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High-performance liquid chromatographic determination of vertilmicin in rat plasma using sensitive fluorometric derivatization

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Abstract

A sensitive and reliable high-performance liquid chromatographic method was developed for the determination of vertilmicin in rat plasma. Derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by C_{18} reversed-phase chromatography allowed the fluorimetric detection of vertilmicin. Optimal conditions for the derivatization of vertilmicin are described. The limit of quantification was 0.02 mg/L. The pharmacokinetics of vertilmicin was studied in 24 rats following intramuscular injection (i.m.) of different doses (4, 8, 16, 32 mg/kg of body weight). The pharmacokinetic parameter values were estimated by use of 3P97 program. In this study, we assessed the dose proportionality of vertilmicin after single intramuscular injection doses and obtained new information on the pharmacokinetics of the compound.

Keywords: Vertilmicin; Aminoglycosides; Fluorescene; High-performance liquid chromatography; 9-Fluorenylmethyl chloroformate; Pharmacokinetics

1. Introduction

Vertilmicin belongs to a class of compounds known as aminoglycoside antibiotics, which inhibit the protein synthesis of microorganisms, especially aerobic gram-negative bacilli, resulting in a rapid, concentration-dependent bactericidal action. However, these drugs can give rise to adverse reactions, including ototoxicity and nephrotoxicity, which is almost always reversible when treatment is discontinued [1]. Vertilmicin, which is a novel drug, was found in the synthesis of netilmicin and is being registered for use in China. It was proved to have better antibiotic potency and less toxicity than netilmicin.

Like many other aminoglycosides, vertilmicin lacks a suitable chromophore for UV or fluorimetric detection (Fig. 1). The analysis of aminoglycosides are usually performed using pre-column [2–8] and post-column derivatization [9] methods, or other detections such as evaporative light scattering detection [10], pulsed electrochemical detection [11–13] and mass spectrometry [12,14,15]. Zhou et al. [16] had reported an analytical method for veritlmicin in plasma and 1-fluoro-2,4-dinitrobenzene was used for derivatization.

The purpose of this study was to develop a simple and rapid method for extraction and HPLC determination of vertilmicin in plasma, and also the method was applied for pharmacokinetic study. We studied the pharmacokinetics of vertilmicin in rats after intramuscular injection of different doses and assessed the dose proportionality of vertilmicin after single intramuscular injection doses.

2. Experimental

2.1. Chemicals

Vertilmicin sulfate (>99%) and netilmicin sulfate (>99%) were offered by Zhejiang Conler pharmaceutical Co. Ltd. (China). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). 9-Fluorenylmethyl chloroformate (FMOC-Cl) was purchased from ALDRICH (97%, 05614BB). Sodium tetraborate and phosphoric acid was

