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Development and validation of a RP-HPLC method with fluorescence detection for simultaneous determination of 10-methoxycamptothecin and its metabolite 10-hydroxycamptothecin in rat plasma

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ABSTRACT

Both 10-methoxycamptothecin (MCPT) and 10-hydroxycamptothecin (HCPT) are the natural bioactive derivatives of camptothecin (CPT) isolated from *Camptotheca acuminata*, and have been confirmed to possess high anti-cancer properties. In the present study, HCPT was identified as the major metabolite of MCPT in rat plasma through HPLC/photodiode array detection (PDA) and LC–MS/MS analysis. A sensitive and reliable RP-HPLC method with fluorescence detection was developed and validated for the simultaneous analysis of MCPT and HCPT in rat plasma. The parental CPT was used as an internal standard (IS). A piecewise linear function was used over lower and higher concentrations, respectively. The calibration curves were linear ($r^2 > 0.999$) over concentrations from 1.25 to 20 ng/mL and 20 to 320 ng/mL for both MCPT and HCPT. The method had an accuracy of 92.24–113.90%, and the intra- and inter-day precision (RSD%) were 10.05% or less for MCPT and HCPT. The stability data showed no significant degradation occurred under the experimental conditions. The mean recoveries at concentrations of 2.5, 40 and 160 ng/mL were 95.09 ± 3.94%, 98.67 ± 1.40% and 95.65 ± 2.15% for MCPT and 84.06 ± 4.39%, 84.85 ± 3.10% and 81.03 ± 4.44% for HCPT, respectively. The lower limit of quantification (LLOQ) using 0.1 mL of plasma was 1.25 ng/mL for both MCPT and HCPT. This method was successfully applied to the pharmacokinetic study of MCPT and its metabolite HCPT in rat plasma after intravenous administration.

1. Introduction

Camptothecin (CPT, Fig. 1) is a naturally occurring pentacyclic indole alkaloid first isolated from a native Chinese tree *Camptotheca acuminata* Decne. (Nyssaceae), by Wall et al. in 1966 [1]. CPT has shown broad-spectrum antitumor activity [2]. Further fractionation of *C. acuminata* resulted in the isolation and identification of minor CPTs, including 10-hydroxycamptothecin (HCPT, Fig. 1) and 10-methoxycamptothecin (MCPT, Fig. 1) [3], both possessing better activities against the cell line 9KB (human nasopharyngeal carcinoma) *in vitro* and P388 lymphocytic leukemia system *in vivo* [4]. The anticancer mechanism of CPTs is based on the inhibition of DNA replication by stabilizing cleavable complexes formed between topoisomerase I and DNA [5]. Topoisomerase I is a ubiquitous enzyme found in all cell types from viruses to human. Moreover, topoisomerase I was found to be over-expressed in advanced stages of human colon adenocarcinoma and other malignancies but not in normal tissues [2]. Therefore CPTs showed high activity in a wide range of cancers. The reduced toxicity of HCPT compared to CPT [6] has brought the growing interest of *in vivo* and clinical studies on the pharmaceutics and pharmacology of HCPT [7–11]. HCPT is currently clinically used against gastric carcinoma, hepatoma, leukemia, and tumors of head and neck in China [8,9]. Apart from the initial isolation and identification of new CPTs and their *in vitro* antitumor activities [4,12–14], few studies were carried out on MCPT due to its higher toxicity [15]. To elucidate the potential of MCPT in the development of new anticancer drugs, we tested its antitumor activity against 2774, an ovarian cancer cell line, and MCPT showed higher cytotoxicity than HCPT. More surprisingly, assaying for MCPT metabolites in plasma after intravenous (i.v.) administration found four new chromatographic peaks (implying four metabolites), of which one of the major metabolites was identified as HCPT. It brings new interest to the potential use of MCPT in the development of novel anticancer drugs.

Generating pharmacokinetic data for MCPT requires the development of an analytical method for the simultaneous detection of both MCPT and HCPT in biological matrices, however no such method has been reported to date. Several methods have been reported for the extraction and detection of CPT and its analogs

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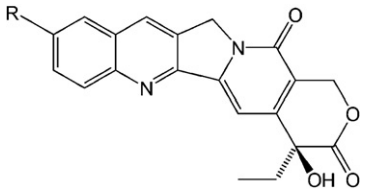

Fig. 1. Chemical structures of CPTs (R: —H, CPT; R: —OH, HCPT; R: —OCH_3, MCPT).

in biological fluids. Biological samples were treated with acetonitrile/methanol to precipitate proteins [16], or extracted with organic solvents using conventional liquid–liquid extraction (LLE) [17] *via* the detection approaches such as high-performance liquid chromatography (HPLC) with fluorescence detection [18,19] or UV detection [20] and mass spectrometry [21–23]. LLE is based on the distinct distribution of analytes between two immiscible solvents [24]. Despite the disadvantages of large volumes of organic solvents and the poor potential for automation [25,26], LLE is widely used for the extraction of aqueous samples [27] due to its fast and effective separation of two phases together with the high efficiency for the preparation of plasma samples [28–30]. The objective of the present study is to establish a validated HPLC method coupled with a fluorescence detector for the pharmacokinetic studies of MCPT and its major metabolite HCPT. CPT was used as the internal standard (IS) while measuring MCPT and HCPT concentrations by HPLC in this study. The method was applied to the pharmacokinetic study of MCPT in rats after i.v. injection.

