Quantitative determination and pharmacokinetics of retinamido-ester in rat plasma by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry

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Abstract: A highly sensitive, rapid and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of retinamido-ester in rat plasma was developed and validated. A simplified protein precipitation with acetonitrile was employed for the sample preparation. The separation was carried out on an Agilent TC C_{18} column (150 mm × 4.6 mm ID, 5 μm particle size) with the mobile phase consisted of methanol-water-formic acid (93 : 7 : 0.1). Simvastatin was used as internal standard. The detection was performed on a trap-quadrupole tandem mass spectrometer by selected reaction monitoring (SRM) scan mode *via* atmospheric pressure chemical ionization (APCI). The range of calibration curve was 0.05 - 50 ng · mL^{-1} and the limit of quantification was 10 pg · mL^{-1}. The intra- and inter-day precision values were between 95.97% and 104.43%, and RSD was between 4.63% and 10.69%, respectively. This method was applied to determine the pharmacokinetic parameters. The main pharmacokinetic parameters of retinamido-ester after oral administration *via* gastric gavage of 2.5, 5, 10 mg · kg^{-1} were as follows, $T_{1/2}$: (11.28 ± 7.23), (8.90 ± 3.82), (8.01 ± 5.65) h; $AUC_{0-\infty}$: (103.41 ± 61.46), (190.23 ± 74.99), (421.66 ± 229.20) ng · h · mL^{-1}; MRT: (6.31 ± 0.75), (5.98 ± 0.71), (6.18 ± 0.97) h; CL/*F*: (30.10 ± 13.67), (29.58 ± 10.59), (31.18 ± 17.51) L · h^{-1} · kg^{-1}; V_d/*F*: (414.94 ± 159.82), (356.16 ± 139.85), (369.28 ± 322.72) L · kg^{-1}, respectively.

Key words: retinamido-ester; LC-APCI-MS/MS; pharmacokinetics

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LC-APCI-MS/MS 法测定大鼠血浆中维胺酯浓度及其药代动力学研究

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摘要: 本文建立一种高灵敏度的液质联用方法用于快速测定大鼠血浆中维胺酯的浓度。采用蛋白沉淀法制备样品; 色谱柱为 Agilent TC C_{18}柱(150 mm × 4.6 mm ID, 5 μm), 流动相为甲醇-水-甲酸(93 : 7 : 0.1), 辛伐他汀作为内标; 在三重四极杆串联质谱仪上, 采用大气压化学离子化(APCI), 正离子方式选择性离子模式进行监测; 血浆浓

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度在 0.05 ~ 50 ng · mL^{-1}线性关系良好，定量限为 10 pg · mL^{-1}，日内、日间精密度分别在 95.97% ~ 104.43%，RSD 在 4.63% ~ 10.69%。本文利用该方法对大鼠进行药代动力学研究，3 个剂量（2.5、5 和 10 mg · kg^{-1}）大鼠灌胃给药后的药代动力学参数分别为 $T_{1/2}$：（11.28 ± 7.23）、（8.90 ± 3.82）、（8.01 ± 5.65）h；$AUC_{0-\infty}$：（103.41 ± 61.46）、（190.23 ± 74.99）、（421.66 ± 229.20）ng · h · mL^{-1}；MRT：（6.31 ± 0.75）、（5.98 ± 0.71）、（6.18 ± 0.97）h；CL/F：（30.10 ± 13.67）、（29.58 ± 10.59）、（31.18 ± 17.51）L · h^{-1} · kg^{-1}；V_d/F：（414.94 ± 159.82）、（356.16 ± 139.85）、（369.28 ± 322.72）L · kg^{-1}。

关键词：维胺酯；LC-APCI-MS/MS；药代动力学

Retinamido-ester (RE), one of the retinoid derivatives, is efficacious in clinics for resisting inflammation, restraining cancer, especially for treating acne by adjusting and controlling the differentiation and development of epithelia, reducing the secretion of sebum and restraining *Propionibacteria* growth of acne. Some works on the chemical properties, pharmaceutical interaction and clinical application have been reported[1,2]. However, few reports involve the methods to analyze RE in rat plasma sample and regarding its pharmacokinetics. Only one reference using HPLC method to study the pharmacokinetic characteristics of RE in rat plasma was reported in 1992 by Tang, et al[3]. But the lower limit of quantification (LLOQ) of this method was 100 ng · mL^{-1}. And the method of administration was by injection into the vein which was different from that used in clinics. Many unanswered questions about metabolism, bioavailability or the intake of RE leave a tremendous amount of work to do with rats as experimental animals.