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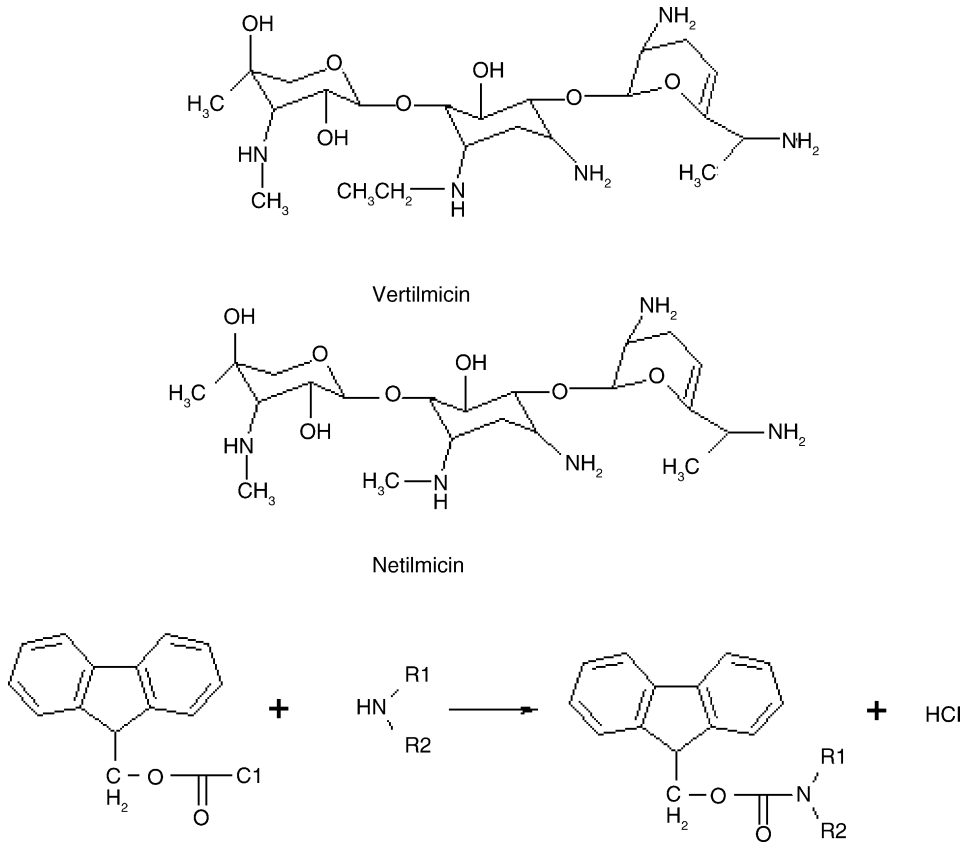

Fig. 1. The structure of vertilmicin and netilmicin and reaction of amines with FMOC-Cl.

obtained from Shanghai Chemical Reagent Co. High-purity water was prepared by a Direct-Q water purification system (Millipore USA).

2.2. Instrumentation and HPLC procedure

The chromatographic system (Agilent HP1100 series high-performance liquid chromatography) (California, USA) consisted of an Agilent G1311A QuatPump fitted with a G1322A Degasser and a column oven. The detector used was an Agilent G1321A FLD. The separation was performed on an Agilent XDB-C_{18} (particle size 5 μm, 150 mm × 4.6 mm i.d.) column. The mobile phase used for the reversed phase chromatographic method consisted of a mixture of water–acetonitrile (5:95, v/v). The mobile phase was filtered through a 0.45-μm pore size membrane filter prior to mixing and ultrasonically degassed after mixing. The flow-rate was 1 ml/min. Chromatography was performed at 30 °C. The analytes were detected at excitation 263 nm and emission 315 nm.

2.3. Solutions

The derivatization reagent consisted of 0.8 M FMOC-Cl in acetonitrile. Borate buffers were prepared by dissolving 19 g sodium tetraborate in 1 L water and adjusted to pH 8.0 with phosphoric acid. Vertilmicin was dissolved in water as stock standard solution (1 g/L). These solutions were all stored at 4 °C before used.

2.4. Sample preparation

A 50-μL volume of plasma was mixed with a 5-μL volume of internal standard (10 mg/L netilmicin dissolved in acetonitrile) and a 100-μL volume of acetonitrile. The solution was vortex-mixed for 1 min and centrifuged for 10 min at 12,000 × *g*. A 100-μL aliquot of the clear supernatant was adjusted to optimal pH by the addition of 50 μL borate buffer (0.2 M in water, pH 8.0) and derivatized with 50 μL FMOC-Cl (8 mM in acetonitrile) at ambient temperature for 15 min. The reaction was stopped by adding 10 μL glycine (0.1 M in water) and vortex-mixed for 1 min. Twenty microlitres of the reaction mixture was injected into the HPLC system.

2.5. Preparation of calibrators and calibration

We prepared the calibrators by diluting each of vertilmicin components in deionized water. The stock solutions were added to blank rat plasma to provide concentrations of 0.02, 0.1, 0.5, 1, 5, 10 and 45 mg/L of individual vertilmicin. The calibrators in plasma were derivatized as described above. The calibration curves were obtained by plotting the peak

area ratios (vertilmicin/netilmicin) as a function of the respective concentrations for verilmicin and calculating the linear regression.