2. Experimental

2.1. Chemicals and reagents

MCPT was synthesized in-house according to reported procedures [31]. CPT and HCPT with purities of 99% were purchased from Harbin Foran High-Tech Development Ltd. (Harbin, Heilongjiang, China).

HPLC-grade acetonitrile (ACN), methanol (MeOH) and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade water (resistivity 18.2 MΩ cm) was produced by purification of distilled water with a Milli-Q gradient system (Billerica, MA, USA). All other reagents or solvents used were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. In vitro cytotoxicity of MCPT and HCPT against human ovarian cancer cell line 2774

Human ovarian cancer cell line 2774 was a generous gift from the CHRISTUS Stehlin Foundation for Cancer Research (Houston, TX, USA). The cells were cultured in RPMI-1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; PAA, UK), L-glutamine (0.29 mg/mL) and 1% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were subcultured every 4–5 days by total replacement using 0.25% (w/v) trypsin. Cells were seeded in 96-well culture plates at a density of 4×10^3 cells in 0.2 mL of growth medium per well and allowed to attach for 24 h. The culture medium was replaced with either MCPT or HCPT at different concentrations in four replicates followed by 72 h of incubation. After incubation, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Amresco, USA) solution at a concentration of 5 mg/mL was added to each well followed by the 4 h of incubation. MTT solution was then aspirated and 150 μL of DMSO was added to each well to dissolve the dark blue crystals thoroughly. The absorbance was measured at 490 nm using a microplate reader (Infinite 200 NanoQuant, Tecan, Austria). The relative growth rate (%) was calculated as (mean absorbance of the sample/mean absorbance of the control) × 100%, considering the optical density of the control as 100% [32,33].

2.3. Chromatographic conditions

2.3.1. Identification of MCPT and HCPT

The identification of MCPT and HCPT in plasma samples was conducted by HPLC with a photodiode array (PDA) detector and a liquid chromatography–tandem mass spectrometry (LC–MS/MS), respectively.

A Waters HPLC system consisting of a 1525 binary pump, a PDA detector (2996) and an analytical Phenomenex Luna C18 column (5 μm, 250 mm × 4.6 mm; Torrence, CA, USA) was used in this study. The column temperature was maintained at room temperature. The lifetime UV spectrum ranges from 210 to 400 nm was scanned by the PDA detector. A flow rate of 1.2 mL/min was used with a 50-μL of injection volume. The binary gradient mobile phases were water (containing 5% ACN) as mobile phase A and ACN as mobile phase B. The time program of the gradient was as follows: phase B was linearly increased from 20% to 60% in the first 12 min, then increased to 90% within 3 min, and then linearly decreased from 90% to 20% in the following 4 min and kept stably at 20% for 4 min. The total data acquisition time was 15 min with a column balance time of 8 min. The Waters Empower software was used for system control and data acquisition.

The LC–MS/MS system consists of a Waters Acquity UPLC unit with a Waters BEH phenyl column (2.1 mm × 50 mm, 1.7 μm, Waters, MA, USA) and a Waters Xevo TQ MS tandem quadrupole mass spectrometer (Waters, MA, USA). The optimized method used binary gradient mobile phases with water (5 mM ammonium acetate, pH 3.0) as mobile phase A and ACN as mobile phase B. A flow rate of 0.25 mL/min was used with a 5-μL of injection volume. The time program of the gradient was as follows: phase B was initially balanced at 20% for 0.2 min, and then linearly increased from 20% to 60% in the following 1.30 min and kept for 0.5 min, then decreased from 60% to 20% in 0.2 min and kept stably at 20% for 0.8 min. The total running time was 3 min. The column temperature was maintained at room temperature. Samples were maintained at 4 °C in the auto-sampler tray until injection. Mass spectral ionization, fragmentation and acquisition parameters were optimized by directly injecting MCPT and HCPT standard solutions in the positive electrospray ionization (ESI) mode. Nitrogen was employed as the desolvation and nebulization gas at the flow rates of 650 L/h and 50 L/h, respectively. Argon was employed as the collision gas. The desolvation temperature was maintained at 450 °C. The source temperature was 150 °C. The capillary voltage was 3.00 kV and the cone voltage was 35 V. The data were acquired under multiple reaction monitoring (MRM) mode. The identification was developed by monitoring the transitions m/z 379.2 → 335.1 and 379.2 → 279.1 for MCPT, and m/z 365.20 → 321.10 and 365.20 → 265.10 for HCPT.