To study the pharmacokinetics of RE in rat plasma, it is necessary to develop a highly sensitive and accurate method to determine the concentration of RE in small volumes of plasma. Nevertheless, to our knowledge, several challenges occurred in developing the analytical method due to the special property of RE. One is that RE is very unstable when exposed to light due to its double bonds. The experiment must be carried out in an environment protected from direct light. And also a rapid sample preparation was required to avoid RE photodegradation. Another is the strong adhesion of RE to LC/MS needle. Tedious work with congruent wash solution should be taken to clean the needle. The third challenge is very low concentration of RE in rat plasma due to the low dose, and the rapid metabolism and distribution.

Our preliminary studies showed that neither HPLC-UV nor LC-MS-Ion Trap is sensitive enough to determine RE in the rat plasma.

To determine RE in rat plasma by ESI(−)/MS/MS, the sample must go through the extraction and concentration procedures because of the sample's high matrix effect. However, these pretreatment procedures for plasma sample are labor intensive, time consuming, and not compatible with the subsequently used ESI(−)/MS/MS method, and finally, they usually require large sample volume to match the sensitivity of the assay and result in the probability of photodegradation and contamination.

Atmospheric pressure chemical ionization (APCI) is characterized by its high selectivity and sensitivity and has been used for the analysis of lipids, retinoid acid[4,5]. Meanwhile, the tripe-quadrupole tandem mass system used here can also provide the necessary high sensitivity and specificity for these analyses[6,7].

In this paper, a rapid, high sensitive and reproducible LC-MS/MS method for the determination of RE in rat plasma was developed and validated. To minimize manual operations and avoid the photodegradation and contamination, a simplified protein precipitation procedure was employed for the sample preparation. The lack of sensitivity in the analysis of RE in small volume rat plasma was overcome by LC/APCI-MS/MS. Pharmacokinetic parameters were determined and discussed.

Materials and methods

Chemicals and reagents RE was kindly supplied by Chongqing Huabang Pharmaceutical Company Ltd. (Chongqing, Sichuan, China). Simvastatin as the internal standard (IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical grade. Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

Sprague-Dawley (SD) rats were purchased from Laboratory Animal Center of Nanjing Medical University (Nanjing, China). Their weights were all

between 220 and 250 g. The heparinized drug-free plasma for the preparation of methodology was obtained from these rats.

Instruments and software The analytical liquid chromatograph system used throughout this work consisted of a Finnigan surveyor LC pump, autosampler equipped with a 25 L injection loop, and column oven enabling temperature control of analytical column. Atmospheric pressure chemical ionization (APCI) (positive ion) source (San Jose, CA, USA) was carried out on a Thermo Finnigan TSQ Quantum Ultra AM tripe-quadrupole tandem mass spectrometer (Thermo Electron Corp.). System control and data acquisition were performed with Xcalibur 1.1 data software. Peak integration and calibration were carried out by using LC Quan software.

LC-APCI-MS/MS conditions The chromatographic separation was achieved on Agilent TC C_{18} column (150 mm × 4.6 mm ID, 5 μm particle size, Wilmington, DE, USA) coupled to a guard column (30 mm × 4.6 mm ID, 5 μm particle size) and with a mobile phase of methanol-water-formic acid (93 ∶ 7 ∶ 0.1) at the flow rate of 1.0 mL · min^{-1}. The column temperature was maintained at 25 ℃, the autosampler was conditioned at 4 ℃, and the injection volume was 20 μL using partial loop mode for sample injection. The entire effluent was directed to the APCI (+)-MS. After each sample run, the autosampler was rinsed with methanol to remove strongly retained residues. In the first 2.5 min of the chromatographic run, a divert valve directed the HPLC-flow run to waste container and afterwards to the ion source.

The mass spectrometer was operated in the positive ion detection mode with the discharge current at 10.0 μA, with the vaporizer at 420 ℃. The high purity nitrogen sheath gas and the auxiliary gas pressure were set to 241 and 34 kPa, respectively. The capillary was heated to 330 ℃, argon was used as the collision-induced dissociation (CID) gas at a pressure of 159.6 mPa. The collision energy in the in-source CID mode was set at 6 eV, and the collision energies for RE and IS in the MS/MS mode were set at 15 eV and 12 eV, respectively. Quantification was performed using selected reaction monitoring (SRM) scan mode. The precursor ions were *m/z* 448.1 for RE ($[M+H]^+$) and *m/z* 419.3 for IS ($[M+H]^+$) and the product ions were *m/z* 283.0 for RE and *m/z* 285.0 for IS, respectively. Both Q1 and Q3 peak widths were set to 0.7 Th.

