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Study on HPLC Determination and Pharmacokinetics of Lenalidomide in Rat Plasma*

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ABSTRACT Objective: To set up a HPLC method for the determination of lenalidomide in rat plasma. **Methods:** The analytical column was a Venusil ASB C18 column (4.6 mm× 250 mm, 5 μm) from Agela Technologies. Separation was achieved by gradient elution using acetonitrile-water containing 0.05 % formic acid, with the flow rate of 1 mL/min. The UV detection wavelength was 254 nm. Thalidomide was used as the internal standard. **Results:** The lower limit of quantification (LLOQ) of the method was 0.1 μg/mL and the linear range was 0.1-5.0 μg/mL. The extraction recovery of lenalidomide was 78.8 ± 3.8, 80.1 ± 3.2 and 79.1 ± 7.6 % at concentrations of 0.2, 1.0 and 5.0 μg/mL (QC samples), respectively. **Conclusion:** The method is accurate and simple with the high sensitivity and will be valuable for the determination of lenalidomide plasma concentrations and the pharmacokinetic study.

Key words: HPLC; Lenalidomide; Plasma concentration; Pharmacokinetics

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Introduction

Lenalidomide,3- (4-amino-1-oxo-1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione is a new member of immunomodulatory drugs (IMiD)[1]. Lenalidomide (LND) is a synthetic derivative of thalidomide. In the 1990s, thalidomide was used in the treatment of multiple myeloma (MM) because of its anti-angiogenic activity [2-5]. However, thalidomide has significant side effects such as teratogenicity[6-8]. The teratogenicity promotes the search for less toxic effects derivatives. LND exhibites more potency and fewer side effects, emerging as a promising orally available agent with lower toxicity. LND has been approved by Food and Drug Administration (FDA) for use in multiple myeloma (MM) and myelodysplastic syndrome (MDS) in December 2005 [9-11]. According to the recent researches, LND exerts more activities including anti-inflammatory and anti-angiogenic properties [12,13], an increase in immune effector cell response, direct induction of tumor apoptosis [14], and synergistic effects in combination therapies with dexamethasone for relapsed or refractory multiple myeloma [15,16]. Although LND have been applied in clinical treatments for a long time, little information on HPLC determination of LND in rat plasma has been reported. This paper developed the HPLC determination of LND in rat plasma.

1 Materials and methods

1.1 Materials

1.1.1 Instruments Waters2690 High Performance Liquid Chromatograph (Waters, Inc.); Vortex Mixer (Labnet International, Inc.); Centrifuge 5418 (Eppendorf AG); BF-2000M Nitrogen blow dry instrument (Beijing Bafang Century Technology Co. Ltd); electronic analytical balance (Shanghai Mettler Toledo Instrument Co. Ltd).

1.1.2 Chemicals and Solvents LND (99.6 %) was supplied by Medicinal chemistry laboratory of Shandong University. Thalidomide was gifted from Changzhou Pharmaceutical Factory Co., Ltd. HPLC-grade methanol and acetonitrile was purchased from Tedia Company. All other reagents were analytical grade. Water was purified with the Mili-Q Plus system and used throughout the study.

1.1.3 Experimental animals Male Wistar rats, weighing 220-240 g, were purchased from the Qingdao Daren Fortune Animal Technology Co. Ltd. These animals were acclimated for at least a week with free access to standard chow and water. The rats were fasted overnight but supplied with water before the experiments.

1.2 Methods

1.2.1 Chromatographic Conditions The HPLC column was a Venusil ASB C18 column (4.6 mm× 250 mm, 5μm) from Agela Technologies. The mobile phase of solvent A was acetonitrile and solvent B was water containing 0.05 % formic acid. Samples were eluted by gradient with solvents A and B. The gradient started

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at the mobile phase A: B (12:88, v/v), changed linearly to A: B (60:40, v/v) until 13 min. The liquid flow rate was set at 1 mL/min. The column was maintained at 26 ℃. LND was detected by UV absorption at 254 nm. As shown in Fig.1, no significant interferences from endogenous substances with analyte or internal standard (I.S.) were detected. Typical retention times for LND and the internal standard were 6.8 min and 10.9 min, respectively.