2.3.2. Quantification of MCPT and HCPT

The HPLC conditions for the quantification of MCPT and HCPT were the same as that used for PDA detection above except for using the 2475 fluorescence detector other than the PDA detector. The eluent was monitored at the excitation and emission wavelengths of 380 and 515 nm, respectively. An injection of ACN with 10% acetic acid was used to wash the column every 3–5 injections.

2.4. Preparation of standard and quality control (QC) samples

A mixed stock solution of MCPT (1 μg/mL) and HCPT (1 μg/mL) was prepared by dissolving 10 mg of MCPT and HCPT in 10 mL of DMSO and further diluting 1000 times with MeOH. Calibration

curves were prepared by spiking 100 μL of the appropriate standard solution with 100 μL of blank rat plasma. The effective concentrations in plasma samples were 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320 ng/mL for both MCPT and HCPT. A stock solution of CPT (IS) was prepared in DMSO at a concentration of 1 mg/mL and further diluted with MeOH given the working solution at the concentration of 50 ng/mL. QC samples were prepared in pools as a single batch at concentrations of 2.5 (low), 40 (medium) and 160 ng/mL (high), divided into aliquots and stored in the freezer at −80 °C until analysis. The spiked plasma samples (standards and quality controls) were treated following the sample processing procedure as for the unknown samples.

2.5. Sample processing

One hundred micro-liters of rat plasma spiked with 100 μL of standard solution and 100 μL of IS working solution (CPT, 50 ng/mL) were mixed in a 2-mL test tube. A 100 μL aliquot of 1% acetic acid and 600 μL of diethyl ether were added to the mixture. After vortexing for 1 min, the mixture was incubated at room temperature for 30 min on a shaker followed by centrifuging at 12,000 × *g* for 10 min. The organic layer was then transferred to a clean tube, and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 200 μL of MeOH. After centrifuged at 12,000 × *g* for 10 min, a 50 μL aliquot was injected into the chromatographic system using a Waters 717 plus auto-sampler.

2.6. Method validation

The analytical method was validated to meet the acceptance criteria as the guidance from Food and Drug Administration (FDA) and per guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with the corresponding spiked plasma.

Plasma samples were quantified using the peak area ratio of MCPT or HCPT to that of the IS. A standard curve in the form of $y = Ax + B$ was used, where x represents MCPT or HCPT concentration in the plasma sample and y represents the ratio of the analyte peak area to that of the IS. A linear least-square regression analysis was conducted to determine the slope, intercept, and coefficient (r^2) to demonstrate the linearity of this method. To evaluate the linearity, plasma calibration curves were prepared and analyzed in triplicates on 5 consecutive days.

The accuracy and precision were also determined by replicate analyses ($n = 6$) of QC samples at three concentrations (2.5, 40 and 160 ng/mL) on different validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision was done by relative standard deviation (RSD%). The concentration of each sample was calculated using the calibration curve and analyzed on the same day.

The extraction recoveries of MCPT and HCPT at three QC levels were evaluated by assaying the samples as described above and comparing the area ratio of the peaks for MCPT or HCPT to that of IS with those for neat solution of standard compounds without extraction.

2.7. Stability

The stability of the standard solutions was tested after standing at room temperature for 6 h and after refrigeration at 4 °C for 30 days. The stability of QC samples was examined by keeping replicates of QC samples of MCPT and HCPT at room temperature for 6 h, in the auto-sampler tray for 24 h, and in a freezer at −80 °C for 30 days; the freeze–thaw stability was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h and then refreezing at −80 °C for 12–24 h. For each concentration and each storage condition, 6 replicates were analyzed in one analytical batch. The concentration of analytes after each storage period was related to the initial concentration, which was determined when the samples were originally prepared and processed.

2.8. In vivo studies

Male Wistar rats (200 ± 20 g) obtained from the Laboratory Animal Center of Jilin University (Changchun, China) were chosen for *in vivo* studies. Animal handling procedures were according to standard operating procedures approved by the institutional animal care and use committee at Northeast Forestry University. All rats were dosed following an overnight fasting (except for water). Formulations of MCPT (25 mg/mL for the identification and 5 mg/mL for the pharmacokinetic studies, respectively) were prepared by mixing MCPT in DMSO/PEG-400/0.01 mol/L H_3PO_4 (5/45/50, v/v/v) [20].

