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Short communication

Rapid quantitative analysis of clarithromycin in rat plasma by UPLC–MS/MS after intravenous injection of the clarithromycin-loaded ultrafine PLGA nanoparticles

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ABSTRACT

Nanoparticles were designed to encapsulate drugs to alter their pharmacological behaviors, therefore, it is very essential to monitor the pharmacokinetic profile of drug encapsulated in nanoparticles in order to clarify and predict their efficacy and side effects. In this paper, we reported a simple, rapid μ-elution 96-well solid phase extraction (μSPE) method combining with ultra high performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for determination of nanoformulated drug in rat plasma. This method presented satisfactory results in terms of sensitivity, precision, accuracy, and recovery, for the first time, of quantitatively analyzing clarithromycin (CLA) in rat plasma after intravenous administration CLA-loaded ultrafine PLGA nanoparticles for pharmacokinetic study. This method has been proved to be fast, reliable and reproducible to accurately analyze drug encapsulated in polymeric nanoparticles sample for a pharmacokinetic study.

1. Introduction

Recently, nanoparticle-based formulations are largely investigated to improve the therapeutic efficacy for drug delivery. For example, biodegradable polymeric nanoparticles can be used to improve the solubility of drug for good bioavailability, reduced systemic side effects and prolonged retention time [1–4]. The pharmacokinetic studies on the drug's blood circulating and clearance time provide vital and important information pertaining the delivery efficiency of the drugs when they are being formulated into the nanoparticles and then administrated into bodies and to describe the behavior of the drug *in vivo*. It is important to note that there is a close relationship between the circulating time and clearance pharmacokinetic profile and the behavior of nanoparticles [5,6].

LC–MS/MS is a standard analytical technique to determine the pharmacokinetic profile of small molecular weight drugs [7,8]. A series of pretreatment steps are usually required for blood samples to extract the drug components prior to analysis [9], and the effort and time length in sample pretreatment will usually be the bottleneck for the throughput of the pharmacokinetic studies. Commonly used pretreatment methods include protein precipitation (PPT), liquid–liquid extraction (LLE), or solid-phase extraction (SPE) [7]. PPT and LLE have their own advantages [7,10–12], and have been mostly used for pharmacokinetic profiling of nanoformulated drugs, however both processes are labor intensive and time-consuming.

Microelution SPE plate (μSPE) has been recently used in a sample pretreatment method for rapid isolation of analytes from complex of biological fluids with ultra-low elution volume, and thus eliminating the need for post-extraction solvent evaporation and reconstitution steps [13,14]. This technique has been successfully applied for drug analysis in biological sample, such as plasma for high throughput preclinical and clinical pharmacokinetic studies [15,16]. Yet, the μSPE method for sample pretreatment in the analysis of nanoformulated drug has not been published in the literature. In this paper, we evaluated the use of μSPE for determination of nanoformulated drug, especially encapsulated into polymer-based nanoparticles with high throughput analysis.

In our previous study, we had developed a new type of polymeric ultrafine poly(lactic-*co*-glycolic acid) nanoparticles (PLGA NPs) [17]. These ultrafine PLGA NPs, with sub-50 nm diameter, exhibited high drug loading content, reduced burst release, and enhanced cell uptake rate compared with traditional PLGA NPs, and show great potential for drug delivery applications. In this study, we synthesized ultrafine PLGA NPs loaded with clarithromycin (CLA), which is a macrolide antibiotic in treating chronic lung and inflammation. [18] This CLA-loaded ultrafine PLGA NPs was a model nanoparticle for the development and validation the μSPE method for rapid quantitation of nanoformulated drug in rat plasma by UPLC–MS/MS.