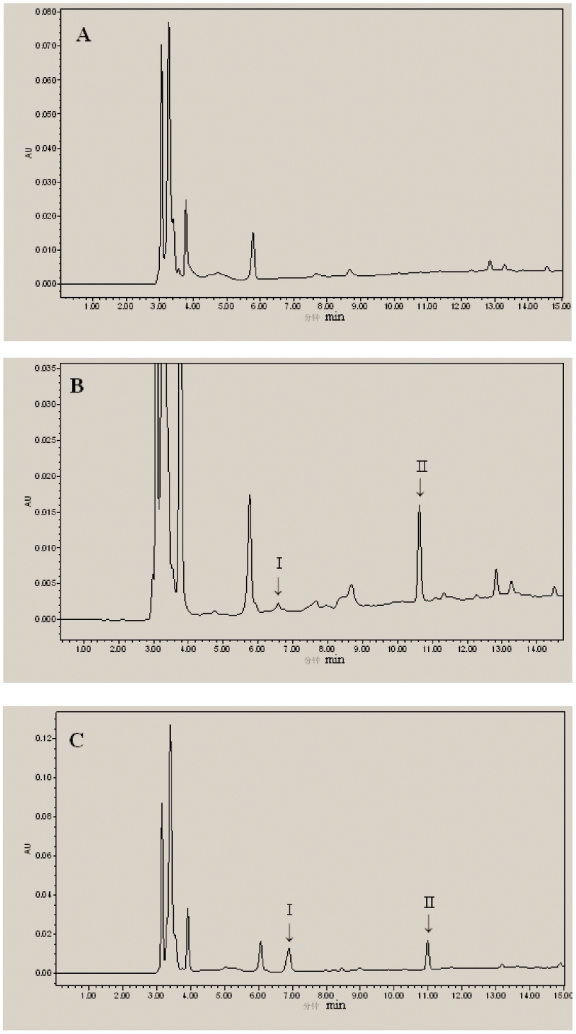

Fig. 1 Representative chromatograms of LND and I.S. in plasma sample: (A) a blank rat plasma; (B) a blank rat plasma spiked with LND of 0.1 μg/mL and internal standard; (C) a real rat plasma sample collected at 1.5 h after oral administration of LND (30 mg/kg). Peak I, LND; Peak II, internal standard

1.2.2 Preparation of standard solutions and quality control (QC) samples The stock solution of LND (1 mg/mL) was prepared in methanol and kept at -20℃. Standard working solutions of LND at concentrations of 1.0, 2.0, 5.0, 10, 20, 50 μg/mL were obtained by a further dilution of the stock solution with acetonitrile-water (20:80, v/v) for the preparation of plasma calibration standards and quality control (QC) samples. The stock solution of thalidomide (1 mg/mL) was prepared in acetonitrile-water-formic acid (50:50:0.1, v/v/v). All working solutions were prepared freshly on each day and stored at 4 ℃. The plasma calibration standards were prepared at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 μg/mL by adding 10 μL working solution of appropriate concentrations to 90 μL blank drug-free rat plasma. Quality control (QC) samples were freshly prepared in a similar manner at concentrations of high (5.0 μg/mL), medium (1.0 μg/mL), and low (0.2 μg/mL). These samples were used to evaluate the intra- and inter-day precision, extraction recovery and stability.

1.2.3 Sample Processing Plasma sample (100 μL) was transferred into 2.0 mL tubes and extracted via protein precipitation with 200 μL 4℃ acetonitrile (containing 15 μg/mL thalidomide) and immediately vortex-mixed for 2 min, centrifuged at 4 ℃ for 3 min at 16,873 × g. Supernatant was removed and evaporated under a gentle stream of nitrogen at 26 ℃. The residue was reconstituted in 100 μL reconstituted solution which consisted of acetonitrile-water-formic acid (50:50:0.1, v/v/v). After vortex mixing for 2 min, A 30 μL of the solution was injected into the HPLC system. The protein precipitation method was also used for QC samples and calibration standards.

2 Results

2.1 Linearity and LLOQ

A standard curve was constructed by using the peak area ratio of LND to I.S. within the concentration range of 0.1-5.0 μg/mL. The mean linear regression equation of calibration curve was: y = 1.7605x -0.0087(r=0.9996), where x is the peak area ratio of LND to thalidomide, and y is the concentration of LND. The calibration curves showed good linearity over the concentration range of 0.1 5.0 μg/mL and the lower limit of quantification (LLOQ) was established at a concentration of 0.1 μg/mL for LND.