2.6. Drug administration and sample collection

Following an overnight fast, 24 male wistar rats (250–300 g) were separated into four groups randomly. Different doses (4, 8, 16, 32 mg/kg) were given to the rats in different groups by intramuscular injection (i.m.). Heparinized venous blood samples, 0.15 mL, were collected from tail vein according to the time schedule, which included a blank blood sample just prior to dosing and then at 5, 10, 15, 30 min, 1, 1.5, 2, 4, 6, 8 and 12 h after drug administration. Fifty microlitres plasma was immediately separated by centrifugation at 8000 rpm for 10 min, then transferred to suitably labeled tubes and stored at –20 °C until assay.

2.7. Pharmacokinetic data analysis

T_{max} and C_{max} were recorded directly from the measured data. AUC_{0-12h} and $AUC_{0-\infty}$ was calculated by noncompartmental methods using the linear trapezoidal rule. The other pharmacokinetic analysis was performed using Practical Pharmacokinetic Program-Version 97 (3P97) published by Chinese Pharmacological Association (Beijing, China).

3. Results and discussion

3.1. Chromatographic separation

Typical chromatograms resulting from the analysis of various plasma samples are showed in Fig. 2. Vertilmicin and netilmicin appear as well resolved peaks with retention times of 15.5 and 12.4 min, respectively. The excellent separation