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2. Experimental

2.1. Material

Clarithromycin (CLA), roxithromycin (ROX) as internal standard (I.S.), poly(D,L-lactic-*co*-glycolic acid) (PLGA, D,L-lactide:glycolide = 50:50, M.W. 40k–75k), and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEGPE-2000) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Oasis WCX μElution SPE plates were products of Waters Co. (Milford, MA, USA). Acetonitrile (ACN), chloroform, and methanol were products of Honeywell International Inc. (Morristown, NJ, USA). Double distilled water was made from Millipore Milli-Q system (Billerica, MA, USA).

2.2. Sample preparation

2.2.1. Preparation of stock solutions and working solution

The stock solution of CLA and ROX was prepared by dissolving each compound in ACN at a concentration of 1.0 mg/mL. A working solution of CLA or ROX was prepared at a concentration of 100 μg/mL by diluting stock solution with 50% ACN (ACN:water = 50:50, v/v). The working solutions were stored at −20 °C until use.

2.2.2. Preparation of calibration standards and quality control (QC) samples

The standard calibration curve samples of CLA at concentrations of 0, 10, 25, 50, 100, 250, 500, 1000, 2000, 2500, and 4000 ng/mL were prepared by serial dilutions with plasma from stock solution. ROX (6 μg/mL) was prepared in 50% ACN as the I.S. QC samples were prepared at concentrations of 10, 50, 250, and 2500 ng/mL by diluting CLA stock solution with plasma.

2.2.3. Preparation of CLA-loaded ultrafine PLGA NPs

As our previous study, 1 mL chloroform containing CLA (7 mg), PLGA (10 mg) and PEGPE-2000 (20 mg) was added into 3 mL H_2O, and the mixed solution was emulsified for 1 min using a microtip probe sonicator, followed by removing residual chloroform by a rotary evaporator. The suspension was centrifuged at 14,000 rpm for 30 min to collect the supernatant. The nanoparticles were washed three times using a 30 kD centrifugal filter (Amicon Ultra-4, Millipore), and were frozen by liquid nitrogen and stored at −20 °C until use. For the assay recovery test of CLA-loaded ultrafine PLGA NPs, samples were prepared at concentrations of 10, 50, 250, and 2500 ng/mL by diluting the nanoparticles solution with plasma.

2.2.4. Plasma sample extraction

For the standard and plasma sample analyses, 10 μL ROX solution (6 μg/mL in 50% ACN) and 60 μL ACN were sequentially added into 50 μL plasma sample pre-loaded PCR tube and then vortexed briefly. After centrifugation at 3500 rpm for 10 min, 15 μL supernatant was transferred to a 96-well μSPE plate conditioned with 200 μL methanol and water. The loaded plate was washed with 600 μL water to remove impurity. Finally the extracted samples were eluted with 150 μL ACN and then evaporated using SpeedVac concentrator. The residue was reconstituted in 150 μL 50% ACN and 5 μL aliquot was injected into the UPLC–MS/MS system.

2.3. UPLC–MS/MS condition

Chromatographic separation was performed on an ACQUITYTM UPLC system (Waters Corp., Milford, MA, USA) with a BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm i.d.; Waters Corp., Milford, MA, USA) maintained at 40 °C. The autosampler temperature was maintained at 10 °C. The linear gradient conditions are shown in Table 1, while the mobile phase was (A) 50 mM ammonium acetate solution and (B) ACN. The injection volume was 5 μL when the full loop mode was selected for sample injection. The UPLC system was connected to Waters ACQUITYTM TQD triple-quadrupole tandem mass spectrometer as an electrospray ionization (ESI) interface monitoring in positive ionization mode with the capillary voltage setting at 0.5 kV. The temperature of the source and desolvation was set at 150 °C and 500 °C. Nitrogen was used as the desolvation gas (1000 L/h) and cone gas (30 L/h), and argon was used as the collision gas for collision-induced dissociation (CID) setting at 0.15 mL/min. All data collected in centroid mode were acquired using MasslynxTM NT 4.1 software. Post-acquisition quantitative analyses were performed using a QuanLynxTM program.

Table 1
Gradient elution program for the analysis of CLA.