2.8.1. Administration for MCPT and HCPT identification

Considering the lower response of UV absorbance than fluorescence, three rats were i.v. administered with a dose of 100 mg/kg MCPT *via* the tail vein, and blood samples were collected in heparinized tubes *via* cardiac puncture 20–30 min after drug administration. Then 300 μL of rat plasma sample was pipetted into a 2-mL test tube and treated with the LLE sample processing method in Section 2.5.

2.8.2. Pharmacokinetic studies in rats

Six rats were taken jugular vein cannulation [34] and i.v. administered with a single dose of 5 mg/kg MCPT *via* the tail vein. Serial blood samples (about 0.3 mL) from the right jugular vein were collected into sodium heparin-containing tubes before and at 0.08, 0.17, 0.33, 0.5, 0.67, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h time points after administration. Plasma was separated by centrifugation at 4000 × *g* for 30 min at 4 °C and stored frozen at −80 °C until analysis.

2.9. Statistical analysis

A non-compartmental pharmacokinetic analysis using the Kinetica™ software package (version 5.0, Thermo Fisher Scientific Inc., MA, USA) was performed to determine the key parameters including the maximum plasma concentration (C_{max}), the time-to-maximum concentration (T_{max}), the elimination half-life time ($T_{1/2}$), the mean residence time (MRT), the area under the plasma concentration–time curve from 0 to 24 h (AUC_{0-24}), and the area under the plasma concentration–time curve from zero to infinity ($AUC_{0-\infty}$).

3. Results and discussion

3.1. Cytotoxicity of MCPT against the human ovarian cancer cell line 2774

To evaluate the cytotoxicity of MCPT, an *in vitro* test against the human ovarian cancer cell line 2774 was performed. The relative proliferation rates of 2774 cells exposed to different concentrations of MCPT and HCPT are shown in Fig. 2. At equivalent concentration, MCPT showed a higher inhibition rate than HCPT, which was more prominent at high concentrations ($p < 0.001$). The IC_{50} value of MCPT, 9.43 ± 0.87 nmol/L, was much lower than that of HCPT (17.70 ± 1.59 nmol/L), which suggests an approximate 2-folds higher in cytotoxicity against 2774 cells than HCPT. Previous

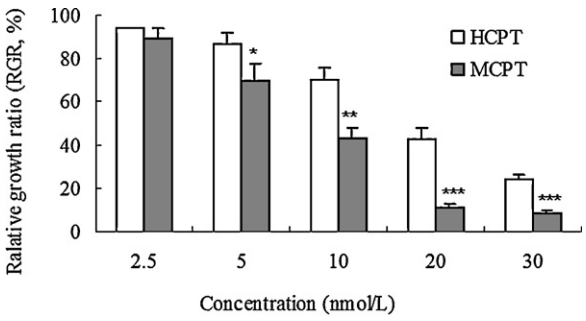

Fig. 2. Relative growth rate of 2774 cells incubated with different concentration of MCPT and HCPT (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

research has shown that MCPT is more cytotoxic than HCPT against a human breast carcinoma cell line BT-20, while an opposite effect was seen in a human breast carcinoma cell line MDA-231 [35], which indicates MCPT may have a selected inhibition to different cell lines.

3.2. Metabolite identification

The ability of the PDA detector to acquire lifetime UV spectrum ranges from 210 to 400 nm allows us to obtain three-dimensional (3D) chromatograms showing the variations of spectral absorbance in relation to wavelength and time. This provides the possibility for chemical identification [36]. Representative 3D and conventional 2D (λ = 254 nm) chromatograms of a blank rat plasma sample, a rat plasma sample after MCPT administration and a blank rat plasma sample spiked with MCPT and HCPT are shown in Figs. 3 and 4, respectively. Besides the peak of MCPT (10.80 min) and an endogenous peak (13.11 min), a major chromatographic peak with the same retention time (6.03 min) and a UV spectrum similar to HCPT was detected along with 3 minor peaks which are underwent further studies (Figs. 3B and 4B).

For further identification of MCPT and HCPT, LC–MS/MS detection in the positive ion MRM mode was used to detect MCPT and the possible metabolite, HCPT. To achieve the optimal sensitivity, direct infusion of standard solution was carried out to optimize MS parameters. The protonated molecular ions were selected as the precursor ions at m/z 379.20 for MCPT and m/z 365.20 for HCPT. The characteristic product ions were produced at m/z 335.10 and 279.10 for MCPT, and at m/z 321.10 and 265.10 for HCPT, which were due to the loss of CO_2 (−44) or CO_2 and C_2H_3CHO (−100) from the protonated precursor ions for both MCPT and HCPT, respectively. As shown in Fig. 5B, two peaks were detected by monitoring the above