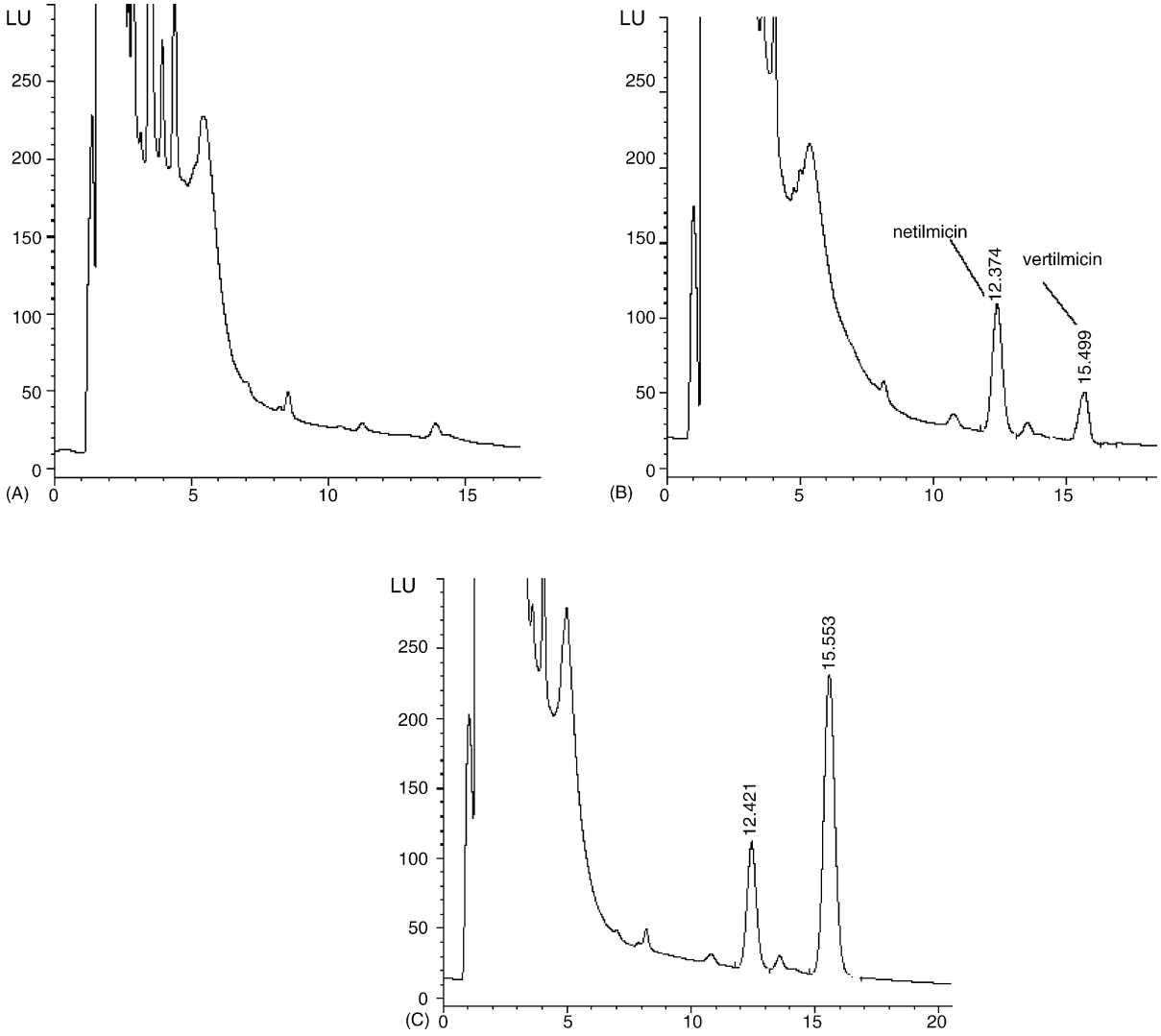

Fig. 2. (A) Chromatogram of a blank plasma sample; (B) plasma sample spiked with vertilmicin (0.5 mg/L) and netilmicin (1 mg/L); and (C) a rat plasma sample 1.0 h after intramuscular administration of 4 mg/kg vertilmicin.

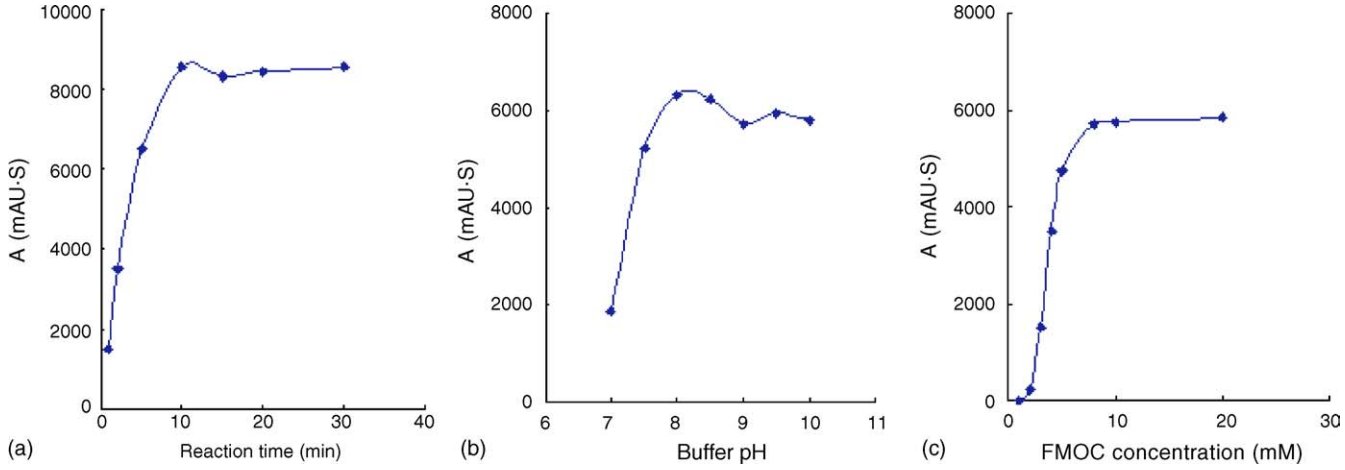

Fig. 3. Condition influence for the reaction of vertilmicin with FMOC-Cl: (a) time course influence; (b) pH influence; (c) FMOC-Cl concentration influence) (other conditions as in Section 2.4).

of the vertilmicin and netilmicin peaks allowed for quantification by simply measuring the peak areas. Other aminoglycosides such as sisomicin, tobramycin and neomycin were added to plasma samples for evaluation of possible interference. No chromatographic interference from these drugs and endogenous substances was found.

