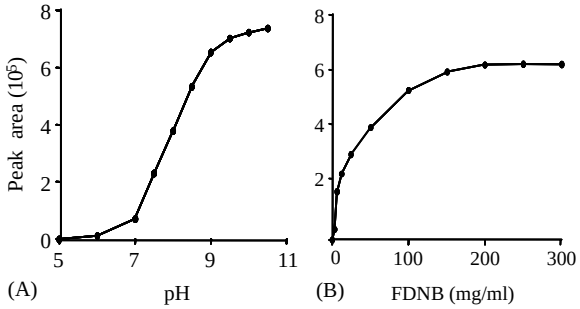


Fig. 2. Effects of pH of the Tris solution (A) and the FDNB concentration (B) on the peak areas of the FDNB-derivatized vertilmicin ($n = 3$).

The experiment showed that the peak areas of the derivatized products increased with higher pH values of the added buffer solution. Above pH 9.0, no significant increase in the peak areas was observed. Therefore, A solution of Tris 20 mg/ml in water (pH 10.5) was selected as buffer. The peak areas of the derivatized products increased with higher reagent concentrations, up to an FDNB concentration of about 100 mg/ml. To ensure complete derivatization, FDNB at 250 mg/ml in acetonitrile was chosen as the drivatization reagent. At the lower temperature, the reaction was found to proceed slowly, whereas at the higher temperature the problem of solvent evaporation was encountered. To ensure stable results, reaction conditions were selected at 80 °C for 45 min. These conditions were consistent with that reported for gentamicin and tobramycin [8,9].

3.2. Characterization of the FDNB-derivatized vertilmicin and etimicin

It was assumed that every amino group, primary as well as secondary, of aminoglycoside reacted with one FDNB molecule and its derivatized products had maximum absorptions at 365 nm [8]. The (+)-ESI-MS^n spectra of the FDNB-derivatized vertilmicin and etimicin are summarized in Figs. 4 and 5. The pseudomolecular ions of vertilmicin and etimicin were at *m/z* 490 and 478,

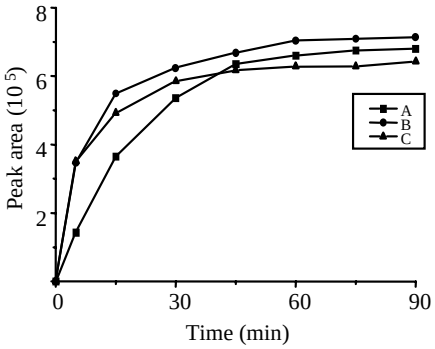


Fig. 3. Effect of the reaction time on the peak areas of the FDNB-derivatized vertilmicin, at reaction temperatures of 65 °C (A), 80 °C (B) and 95 °C (C) ($n = 3$).

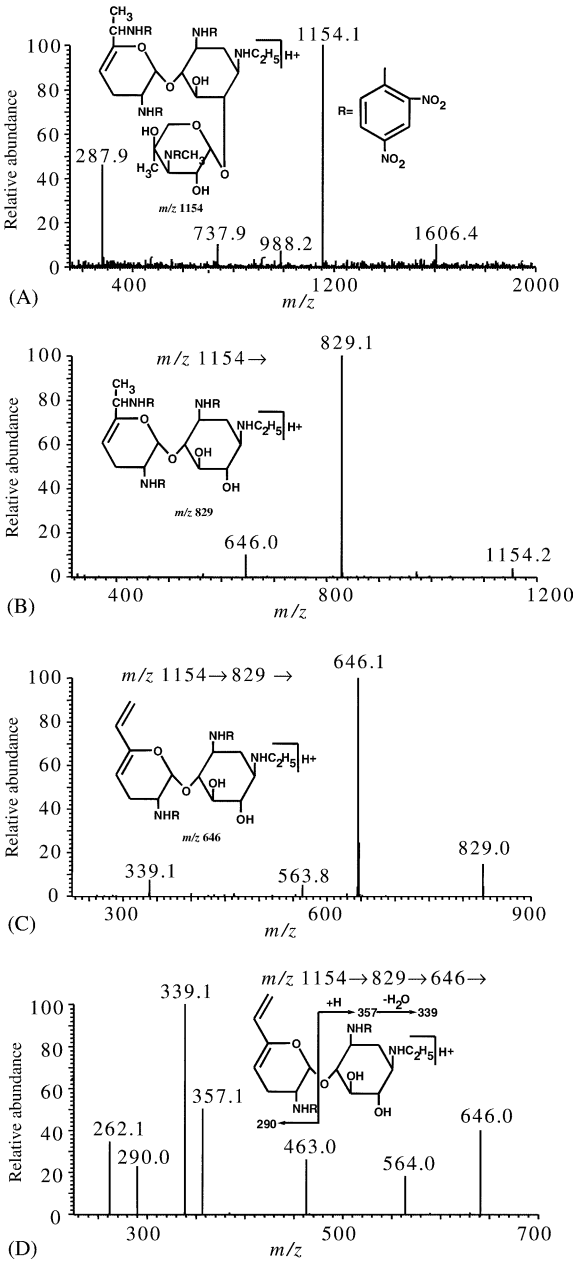


Fig. 4. (+)-ESI-MS^n spectra of the FDNB-derivatized vertilmicin (A: MS scan, B: MS^2 scan, C: MS^3 scan, D: MS^4 scan).