Preparation of standard solutions RE is insoluble in water and soluble in methanol. Stock solutions of 0.5 mg · mL^{-1} in methanol for RE and internal standard solution were prepared, separately. A series of working standard solutions at 2, 4, 10, 20, 40, 100, 200, 600, 2 000 ng · mL^{-1} for calibration standards and at 4, 40, 600 ng · mL^{-1} for quality control (QC) samples were just diluted with RE stock solution with methanol prior to use. Simvastatin stock solution was diluted with acetonitrile to 50 ng · mL^{-1} and used as the internal standard as well as the precipitator of protein. All the solutions were stored at −20 ℃.

Sample preparation Before analysis, the plasma sample was thawed to room temperature, and mixed in a moment with vortex agitator. 300 μL of precipitator was added to 100 μL aliquot of rat plasma sample in 1 mL centrifugal tube. The mixture was vortexed for 1 min and then centrifuged at 15 000 r · min^{-1} for 10 min, the clear supernatant was transferred to a brown conical autosampler vial. 20 μL of the supernatant sample was injected into the HPLC system for analysis. Those samples with concentrations greater than the maximum standard in the calibration curve were quantitatively diluted by blank plasma.

Method validation

Selectivity Selectivity was investigated by comparing chromatograms of blank plasma with those corresponding to standard plasma samples spiked with RE and IS (4 ng · mL^{-1}) and to plasma samples after oral administration of RE in rat.

Calibration curve and sensitivity Five independent calibration curves based on nine spiked plasma samples (0.05 − 50 ng · mL^{-1}) were prepared to validate the linearity of the method. Each calibration curve was constructed by plotting the peak area ratios of RE to IS *versus* the nominal concentrations in the standard plasma samples by the weighted ($1/x^2$) least square linear regression.

Limit of detection (LOD) LOD was defined as the concentration of drug giving a signal-to-noise ratio of 3∶1 and the limit of quantification (LOQ) was defined as the lowest concentration of the analytes quantified.

Precision and accuracy Both repeatability and reproducibility were determined. They were evaluated by analyzing the QC samples at three concentration levels (lower, intermediate and higher concentration level of the calibration curve, i.e. 0.4, 4 and 60

ng · mL^{-1}) of RE. A replicate analysis of QC samples performed on the same day determined the intra-day accuracy and precision (n = 5), and inter-day accuracy and precision were assessed by repeated analysis on three consecutive days (n = 5 series per day). Mean and relative standard deviation were calculated and used to judge accuracy and precision of the method. The RSD should be less than 15%, except at the LOQ where it should not exceed 20%.

Recovery and matrix effect The recovery of RE was evaluated by comparing the peak areas obtained from QC samples treated by precipitation with those of post-precipitation blank plasma spiked with same nominal analyst concentration. This procedure was performed at three concentrations of 0.4, 4 and 60 ng · mL^{-1} for five replicates. The recovery of IS was determined similarly.

The matrix components often affected the ionization of analyst, either suppressing or enhancing the signal. The matrix effects on RE were evaluated by spiking blank plasma at three different concentrations of 0.4, 4 and 60 ng · mL^{-1}. The corresponding peak areas were compared with those of the RE standard solutions dried directly and reconstituted with acetonitrile. The ratio was used to evaluate the matrix effects. The matrix effect of internal standard was also evaluated using the same method.

Stability The stability of RE solution was evaluated using recovery samples stored at room temperature for 12 h. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 8 h. Freeze and thaw stability was performed by subjecting the QC plasma samples to three freeze-thaw cycles. The concentration analyzed was compared with the nominal values.

Application to pharmacokinetic study The method described above was applied to quantify RE concentration in rat plasma in a single-dose pharmacokinetic study. Eighteen Sprague-Dawley rats were used. They were randomly assigned to three groups, each of which consisted of 6 rats. These SD rats were fasted 12 h before drug sampling.