Chromatographic gradient program over UPLC–MS/MS analysis time (2 min)			
Time (min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)	Flow rate (mL/min)
0.0	50	50	0.3
1.5	10	90	0.3
1.7	10	90	0.3
1.8	50	50	0.3
2.0	50	50	0.3

[a] 50 mM ammonium acetate.
[b] ACN.

2.4. Method validation

2.4.1. Precision and accuracy test

The assay was confirmed by measuring QC plasma samples for different dynamic ranges, including 10, 50, 250, and 2500 ng/mL. Intra-day and inter-day precision and accuracy were all calculated from three replicate values at the peak area ratio of CLA *versus* I.S. for each concentration.

2.4.2. Extraction recovery test

The intra-day and inter-day extraction recovery were evaluated to cover the corresponding dynamic range of each assay at nanoformulated CLA concentrations of 10, 50, 250, and 2500 ng/mL with an I.S. concentration of 6 μg/mL. The data were reported as a mean of three replicate measurements at each concentration level.

2.5. Pharmacokinetic study

2.5.1. Drug administration and plasma sample collection

Male Sprague-Dawley rats (250–300 g) were used to study the pharmacokinetics of nanoformulated CLA. The day before the pharmacokinetic experiment, the jugular vein of all rats was catheterized with polyethylene tubing under zoletil (25 mg/kg i.m.) and xylazine (10 mg/kg i.m.) anesthesia. Rats were administered a single intravenous dose (equivalent 20 mg/kg CLA) of CLA-loaded ultrafine PLGA NPs. Blood samples (500 μL) were collected into K_2EDTA tubes *via* the jugular vein at the following times: 0.05, 0.25, 0.5, 1, 2, 4, 8, and 24 h. Plasma samples were obtained after centrifugation at 4000 rpm for 15 min, and stored at −80 °C until analysis.

2.5.2. Pharmacokinetic analysis

The plasma concentration-*versus*-time data of CLA from three rats were analyzed to determine the pharmacokinetic parameters using a non-compartmental model in WinNonlin Professional version 5.2 (Pharsight, Mountain View, CA).

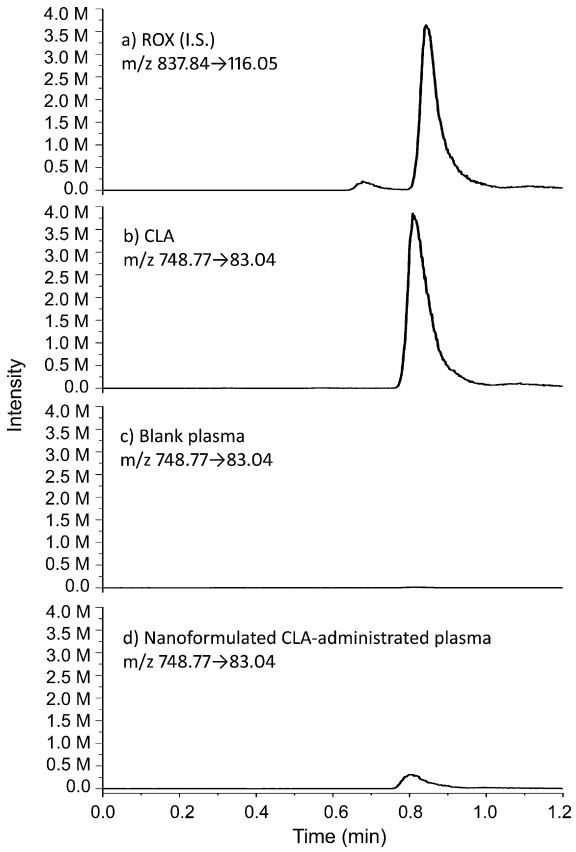

Fig. 1. Representative MRM chromatograms of (a) internal standard of ROX (1000 ng/mL), (b) standard of CLA (1000 ng/mL), (c) blank of rat plasma, and (d) rat plasma sample obtained 8 h after i.v. administration of nanoformulated CLA at a dose of 20 mg/kg.