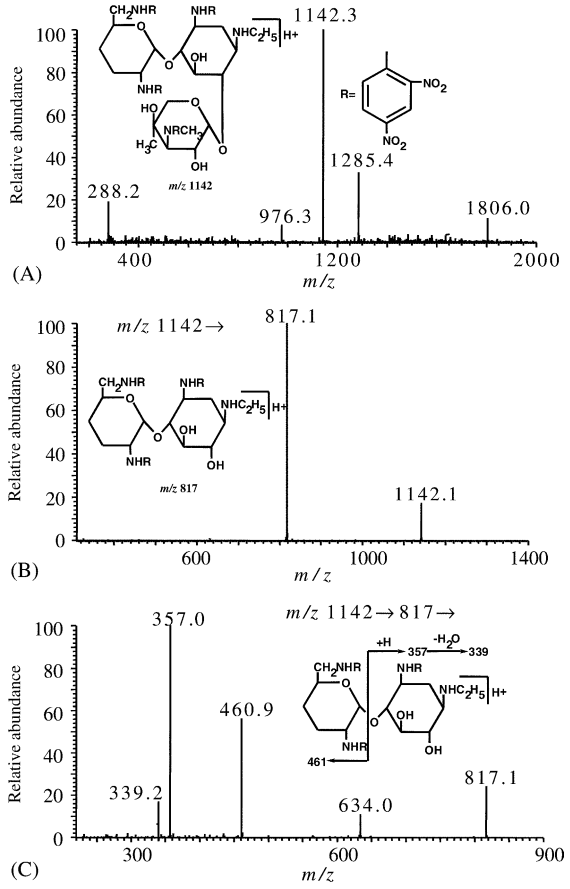


Fig. 5. (+)-ESI-MS^n spectra of the FDNB-derivatized etimicin (A: MS scan, B: MS^2 scan, C: MS^3 scan).

respectively. While the pseudomolecular ions of their derivatized products were at *m*/*z* 1154 and 1142 (Figs. 4A and 5A), respectively, an increase of 664 Da and indicative of the addition of four FDNB molecules to vertilmicin and etimicin molecule (FDNB MW 186 Da). Figs. 4B and 5B demonstrated abundant product ions at *m*/*z* 829 and 817, respectively, which were formed from the ions at *m*/*z* 1154 and 1142 by loss of an identical neutral fragmentation 325, 166 Da more than that of before derivatization, suggesting that the secondary amino group of the lost pyranosyl ring was derivatized by one FDNB molecule. Fig. 4C gave a prominent fragmentation ion at *m*/*z* 646, with loss of 183 Da (H_2NR) from the ion at *m*/*z* 829. Fig. 4D showed product ions at *m*/*z* 463, 357, 339 and 290, respectively. The former was formed by loss of a H_2NR molecule from the ion at *m*/*z* 646, and the ion at *m*/*z* 339 was formed by loss of H_2O from the ion at *m*/*z* 357, while the ions at *m*/*z* 357 and 290 were inferred to be formed by the cleavage of the C–O bond between the enopyranosyl and oxygen. As shown in Fig. 5C, it gave product ions at *m*/*z* 357, 339, 461 and 634, respectively, which were formed by the same cleavage patterns as the ion at *m*/*z* 646 in Fig. 4D. These results led to the conclusion that three primary and one secondary amino groups of vertilmicin and etimicin were derivatized by four FDNB molecules, respectively, and another secondary amino group kept free, under the experimental conditions.

3.3. Assay specificity

The typical chromatograms of derivatized products of vertilmicin and the internal standard are shown in Fig. 6. The peaks for their derivatized products were not interfered by endogenous substances. The retention times for derivatized products of vertilmicin and the internal standard were approximately 12.0 and 17.0 min, respectively.

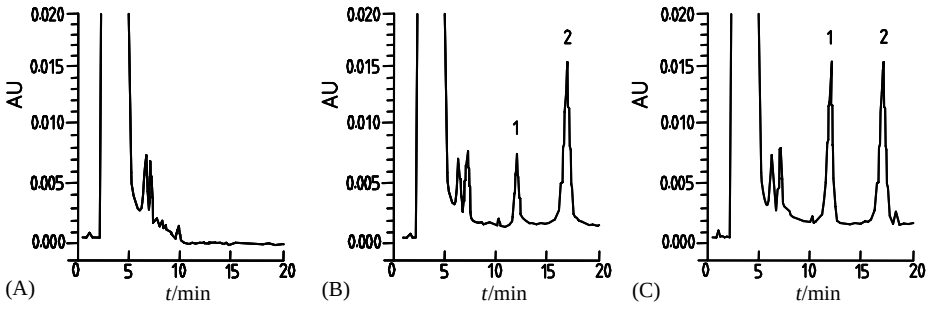


Fig. 6. Representative chromatograms of the FDNB-derivatized products. (A) A blank rat serum sample; (B) a blank rat serum sample spiked with vertilmicin (concentration at 2.0 μg/ml) and the internal standard (concentration at 20.0 μg/ml) and (C) a rat serum sample 2.0 h after intravenous administration of 40 mg/kg vertilmicin. Peaks 1 and 2 refer to the FDNB-derivatized products of vertilmicin and the internal standard, respectively.