2.2 Recovery

The extraction recovery of LND was carried out in plasma QC samples at high, medium and low levels and determined by comparing peak areas obtained from plasma samples with those found by direct injection of a standard working solution at equivalent concentrations.

The extraction recoveries of LND under the protein precipitation conditions were 78.8± 3.8, 80.1± 3.2 and 79.1± 7.6% at concentrations of 0.2, 1.0 and 5.0 μg/mL (QC samples), respectively. The recovery of I.S. was 77.6± 4.5 % in rat plasma (n = 6).

2.3 Precision

The precision of the assay were evaluated by analyzing six repli cates of QC samples at three concentrations on the same day and three consecutive days. The intra- and inter-day precision of lenalidomide determinations in plasma are summarized in Table 1.

Table 1 Precision of lenalidomide detected by HPLC (n=6)

Nominal concentration (μg/mL)	Intra-day		Inter-day	
	measured concentration (Mean± SD, μg/mL)	RSD (%)	measured concentration (Mean± SD, μg/mL)	RSD (%)
0.2	0.18± 0.011	6.27	0.21± 0.013	5.99
1.0	1.07± 0.051	4.75	0.94± 0.054	5.73
5.0	4.87± 0.161	3.31	5.13± 0.237	4.62

2.4 Stability

The LND stability experiments in plasma was assessed by analyzing six replicates of QC samples at the concentrations of 0.2, 1.0 and 5.0 μg/mL. Stability of LND in plasma during sample preparation was assessed by detecting samples after storage for 2 h at 26 ℃, which exceeded the time of the sample processing procedures. Long-term stability was assessed by analyzing samples kept at -80℃ for two weeks. Freeze-thaw stability was evaluated over three freeze (-80 ℃)/thaw (26℃) cycles. Autosampler storage stability was evaluated by analyzing samples kept in the autosampler at room temperature for 12 h (Table 2).

Table 2 Stability of lenalidomide in rat plasma (n=6)

Storage conditions	Concentration(μg/mL)		RSD(%)
	Nominal	Measured(Mean± SD)	
Short-term stability (2h,room temperature)	0.2	0.19± 0.02	10.52
	1	0.96± 0.09	9.38
	5	4.82± 0.23	4.77
Long-term stability (two weeks,-80℃)	0.2	0.185± 0.013	6.86
	1	0.97± 0.03	2.95
	5	4.87± 0.18	3.63
Freeze and thaw stability (three cycles,-80℃/26℃)	0.2	0.186± 0.018	9.36
	1	0.97± 0.05	5.31
	5	4.72± 0.41	8.68
Autosampler stability (10h,room temperature)	0.2	0.18± 0.013	7.22
	1	0.95± 0.015	1.58
	5	4.86± 0.17	3.49

2.5 Application to pharmacokinetic study

Six male Wistar rats, weighing220 g -230 g, were fasted 12 h before received an oral administration of 30 mg/kg. The dosage preparation was made by suspending the appropriate amounts of LND in 0.5 % carboxymethylcellulose sodium (CMC-Na). Blood samples were collected in heparinized EP tubes from oculi chorioideae vein at 0.083, 0.167, 0.25, 0.333, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 10 h after oral administration. The blood samples were centrifuged immediately at 8609 × g for 5 min to obtain plasmas samples which were immediately processed following the method described above. The plasma concentrations of LND at different time points were expressed as the mean ± SD and the mean plasma concentration-time profiles of LND in rats are shown in Fig.2. The peak plasma concentration (Cmax) and the time to reach Cmax (Tmax) were directly obtained from the experimental data and other pharmacokinetic calculations were performed by non-compartmental method using the DAS 2.0 statistical software (Pharmacology Institute of China). The main pharmacokinetic parameters are presented in Table 3.

Table 3 Pharmacokinetic parameters after oral administration of LND (30 mg/kg) to rats (n = 6)

Parameters	Mean± SD
Cmax(μg/mL)	3.48± 0.71
Tmax(h)	1.58± 0.38
t 1/2(h)	1.71± 0.78
MRT 0-t(h)	2.83± 0.23
MRT 0-∞ (h)	3.02± 0.60
AUC 0-t(μgh/ mL)	12.01± 1.64
AUC 0-∞ (μgh/ mL)	12.33± 1.48
AUMC 0-t(μgh^2/ mL)	33.80± 3.29
AUMC 0-∞ (μgh^2/ mL)	38.96± 6.29

C_{max}: the maximum plasma concentration; T_{max}: time to peak value; t1/2: elimination half-live; MRT(0-t): mean residence time from time zero to the last sampling time point; MRT 0-∞ : mean residence time from time zero to the infinity; AUC 0-t: area under the plasma concentration-time curve from time zero to the last sampling time point; AUC 0-∞ : area under the plasma concentration-time curve from time zero to the infinity; AUMC 0-t: the area under the first moment of the plasma concentration-time curve from time zero to the last sampling time point; AUMC 0-∞ : the area under the first moment of the plasma concentration-time curve from time zero to the infinity.