3.2. Optimization of derivatization conditions

Vertilmicin was reacted with FMOC-Cl, as described above, but the pH, reaction time, concentrations of acetonitrile, borate and FMOC-Cl were independently varied in turn. The effects of these parameters on the peak areas (A) of vertilmicin were studied and shown in Fig. 3.

Optimal reaction of vertilmicin with FMOC-Cl occurred at 8.0–8.5 and was complete after 10 min. Maximal yields of the derivatives were detected following reaction in 60% (v/v) acetonitrile, and at concentrations of FMOC-Cl above 8 mM.

It was important to maintain a concentration of acetonitrile of around 60% (v/v) in the reaction mixture. FMOC-Cl was precipitated if the proportion of acetonitrile fell below 30%. While vertilmicin and borate buffer are not soluble in higher acetonitrile proportion solution.

The pH dependence of the reaction was consistent with that reported for aminoglycosides [4]. A sufficiently high concentration of FMOC-Cl was required for the reaction to proceed efficiently.

3.3. Method validation

The method was validated for rat plasma. Accuracy and precision data for the described method using spiked vertilmicin are shown in Table 1. Intra- and inter-day reproducibility data for vertilmicin are present in Table 2. Relative standard deviations were all below 6% for intra-day precision as well as inter-day precision.

Table 1
Accuracy and precision of vertilmicin added to plasma

Added (mg/L)	Found (mg/L)	Recovery (%)	R.S.D. (%) ($n = 3$)
0.02	0.02 ± 0.002	95.6	9.31
0.1	0.1 ± 0.003	99.1	2.87
1.0	1.0 ± 0.1	102	6.02
10.0	9.8 ± 0.1	98.1	5.44

The calibration curve for vertilmicin was linear from 0.02 to 45 mg/L (0.02, 0.1, 0.5, 1, 5, 10, 45 mg/L) [$y = 0.8835x + 0.0063$, $r = 0.9999$, $n = 7$]. The mean of slope ($n = 7$) was 0.8835 (R.S.D. = 2.75%) and the y-intercepts were determined to be not significantly different from zero. The limit of detection was evaluated as 0.01 mg/L for vertilmicin (S/N = 3). The limits of quantification for vertilmicin was 0.02 mg/L (S/N = 10) (R.S.D. = 9.31%, $n = 6$). The stability of vertilmicin in rat plasma was investigated at −20 °C and the results showed that vertilmicin was stable for at least 14 days. The derivatized products of vertilmicin and the internal standard at room temperature were stable for 24 h.

Table 2
Intra- and intra-day reproducibility data

Added (μg/ml)	R.S.D. (%)
Intra-day ($n = 3$)	
0.02	6.76
0.1	2.91
1.0	0.31
10.0	2.88
Inter-day ($n = 9$)	
0.02	8.54
0.1	5.08
1.0	2.95
10.0	4.01