RE was minced and dispersed by 0.1% sodium carboxymethylcellulose to 0.5 mg · mL^{-1}. Then 2.5, 5, 10 mg · kg^{-1} of RE were given ig. The blood samples (about 0.3 mL) were collected by the puncture of the retro-orbital sinus into heparinized plastic tubes at 0.25, 0.5, 1, 3, 6, 8, 10, 12, 14, 16, 18, 24, 36 and 48 h after RE administration. After collection, each blood sample was immediately centrifuged at 4 000 r · min^{-1} for 5 min. The obtained supernatant plasma layer were transferred into a 1 mL tube and stored in the dark at −20 ℃ until analysis.

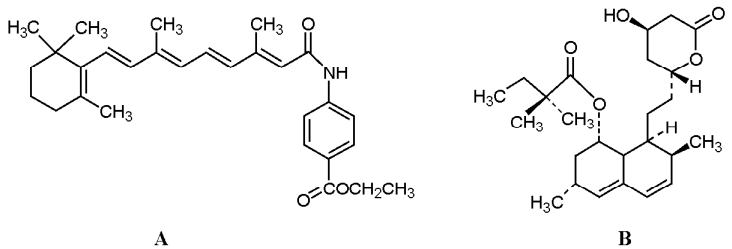
The RE plasma concentration data were analyzed *via* the noncompartmental method with the aid of the Drug and Statistics 2.1.1 software (The Chinese Mathematical Pharmacology Society) to obtain the appropriate pharmacokinetic parameters.

Results and discussion

1 Method development

RE (Figure 1) can easily isomerize as retinol. Light is the most important catalyst[8]. To minimize RE photoisomerization, the acquisition of plasma and all subsequent processing steps must be carried out in a room protected from direct sunlight and only lightened by a low-intensity red light source.

As a derivative of retinol, RE is a lipophilic compound. Several methods for retinol determination in human serum or plasma have been reported[9-11]. Most of them required protein precipitation, followed by liquid-liquid extraction, then dried by nitrogen and re-dissolved before analysis. Several precipitators such as ethanol, methanol, acetonitrile and acetone and several extraction solvents such as cyclohexane, hexane, ether and ethyl acetate were investigated. Although the extraction with ethanol-cyclohexane can be successfully used to determine RE concentration in rat plasma, the procedures are quite complex, time-consuming and can meet the risk of artificial light



A B

Figure 1 Structures of retinamido-ester (A) and IS (B)

degradation and another contamination. The sensitivity of RE detected by APCI (+)/MS/MS was high enough, and the method reported here is very simple, time-saving and can avoid the photodegradation.

The strong adhesion of RE to LC-MS needle leads to the difficulty of the analysis. Although the needle washing was carried out with the mixture of methanol-water (50 : 50) for several times as recommended by the LC-MS instrument manufacturer for several times, the leftover still existed in needle. When absolute methanol was used instead, the adhesion was greatly eliminated.

The choice of internal standard is a key to analysis. In order to separate the photoisomer from RE, C_{18} column was chosen. However, the strong retention of RE to C_{18} column made it very hard to select the internal standard. Simvastatin, a steady ester with similar structure as RE, was chosen as the internal standard considering its high recovery and appropriate retention.

Blank plasma from four rats, treated with the proposed sample preparation procedure, did not show any significant interfering peak at the retention times of RE and IS. The background noise was very low after the plasma samples were precipitated with acetonitrile (Figure 2, 3).

2 Linearity, LOD and LOQ

The calibration standards were prepared as follows: a series of 10 μL working standard solutions were spiked into 1 mL centrifugal tubes. After solvent was evaporated by nitrogen, 100 μL drug-free rat plasma was added and vortexed for 1 min. And then 300 μL precipitator (including 50 ng · mL^{-1} IS) was added and vortexed for 3 min. After centrifuged at 15 000 r · min^{-1} for 10 min, 20 μL clear supernatant was injected into the HPLC system for analysis. It showed a linear response over the mean concentration ($n = 5$) ranging from 0.05 to 50 ng · mL^{-1}. And calibration curves were constructed by weighed ($1/C^2$) least-squares linear regression analysis of analyte/