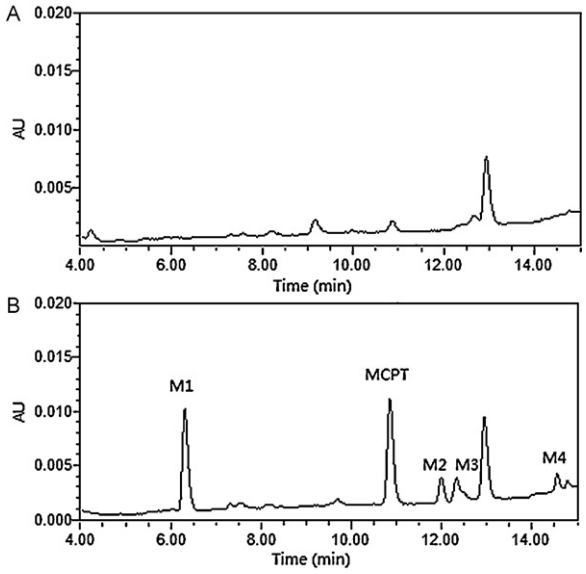

Fig. 4. Representative chromatograms at λ = 254 nm of (A) a blank rat plasma sample; (B) a rat plasma sample at 0.5 h after i.v. administration of MCPT at dose of 100 mg/kg.

four pairs of transitions (m/z 379.20 → 335.10 and 379.20 → 279.10 for MCPT, and 365.20 → 321.10 and 365.20 → 265.10 for HCPT) in rat plasma samples 0.5 h after i.v. administration of MCPT at a dose of 5 mg/kg. By comparing the retention time of analytes with HCPT and MCPT standards (Fig. 5C), the peak at 1.43 min of retention time was confirmed to be HCPT, the demethylated metabolite of MCPT.

3.3. Method development

The parental compound CPT was generally selected as the IS for the structural similarity to its derivatives [18–22], which also showed a satisfied extraction recovery and proper retention time in the present study.

For differences in fluorescent characteristics of CPT, MCPT and HCPT, different detective excitation (Ex) and emission (Em) wavelengths were estimated. At the optimal Ex and Em wavelengths of 347 and 446 nm for MCPT or that of 383 and 553 nm for HCPT [7], the other compound was undetectable, respectively. To solve this problem, different Ex and Em wavelengths were tested for the simultaneous detection of MCPT, HCPT and CPT (Ex: 370 nm, Em: 434 nm) [37], which yielded optimal Ex and Em wavelengths of 380 and 515 nm for all three compounds.

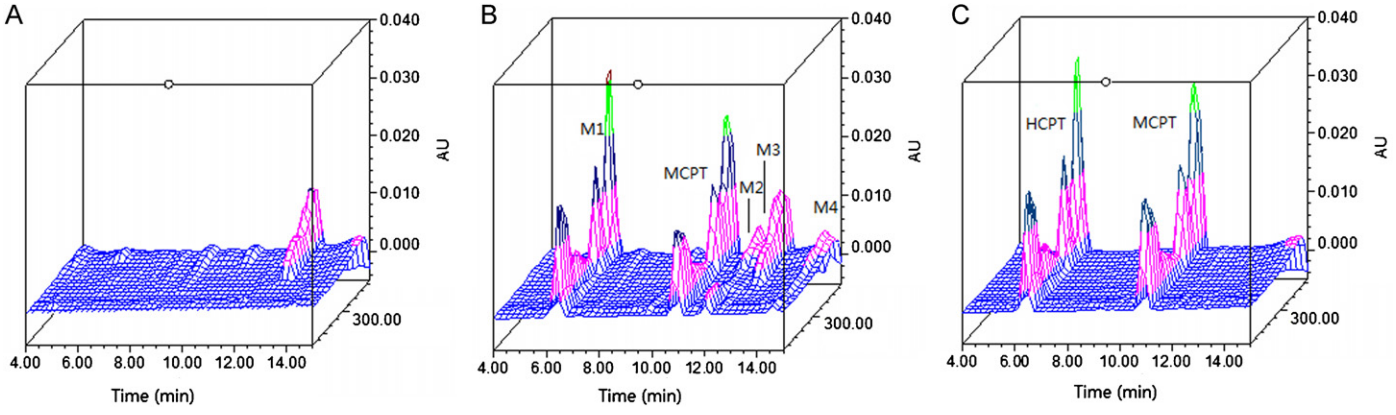

Fig. 3. Representative 3D chromatograms of (A) a blank rat plasma sample; (B) a rat plasma sample at 0.5 h after i.v. administration of MCPT at a dose of 100 mg/kg; (C) a blank rat plasma sample spiked with MCPT (5 μg/mL) and HCPT (5 μg/mL).