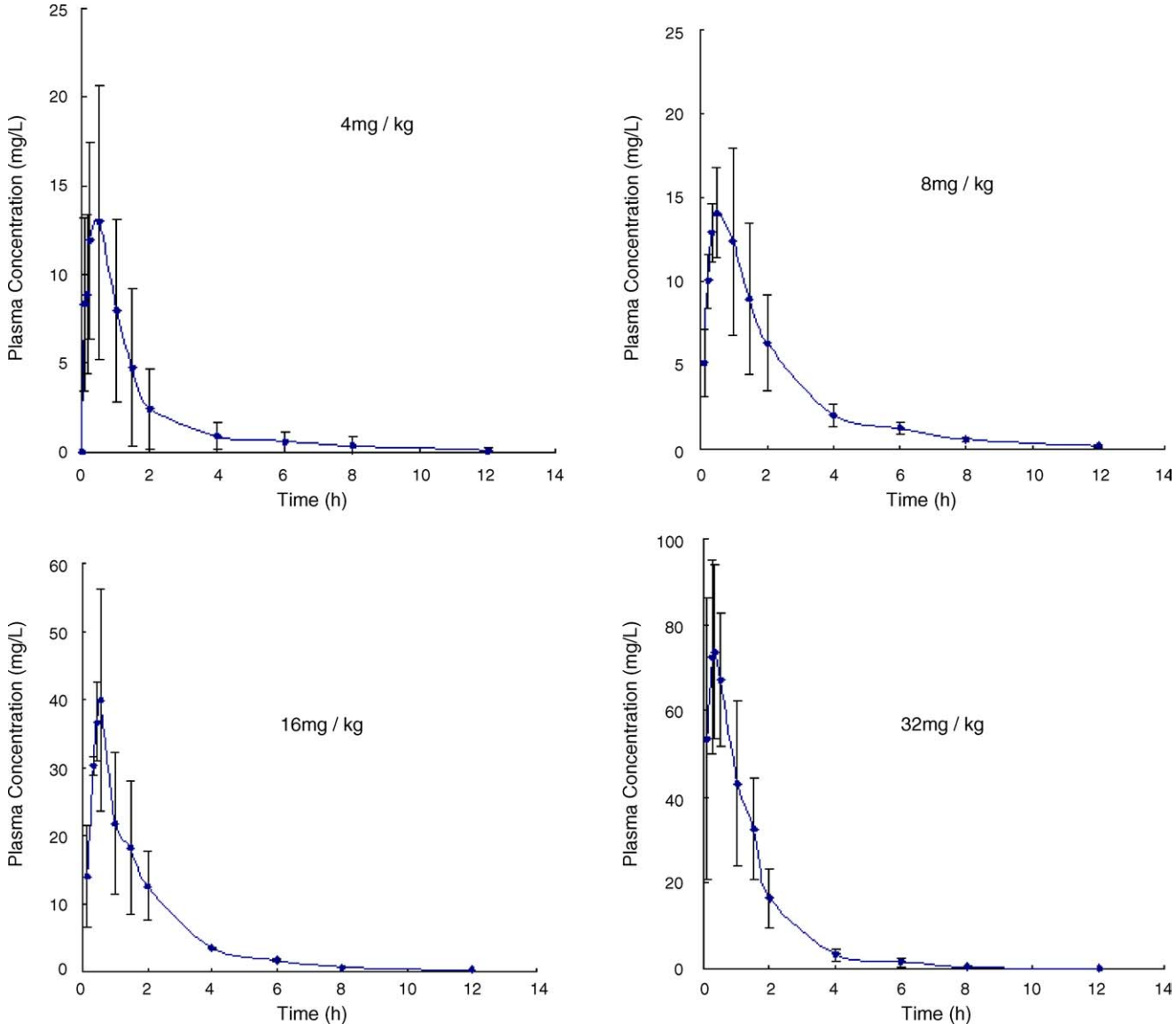

Fig. 4. Mean plasma concentration–time profiles of vertilmicin after single intramuscular injection to rats.

3.4. Pharmacokinetics

A plot of the mean plasma vertilmicin concentrations is presented in Fig. 4. Pharmacokinetic parameters after single doses are summarized in Table 3. Vertilmicin was rapidly absorbed with a mean t_{max} between 0.31 and 0.62 h for all four cohorts. Mean vertilmicin C_{max} ranged from 14.24 (5.23–25.94) to 78.26 (62.92–98.01) mg/L after 4 and 32 mg/kg, respectively. $AUC_{0-\infty}$ was 24.07 (17.99–34.49) to 118.14 (80.34–140.93) mg h/L, respectively. No significant difference was found in the half-life at β phase ($t_{1/2\beta}$), which ranged from 2.35 to 3.06 h.