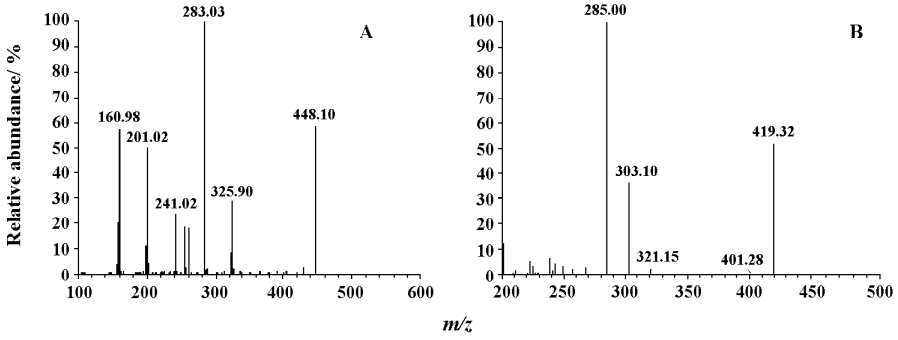


Figure 2 Product ion scan mass spectra of retinamido-ester (A) and IS (B)

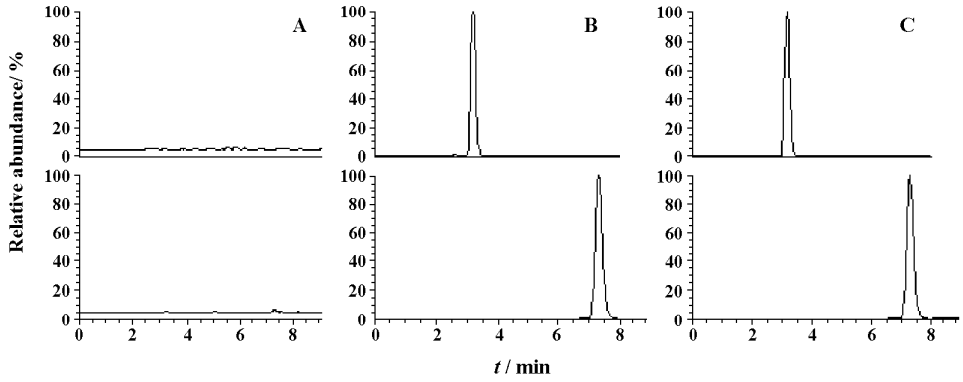


Figure 3 SRM chromatograms. A: Rat blank plasma; B: Blank plasma spiked with 60 ng · mL^{-1} retinamido-ester and 50 ng · mL^{-1} IS; C: Plasma sample from a subject 3 h after the administration of retinamido-ester, in which the concentration of retinamido-ester was found to be 60.8 ng · mL^{-1}. The retention time for retinamido-ester is about 7.27 min and for IS about 3.19 min

internal standard peak area ratio *versus* the concentrations of analyte. The mean regressive equation was $Y = 0.064\,93X - 0.001\,075$, $r = 0.994\,5$. The linear range was 0.05 – 50 ng · mL^{-1}, the LOD and LOQ for RE was 3 pg · mL^{-1} and 10 pg · mL^{-1} ($S/N = 41$), respectively. The acceptable accuracy within ± 20% and RSD under 20% were satisfied.

3 Precision and accuracy

The precision and accuracy of the method were performed by calculating the variation of five replicates of each QC samples (0.4, 4 and 60 ng · mL^{-1}) on the intra- and inter-day. The intra-assay precision was from 4.63% to 10.49% and accuracy was from 97.36% to 102.21%. The inter-assay precision was from 4.89% to 10.69% and accuracy was from 95.97% to 104.43% (Table 1). These results indicated that the method was reproducible and accurate.

Table 1 Precision and accuracy of RE in quality control samples ($n = 5$)

Concentration /ng · mL^{-1}	Intra-day/%		Inter-day/%	
	Accuracy	RSD	Accuracy	RSD
0.4	97.36	10.49	95.97	10.69
4	102.21	5.40	104.40	5.99
60	100.71	4.63	104.43	4.89

4 Recovery and matrix effect

Each QC sample (10 μL) was added to the 1 mL centrifugal tube, after evaporating solvent by nitrogen, 100 μL drug-free rat plasma was added and the mixture was vortexed for 1 min. And then 300 μL precipitator (including 50 ng · mL^{-1} IS) was added and vortexed for 3 min. After centrifuged at 15 000 r · min^{-1} for 10 min, 20 μL clear supernatant was injected into the HPLC system for analysis.

The absolute recovery of the method was measured by comparing the peak areas obtained from the plasma sample with those from standard solutions at the same concentration (0.4, 4 and 60 ng · mL^{-1}). As shown in Table 2, the RSD of recovery of RE ranged from 1.88% to 11.39% and IS was 4.69%, and no obvious matrix effect was observed.