3. Results and discussions

3.1. Characterization of CLA-loaded ultrafine PLGA NPs

The average size of CLA-loaded ultrafine PLGA NPs measured from the TEM image (data not shown) was 32.5 ± 6.2 nm. The mean zeta potential was −34.2 ± 0.8 mV measured by ZetaPALS (Brookhaven, New York, USA). These nanoparticles were well-dispersed and spherical in shape.

3.2. Method development

This paper describes an analytical method and the validation results for the determination of CLA in rat plasma using ROX as an I.S. After the pretreatment with a rapid 96-well μSPE extraction method which was used to extract the nanoformulated drug from rat plasma samples and were separated by UPLC–MS/MS with ESI^+ mode.

3.2.1. UPLC condition

Several assays have been reported on the quantitative analysis of CLA in plasma using LC–MS techniques [19,20]. A modified chromatographic condition is shown in Table 1. MRM chromatograms for ROX and CLA are shown in Fig. 1a and b, respectively. Fig. 1c and d shows the chromatograms from the elution of extracted plasma samples of blank plasma and plasma sample obtained 8 h after administration of nanoformulated CLA, respectively. The retention times for CLA and ROX were 0.8 min and 0.9 min, respectively. The peak shapes were satisfactory and suitable for quantitative analysis.

Table 2
Intra-day and 3-day inter-day validation of the method for the determination of CLA in rat plasma.

Expected concentration (ng/mL)	Measured concentration ± S.D. (ng/mL)	Precision (%)	Accuracy (%)
(A) Intra-day, $n = 3$			
10	9.7 ± 0.296	3.0	97.0
50	52.3 ± 4.152	8.3	104.6
250	241.1 ± 14.412	5.8	96.5
2500	2517.9 ± 12.435	0.5	100.7
(B) Inter-day, $n = 3$			
10	9.7 ± 0.424	4.2	94.1
50	48.9 ± 1.792	3.6	102.0
250	245.6 ± 18.385	7.4	98.2
2500	2529.7 ± 131.137	5.2	101.0

Assay LOD = 3.072 ng/mL.

3.2.2. Mass spectrometry

MRM mode was investigated to detect CLA and ROX because their similarity in chemical structure. The corresponding mass spectrometry parameters were optimized by tuning the instrument with the infusion of CLA and ROX solutions dissolved in 50% ACN. Quantitation was performed using the MRM to study precursor → product ion transitions of the analyte and I.S. which were of m/z 748.77 → 83.04 and 837.84 → 116.05 for CLA and ROX, respectively.

3.3. Sample pretreatment and extraction

All plasma samples mixed with ROX solution were added with 50% ACN followed by sonication to precipitate the protein and collapse the PLGA NPs to extract CLA in plasma. μSPE was used for extraction since the resins can exert both the hydrophobic interaction and ionic interaction with the amine groups from CLA and ROX. This μSPE is easy and rapid to operate comparing to the traditional liquid–liquid extraction method.

3.4. Method validation

3.4.1. Selectivity

Selectivity of CLA was determined by averaging the chromatograms of six different runs of blank human plasma samples. As shown in Fig. 1c, no interference was observed at the retention time of CLA.

3.4.2. Calibration curve, linearity, and sensitivity

The calibration curve equation was: $y = 1.2263x + 4.4741$, $R^2 = 0.9999$ (RSD = 3.20%), where y is the peak area ratio of CLA to I.S., and x is the concentration of CLA over the range of 10–4000 ng/mL in rat plasma. The LLOQ of the assay was 3 ng/mL.

3.4.3. Precision and accuracy

The intra-day and 3-day inter-day precision and accuracy were evaluated by analyzing three replicate QC samples in rat plasma at each of four concentration levels. As shown in Table 2, the intra-day and inter-day precision (RSD) for QC samples at low, medium, and high concentrations were in the range of 0.5–8.3% and 3.6–7.4%, while the accuracy (RE) ranged from 96.5% to 104.6% and 94.1% to 102%. This method was demonstrated to be accurate and reproducible in rat plasma. All these data were acceptable for analysis of CLA in plasma sample for pharmacokinetic study.