3.4. Linearity of calibration curve and lower limit of quantification (LLOQ)

The linearity test of calibration range was carried out in the range of 0.5–250 μg/ml in rat serum. Calibration curves were linear in the studied range with correlation coefficient ($r = 0.9967$). A typical linear regression equation for the corresponding curves was $y = 0.0906x + 0.0032$, where y represents the peak area ratio of derivatized products of vertilmicin to the internal standard, and x represents concentration of vertilmicin.

Lower limit of quantification (LLOQ) was defined as the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision ≤15% and accuracy within ±15% of the true concentration. LLOQ of vetilmicin was 0.5 μg/ml for the determination of vertilmicin in rat serum.

3.5. Assay precision and accuracy

Table 1 summarizes the intra- and inter-day precision and accuracy for vetilmicin from QC samples. In this assay, the intra- and inter-day precisions ranged from 4.8 to 6.4% and from 2.1 to 7.4%, respectively. The accuracy ranged from −0.1 to 3.6%. These data suggested that the method was accurate and reproducible for the determination of vetilmicin in rat serum.

3.6. Absolute recovery and analyte stability

The absolute recoveries of vertilmicin, at concentrations of 1.0, 50.0, and 200.0 μg/ml, were 95.5 ± 5.2, 90.5 ± 4.5, and 91.6 ± 4.8%, respectively. The absolute recovery of the internal standard recovery was determined at the concentration used in the assay procedure and was found 92.6 ± 5.1%.

Vertilmicin was found to be stable (>90%) in rat serum after three cycles of freeze (−20 °C)–thaw (room temperature). Under −20 °C freezer, the analyte was found to be stable in rat serum for at least 1 month. The derivatized products of vertilmicin and the internal standard were also shown to be stable for at least 24 h at room temperature.

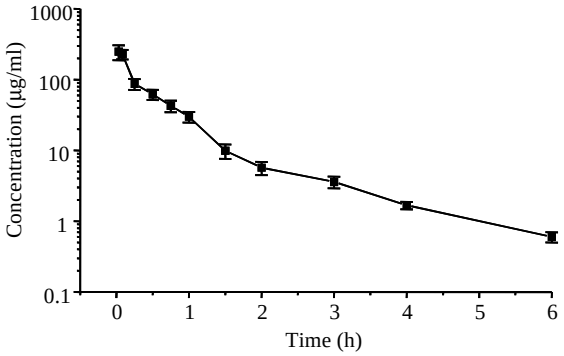


Fig. 7. Mean serum concentration-time profile of the vertilmicin after intravenous administration of 40 mg/kg to six Wistar rats.

3.7. Application of the analytical method in pharmacokinetic studies

After intravenous injection of 40 mg/kg vertilmicin to rats, serum concentrations of vertilmicin were determined by the described HPLC method. Fig. 7 shows the mean serum

Table 1
Precision and accuracy for analysis of the vertilmicin in rat serum ($n = 6$)

Concentration spiked (μg/ml)	Intra-day concentration found (Mean ± S.D.)	R.S.D. (%)	Inter-day concentration found (Mean ± S.D.)	R.S.D. (%)	Accuracy RE (%)
1.0	1.04 ± 0.05	4.8	1.00 ± 0.02	2.1	3.6
50.0	49.96 ± 1.42	2.8	51.32 ± 1.94	3.8	−0.1
200.0	205.7 ± 13.2	6.4	208.8 ± 15.3	7.4	2.8

concentration-time curve of vertilmicin after intravenous administration ($n = 6$). Serum concentrations of vertilmicin in rat were detectable for at least 6 h after intravenous administration. The main pharmacokinetic parameters of vertilmicin, calculated by non-compartmental analysis, were as follow: the elimination half-life ($T_{1/2}$) was 1.21 h, elimination rate constant (K_e) was 0.57 h, the area under the serum concentration-time curve from zero to time of the last measurable concentration ($AUC_{0\text{-}t}$) was 111.9 μg h/ml, and total clearance (CL_{tot}) was 5.96 ml/min.

4. Conclusion

The developed bioanalytical method for the determination of vertilmicin in rat serum was sensitive and specific. The linear calibration curves were obtained in the concentration range of 0.5–250 μg/ml. The LLOQ obtained with the proposed method was 0.5 μg/ml. Both intra- and inter-day precisions of vertilmicin were less than 7.4%. The accuracy ranged from −0.1 to 3.6%. In addition, the structures of the FDNB-derivatized vertilmicin and etimicin were investigated by (+)-ESI-MS^n. The method was shown to be successful in application of pharmacokinetic study for in rats.

Acknowledgements

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