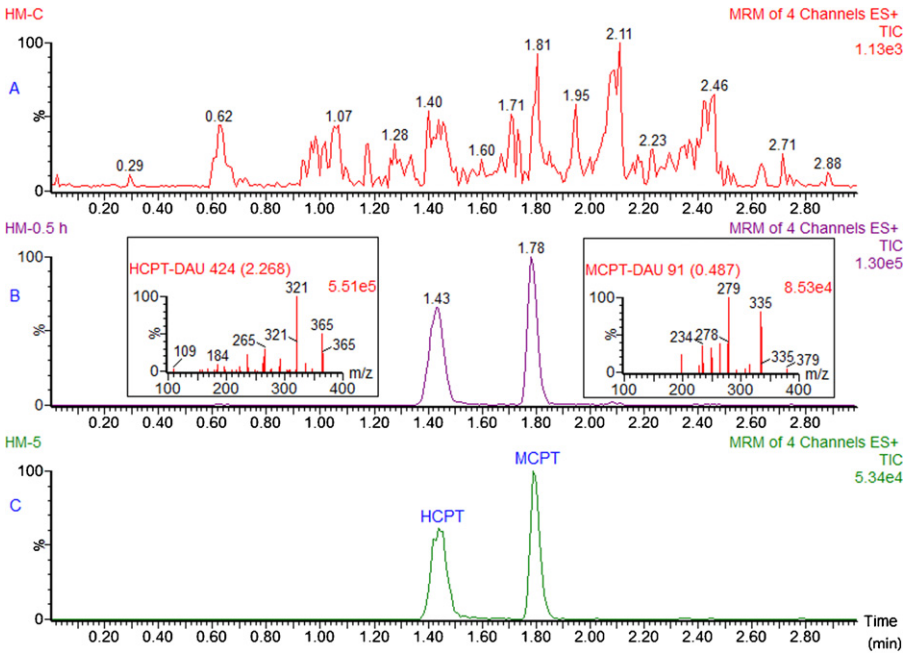

Fig. 5. LC–MS/MS chromatograms for MCPT and HCPT identification of (A) a blank rat plasma sample; (B) a rat plasma sample 0.5 h after i.v. administration of MCPT at a dose of 5 mg/kg; (C) a blank rat plasma spiked with MCPT (5 ng/mL) and HCPT (5 ng/mL).

Water consisting 5% ACN (v/v) was used as mobile phase A in the present study, which showed good bacteria resistance and benefited the instrument subsequently. Under the selected chromatographic conditions, the data acquisition time for each sample was 15 min followed by an equilibration time of 5–8 min. The typical retention times were 11.44, 7.02 and 10.24 min for MCPT, HCPT and CPT, respectively.

3.4. Method validation

3.4.1. Selectivity

The selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma samples with the corresponding spiked plasma samples. Fig. 6 shows the typical chromatograms of a blank rat plasma sample (A), a blank rat plasma spiked with standard MCPT (10 ng/mL), HCPT (10 ng/mL) and IS (50 ng/mL) (B), a rat plasma sample 1 h after i.v. administration of MCPT at a dose of 5 mg/kg (*C*). They showed well-separated analytes with no significant interference from endogenous substances observed at their corresponding retention times under current HPLC conditions.

3.4.2. Linearity of calibration curves and lower limits of quantification (LLOQ)

The standard curve was established by plotting the ratio of the peak area of MCPT or HCPT to that of the IS. The linear-regression correlation coefficients were greater than 0.999 in all validation runs. In this study, a piecewise linear function was used under the consideration of the differences in signal response among the lower and higher concentrations, and the concentration ranges were 1.25–20 ng/mL and 20–320 ng/mL, respectively. The typical standard curves were $y_1 = 2.46 \times 10^{-2}x_1 + 1.40 \times 10^{-2}$ for MCPT and $y_2 = 2.73 \times 10^{-2}x_2 + 1.03 \times 10^{-2}$ for HCPT over the range of 1.25–20 ng/mL; and $y'_1 = 1.98 \times 10^{-2}x'_1 + 0.19$ for MCPT and $y'_2 = 2.17 \times 10^{-2}x'_2 + 2.17 \times 10^{-2}$ for HCPT at 20–320 ng/mL, where y represents the ratio of the peak area of MCPT or HCPT to IS and x represents MCPT or HCPT concentration.