Table 2 Matrix effect and absolute recoveries of RE from spiked rat plasma ($n = 5$)

Concentration /ng · mL^{-1}		Matrix effect/%		Absolute recovery /%	
		$\bar{x} \pm s$	RSD	$\bar{x} \pm s$	RSD
RE	0.4	99.52 ± 16.08	16.16	95.20 ± 10.85	11.39
	4	110.18 ± 5.41	4.91	101.75 ± 7.62	7.48
	60	97.74 ± 4.28	4.38	100.61 ± 1.89	1.88
IS	50	100.33 ± 2.92	2.91	101.62 ± 4.76	4.69

5 Stability

The recovery samples stored at room temperature for 12 h did not suffer any appreciable changes in assay value. The change of QC plasma samples kept at room temperature for 8 h was very slight. And the QC plasma samples after three freeze-thaw cycles were stable.

6 Pharmacokinetic study

The plots of mean plasma concentration ($n = 6$) *versus* time after administration of three dosages were shown in Figure 4. The main pharmacokinetic parameters of RE in rats' plasma were determined by using noncompartmental analysis of DAS software and listed in Table 3.

RE in the plasma can be detected at 0.25 h, and peaked at 3.5 h after administration. RE has long terminal half life (8.0 – 11.3 h). The clearance was about 30 L · h^{-1} · kg^{-1}, and the volume of steady state distribution was about 356.2 – 414.9 L · kg^{-1}.

Table 3 Mean pharmacokinetic parameters of RE in rats after an oral administration

Parameter	Dosage of RE/mg · kg^{-1}		
	2.5	5	10
C_{max}/ng · mL^{-1}	16.50 ± 11.34	27.61 ± 7.74	68.91 ± 31.22
T_{max}/h	3.50 ± 1.22	3.50 ± 1.22	3.50 ± 1.22
$T_{1/2}$/h	11.28 ± 7.23	8.90 ± 3.82	8.01 ± 5.65
MRT/h	6.31 ± 0.75	5.98 ± 0.71	6.18 ± 0.97
$AUC_{0-48\,h}$/ng · h · mL^{-1}	102.04 ± 59.23	188.13 ± 72.28	420.06 ± 230.25
$AUC_{0-\infty}$/ng · h · mL^{-1}	103.41 ± 61.46	190.23 ± 74.99	421.66 ± 229.20
(CL/F)/L · h^{-1} · kg^{-1}	30.10 ± 13.67	29.58 ± 10.59	31.18 ± 17.51
(V_d/F)/L · kg^{-1}	414.94 ± 159.82	356.16 ± 139.85	369.28 ± 322.72

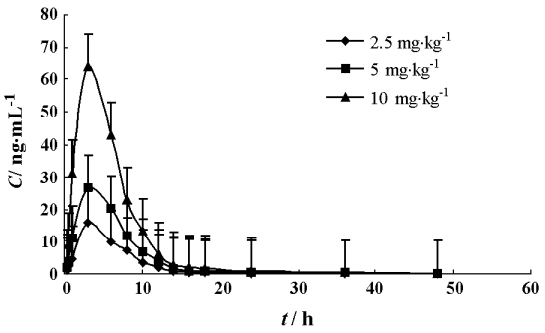


Figure 4 Mean plasma concentration-time profiles of retinamidoester in rats. The above values are mean plasma concentration obtained from six rats which were administered with a single oral dose of 2.5, 5, 10 mg · kg^{-1}, separately. $n=6$, $\bar{x} \pm s$

These parameters showed that the linearity between C_{max} (ng · mL^{-1}), $AUC_{0-48\,h}$ (ng · h · mL^{-1}), $AUC_{0-\infty}$ (ng · h · mL^{-1}) and dosages were good (correlation coefficient $r > 0.99$), suggesting that RE may have linear pharmacokinetic characteristics in rat within the dose ranges tested.

Conclusion

With the protein precipitation, the analyte from sample was accurately determined and no matrix ion suppression or ion enhancement could be observed. The sample preparation is very simple, time-saving and can avoid artificial transformation.

The strong adhesion of RE was greatly eliminated by using absolute methanol to wash the needle of autosampler instead.

The LC/APCI-MS/MS method developed for the determination of RE in rat plasma is highly selective, sensitive and rapid. This method was validated for a concentration range from 0.05 - 50 ng · mL^{-1} with LOQ of 10 pg · mL^{-1} of RE.

The proposed method allows plentiful sample throughput to be applied to pharmacokinetic studies of RE. The pharmacokinetic parameters may be helpful to establish appropriate dose and frequency of clinical study.

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