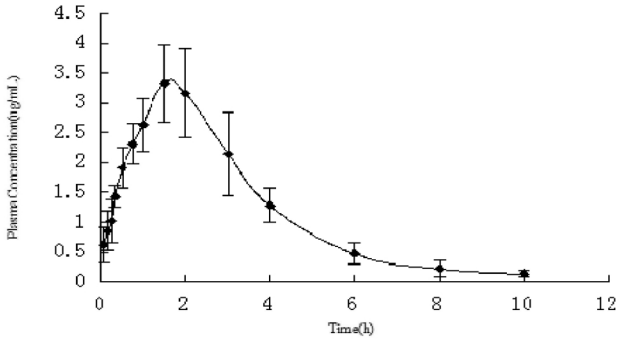

Fig. 2 Mean plasma concentration-time profile of LND after an oral administration of 30 mg/kg (mean ± SD, n = 6)

3 Discussion

3.1 Optimization of chromatographic condition

The different mobile phase compositions had been tried with methanol/water and acetonitrile/water solvent system using different buffers such as formic acid to achieve good resolution, improve peak shape and decrease run time[17,18]. In comparing isocratic elution with gradient elution, For example, the isocratic elution using acetonitrile-water containing 0.05 % formic acid (20:80, v/v) showed that the retention times for LND and the I.S. were 4.6 min and 19.1 min, respectively. However, the use of gradient elution led to the retention times for LND and the I.S. were 6.8 min and 10.9 min. The mobile phase consisted of acetonitrile-water containing 0.05 % formic acid with a gradient elution was found to be optimal for this study. Under the optimized conditions, there was little interference peak close to the retention positions of the analytes and I.S. in the rat plasma.

3.2 Optimization of sample processing

One-step protein precipitation was selected for plasma sample preparation due to the high polarity of LND. In pharmacokinetic study, many samples need to be processed and analyzed. Thus, the one-step protein precipitation is the best choice. Acetonitrile as a suitable protein precipitation solvent was adopted due to preferable recovery and excellent precipitation [19,20]. By comparison, 200 μL acetonitrile for 100 μL plasma protein precipitation was the optimal method for the sample preparation.

3.3 Selection of reconstituted solution

The optimal reconstituted solution can not only improve the autosampler storage stability of LND but also obtain better peak shape. By comparing a series of different ratios of acetonitrile/water and methanol/water, in presence or absence of formic acid, we found that the reconstituted solution which consisted of acetonitrile-water-formic acid (50:50:0.1, v/v/v) was the best choice.

In conclusion, a simple, accurate HPLC method with the determination of LND has been successfully developed and validated for the quantification of LND in rat plasma. The protein precipitation method was very simple and applied for the pretreatment of plasma samples. This validated method was shown to be valuable for pharmacokinetic study with the oral administration in rats.

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高效液相色谱法测定大鼠血浆中来那度胺的浓度及其药动学 *

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摘要 目的：建立高效液相色谱法测定大鼠血浆中来那度胺的浓度。**方法：**色谱柱采用 Venusil ASB C18 column (4.6 mm× 250 mm, 5 μm) 购自于博纳艾杰尔科技有限公司。样品采用梯度洗脱，流动相为乙腈和 0.05%甲酸溶液，流速为 1.0 mL/min，检测波长为 254 nm，以沙利度胺为内标。**结果：**来那度胺血药浓度的线性范围为 0.1-5 μg/mL，最低检测限为 0.1 μg/mL，三个浓度的 QC 样品 (0.2, 1 and 5 μg/mL)的提取回收率分别为 78.8± 3.8, 80.1 ± 3.2 and 79.1 ± 7.6 %。**结论：**此方法准确、简便、灵敏度高，对来那度胺的血药浓度测定和药物代谢动力学研究极具价值。

关键词：高效液相色谱法；来那度胺；血药浓度；药物动力学

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