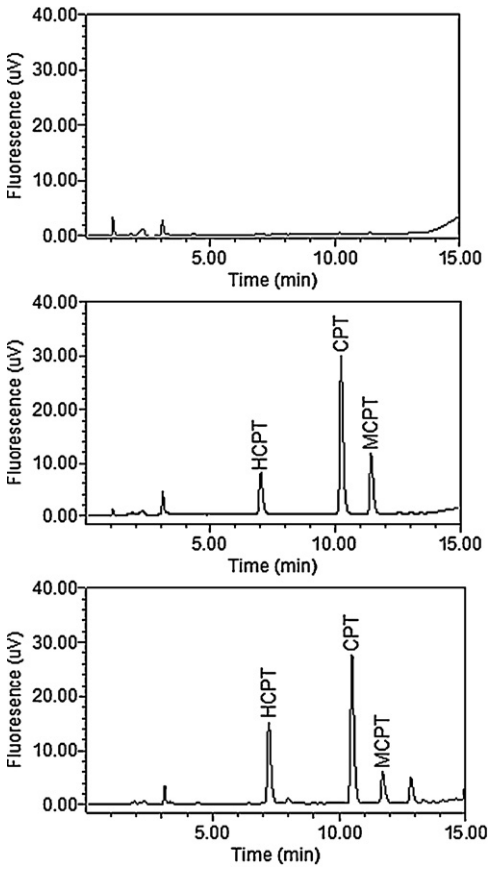

Fig. 6. Representative chromatograms of (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with MCPT (10 ng/mL), HCPT (10 ng/mL) and IS (CPT 50 ng/ml); (C) a rat plasma sample at 1 h after i.v. administration of MCPT at a dose of 5 mg/kg and spiked with IS (CPT 50 ng/mL).

Table 1
Accuracy and precision for the analysis of MCPT in rat plasma.

Added, *C* (ng/mL)	Found, *C* (ng/mL)	RSD (%)	Relative error (%)	Accuracy (%)
Intra-day ($n = 18$)				
2.5	2.39	3.30	−4.12	95.88
40	41.15	1.81	2.88	102.88
160	159.83	1.14	−0.11	99.89
Inter-day ($n = 30$)				
2.5	2.39	4.94	−4.35	95.65
40	40.34	2.06	0.86	100.86
160	159.24	1.33	−0.47	99.53

Table 2
Accuracy and precision for the analysis of HCPT in rat plasma.

Added, *C* (ng/mL)	Found, *C* (ng/mL)	RSD (%)	Relative error (%)	Accuracy (%)
Intra-day ($n = 18$)				
2.5	2.48	4.38	−0.72	99.27
40	41.31	2.96	3.28	103.28
160	159.78	2.24	−0.14	99.86
Inter-day ($n = 30$)				
2.5	2.43	4.48	−2.69	97.31
40	40.35	2.59	0.88	100.88
160	159.47	2.24	−0.33	99.67

The LLOQ was defined as the lowest concentration on the calibration curve with an acceptable accuracy of ±15% and a precision below 15% [30]. The present method offered a LLOQ of 1.25 ng/mL for both MCPT and HCPT. MCPT and HCPT in rat plasma samples were detectable till 24 h after i.v. injection of 5 mg/kg MCPT. Thus this method provides a sufficient sensitivity to investigate the pharmacokinetic behaviors of MCPT and its major metabolite HCPT after administration of MCPT in rats.

3.4.3. Accuracy and precision

Tables 1 and 2 summarize the precision and accuracy results. The intra- and inter-day precision and accuracy for MCPT and HCPT were evaluated by assaying the QC samples (2.5, 40 and 160 ng/mL). The precision is indicated by relative standard deviation (RSD%). For each QC level of MCPT, the intra-day precision was 3.30% or less, and the accuracy was between 95.88% and 102.88%. The inter-day precision was 4.94% or less, and the accuracy was between 95.65% and 100.86%. For each QC level of HCPT, the intra-day precision was 4.38% or less, and the accuracy was between 99.27% and 103.28%; while the inter-day precision was 4.48% or less, and the accuracy was between 97.31% and 100.88%. For LLOQ samples (1.25 ng/mL), the corresponding accuracy and precision for MCPT and HCPT were also tested on five validation days. The accuracy for MCPT and HCPT was 92.24% and 113.90%, respectively, and the precision were 10.05% and 5.21%, respectively. These data suggest that the method is accurate and precise for the quantitative analysis of MCPT and HCPT in rat plasma.

3.4.4. Recovery and stability

Recoveries were determined at three QC sample concentrations (2.5, 40 and 160 ng/mL). For MCPT, the values were 95.09 ± 3.94%, 98.67 ± 1.40% and 95.65 ± 2.15% ($n = 6$), respectively, and for HCPT, the values were 84.06 ± 4.39%, 84.85 ± 3.10% and 81.03 ± 4.44% ($n = 6$), respectively. The recoveries of IS (50 ng/mL), MCPT (2.5 ng/mL) and HCPT (2.5 ng/mL) were also calculated by comparing the peak areas with those for neat solution of standard compounds without extraction. The value for IS was 53.87 ± 6.40% ($n = 6$), very consistent with that of MCPT (52.08 ± 6.63%) and HCPT (45.98 ± 5.81%).