Dose-proportionality for the fasted treatment groups (4, 8, 16 and 32 mg/kg) was investigated by fitting the power model: $\ln y = a + b \ln \text{dose}$, where y is the response variable ($AUC_{0-\infty}$ or C_{max}) and where $b = 1$ indicates dose-proportionality. The dose-proportionality constant for $AUC_{0-\infty}$ and C_{max} is estimated as 0.79 and 0.83, respectively.

Analysis of variance for T_{max}, C_{max} and $AUC_{0-\infty}$ were performed by using software SPSS 11.0. There were no significant difference among different doses ($p > 0.05$). The results of power model and analysis of variance show that C_{max} and AUC were dose proportional. From the plot of C_{max} (or $AUC_{0-\infty}$) versus dose, there also appears to be a proportional rise (Figs. 5 and 6).

After single doses, a trend of proportional drug exposure was observed in the dose range of 4–32 mg/kg in rat and our study results suggest that vertilmicin has a relatively short clearance $t_{1/2}$.

Table 3
Pharmacokinetics parameters of vertilmicin after intramuscular injection of single escalating doses to healthy male rats under fasting conditions

Parameter	Dose of vertilmicin (mg/kg)			
	4	8	16	32
A (mg/L)	69.1 ± 50.0	54.4 ± 47.8	107 ± 46.7	109 ± 36.8
α (1/h)	1.89 ± 0.55	1.46 ± 0.37	2.05 ± 1.50	1.06 ± 0.06
B (mg/L)	2.08 ± 2.29	7.05 ± 3.56	8.84 ± 5.94	5.55 ± 5.08
β (1/h)	0.33 ± 0.12	0.30 ± 0.08	0.27 ± 0.10	0.24 ± 0.07
Ka (1/h)	5.51 ± 4.06	3.83 ± 2.19	4.50 ± 1.43	19.2 ± 15.8
$t_{1/2(\alpha)}$ (h)	0.39 ± 0.10	0.50 ± 0.13	0.47 ± 0.29	0.66 ± 0.04
$t_{1/2(\beta)}$ (h)	2.35 ± 0.90	2.48 ± 0.78	2.88 ± 1.34	3.06 ± 0.86
$t_{1/2(\mathrm{Ka})}$ (h)	0.18 ± 0.09	0.22 ± 0.12	0.17 ± 0.06	0.07 ± 0.07
k_{21} (1/h)	0.44 ± 0.15	0.57 ± 0.19	0.56 ± 0.41	0.28 ± 0.10
k_{10} (1/h)	1.41 ± 0.43	0.77 ± 0.21	1.00 ± 0.26	0.92 ± 0.10
k_{12} (1/h)	0.37 ± 0.34	0.41 ± 0.29	0.76 ± 0.92	0.10 ± 0.02
CLs (L/h)	0.32 ± 0.23	0.25 ± 0.06	0.25 ± 0.06	0.31 ± 0.10
$T_{\max}$ (h)	0.37 ± 0.12	0.62 ± 0.34	0.47 ± 0.08	0.31 ± 0.16
$C_{\max}$ (mg/L)	14.2 ± 8.0	14.9 ± 3.7	42.0 ± 14.5	78.3 ± 18.0
$\mathrm{AUC}_{0-12\mathrm{h}}$ (mg h/L)	21.9 ± 15.3	33.1 ± 12.8	69.3 ± 15.9	117 ± 31.9
$\mathrm{AUC}_{0-\infty}$ (mg h/L)	22.4 ± 16.0	33.9 ± 12.4	70.7 ± 16.4	118 ± 33.0

Values are mean ± S.D. for $n = 6$ per dose group.

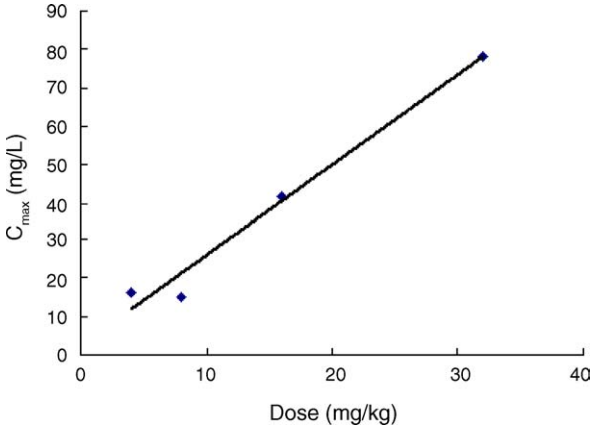

Fig. 5. Regression plot of peak plasma concentration ($C_{\max}$) of vertilmicin vs. dose after intramuscular injection of single doses to rats.