3.4.4. Assay extraction recovery

The recovery of CLA in rat plasma spiked with nanoformulated CLA by the current μSPE method was evaluated by comparing peak

Table 3
Intra-day and 3-day inter-day validation of the method for the determination of nanoformulated CLA in rat plasma.

Expected concentration (ng/mL)	Measured concentration ± S.D. (ng/mL)	Precision (%)	Accuracy (%)
(A) Intra-day, $n = 3$			
10	10.5 ± 0.408	4.1	104.6
50	49.9 ± 1.092	2.2	99.9
250	245.9 ± 0.753	0.8	98.4
2500	2499.4 ± 1.586	0.5	100.0
(B) Inter-day, $n = 3$			
10	10.5 ± 1.225	12.2	104.9
50	49.5 ± 0.493	1.0	99.0
250	247.0 ± 0.358	0.1	98.9
2500	2510.7 ± 3.048	0.1	100.4

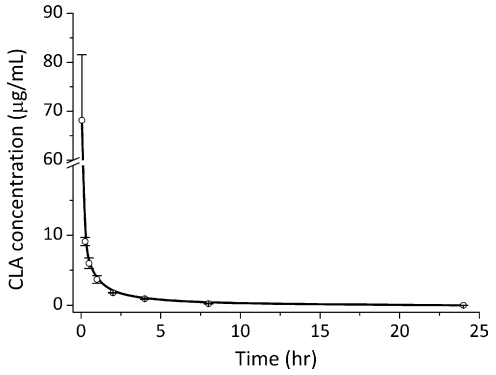

Fig. 2. Mean plasma concentration–time profile after single i.v. administration nanoformulated CLA at a dosage of 20 mg/kg in rats ($n = 3$).

area ratios (analyte/I.S.) of QC samples. For extraction of nanoformulated CLA in plasma, the inter-day recoveries were 104.9%, 99%, 98.9%, and 100.4% at the concentrations of 10, 50, 250, and 2500 ng/mL, respectively, and the intra-day recovery ranged from 98.4% to 104.6%, shown in Table 3. These results suggested that there was no significant difference in extraction recovery at different concentrations of nanoformulated CLA in plasma samples.

3.5. Application to pharmacokinetic study of nanoformulated CLA

The applicability of this method was demonstrated *in vivo* by the determination of CLA concentrations in plasma samples. Fig. 2 reports the mean plasma concentration–time profiles of CLA after a single intravenous administration of nanoformulated CLA (20 mg/kg) into rats. Pharmacokinetic parameter values of half-life ($t_{1/2}$), area under curve (AUC), clearance (CL), steady-state volume of distribution (V_{ss}), and mean residence time (MRT) were 2.257 h, 26.310 h μg/mL, 760.959 mL/h/kg, 1192.947 mL/kg, and 1.554 h, respectively. From the results of the presented study, this method is applicable to pharmacokinetic studies.

4. Conclusions

A rapid, sensitive and selective 96-well μSPE by UPLC MS/MS method has been developed and validated for the determination of nanoformulated drug in rat plasma. The method has been applied successfully, for the first time, to quantitatively analyze CLA in rat plasma after intravenous administration of CLA-loaded ultrafine PLGA NPs for pharmacokinetic study. This μSPE extraction method resulted in a consistent and reproducible recovery of the CLA from rat plasma. The method was linear ($R^2 = 0.9999$) over the concentration range of 10–4000 ng/mL. No matrix interference at the retention time of CLA was observed when the method was tested using rat plasma. The validated method was successfully utilized to accurately analyze nanoformulated drug sample for a pharmacokinetic study. The use of μSPE for determination of nanoformulated drug may provide a rapid analysis of large quantities of samples of pharmacokinetic study for further clinical investigations.

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