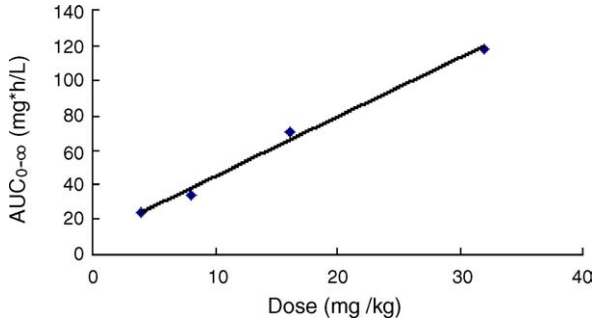

Fig. 6. Regression plot of $\mathrm{AUC}_{0-\infty}$ of vertilmicin vs. dose after intramuscular injection of single doses to rats.

3.5. Comparison of methods

Most HPLC methods involved 1-fluoro-2,4-dinitrobenzene (FNDB) [2,3], 9-fluorenyl-methyl chloroformate (FMOC-Cl) [4,18,19], phenylisocyanate (PITC) [8], 1-naphthoquinone-4-sulfonate (NQS) [9] and *o*-phthalaldehyde (OPA) [5–7] as derivatization reagents to react with aminoglycosides. In this study, FNDB, FMOC-Cl, PITC and OPA were all tested to react with vetilmicin and we found that FMOC-Cl derivates of vertilmicin had advantages of higher sensitivity, less interference and simpler react condition than others.

Several high-performance liquid chromatography (HPLC) methods [2–6,17–19] have been developed for the determination of aminoglycosides in plasma. However, most published methods for quantifying aminoglycosides in plasma samples involved the time-consuming sample pretreatments

Table 4
Comparison of pre-column derivatization HPLC assays for aminoglycosides

Drug	Derivatives	Extraction method	LOQ (mg/L)	I.S.	Plasma volume (mL)	Reference
Vertilmicin	FMOC-Cl	1-Step liquid extraction	0.02	Netilmicin	0.05	*
Gentamicin	FNDB	Solid-phase extraction	0.1	None	1	[2]
Paromomycin	FNDB	3-Step liquid extraction	0.5	Kanamycin	0.3	[3]
Neomycin	FMOC-Cl	Solid-phase extraction	0.01	None	1	[4]
Sisomicin	OPA	Online clean-up	0.1	None	1	[5]
Tobramycin	NITC	2-Step liquid extraction	0.93	Anthracene	0.4	[16]
Vertilmicin	FNDB	1-Step liquid extraction	0.5	Etimicin	0.05	[17]

Note: (*) means the current article.

[2–6], which are not conveniently available for routine drug monitoring. And many currently available HPLC assays for aminoglycosides require large sample volumes [2–6].

Only one method was reported to determine vertilmicin in plasma [16], which needed a long-time sample preparation and a complex derivatization. The present method used netilmicin as internal standard, for the determination of vertilmicin in a small volume of plasma sample as 50 μL, with one-step extraction. The limit of detection of the assay was evaluated as 0.01 mg/L for vertilmicin. It is simple, convenient, feasible and very useful for carrying out drug monitoring and simultaneous studies of the pharmacokinetics of vertilmicin. Table 4 provides a comparison of the analytical performance of the present method with those reported for other pre-column derivatization HPLC assays for aminoglycosides.

In conclusion, the present method allows rapid analysis of vertilmicin in plasma and it can be used to determination vertilmicin in plasma for pharmacokinetic studies.

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