The stability of MCPT and HCPT was evaluated as described in the method section. Data shown in Table 3 suggest that no significant degradation occurred under the experimental conditions.

3.5. Application to a pharmacokinetic study

In this study, the mean plasma concentration–time curves of MCPT and its metabolite HCPT were quantified in six rats each receiving a single 5 mg/kg i.v. dose of MCPT *via* the tail vein. The curve and the pharmacokinetic parameters of MCPT and HCPT are shown in Fig. 7 and Table 4.

After i.v. administration, the level of MCPT reached the maximum concentration, and declined immediately. HCPT concentration reached the maximum within the first 5–10 min after administration, and declined promptly. This indicates a spontaneous conversion from MCPT to HCPT *in vivo*. As shown in Fig. 7, the concentration of HCPT was higher than that of MCPT, and the

Table 3
Stability of HCPT and MCPT in rat plasma samples ($n = 6$).

Experimental condition	Added, *C* (ng/mL)	MCPT		HCPT	
		Found, *C* ± S.D. (ng/mL)	Relative error (%)	Found, *C* ± S.D. (ng/mL)	Relative error (%)
Standard solutions 6 h at RT	2.5	2.39 ± 0.07	−4.47	2.39 ± 0.03	−4.29
	40	39.95 ± 0.48	−0.12	39.83 ± 0.45	−0.42
	160	158.73 ± 1.83	−0.80	159.06 ± 3.64	−0.59
Standard solutions 30 days at 4 °C	2.5	2.39 ± 0.04	−4.46	2.42 ± 0.14	−3.07
	40	39.93 ± 0.63	0.17	39.91 ± 0.61	−0.21
	160	159.17 ± 2.78	−0.52	159.58 ± 4.14	−0.26
QC samples 6 h at RT	2.5	2.32 ± 0.06	−8.80	2.80 ± 0.11	12.00
	40	36.82 ± 0.61	−7.95	36.57 ± 0.54	−8.58
	160	148.55 ± 5.10	−7.16	169.09 ± 3.23	−5.68
QC samples auto-sampler 24 h	2.5	2.38 ± 0.05	−4.98	2.38 ± 0.05	−4.68
	40	40.04 ± 1.2	0.09	39.71 ± 1.15	−0.72
	160	166.22 ± 4.69	3.89	165.40 ± 4.33	3.38
QC samples 30 days at −80 °C	2.5	2.42 ± 0.09	−3.02	2.41 ± 0.05	−3.64
	40	40.57 ± 0.83	1.43	40.27 ± 0.50	0.67
	160	162.63 ± 4.33	1.64	162.37 ± 4.65	1.48
QC samples 3 freeze–thaw cycles	2.5	2.36 ± 0.03	−5.45	2.34 ± 0.15	−6.02
	40	37.13 ± 3.58	−7.17	37.07 ± 2.04	−7.32
	160	156.19 ± 6.09	−2.38	155.61 ± 3.69	−2.74

Table 4
The pharmacokinetic parameters of MCPT and its major metabolite HCPT after i.v. administration with 5 mg/kg of MCPT.

Parameters	MCPT	HCPT
AUC_{0-24} (ng h/mL)	112.76 ± 26.45	184.63 ± 107.46
$AUC_{0-\infty}$ (ng h/mL)	157.99 ± 18.47	279.65 ± 118.08
C_{max} (ng/mL)	–	85.64 ± 57.70
T_{max} (h)	–	0.15 ± 0.04
$T_{1/2}$ (h)	14.24 ± 6.83	16.53 ± 9.21
MRT (h)	19.76 ± 8.64	23.68 ± 9.17

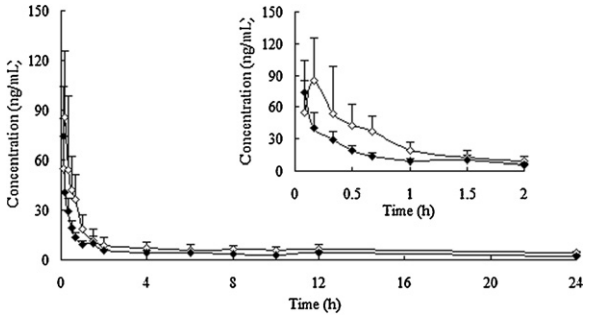

Fig. 7. Mean concentration *vs.* time profiles of MCPT (♦) and HCPT (◊) in rat plasma samples from four subjects each receiving a single dose of 5 mg/kg MCPT.

ratio of $AUC_{0-\infty}$ of HCPT to MCPT was 151.47%, demonstrating that formation of demethylated HCPT may be a dominant metabolic reaction for MCPT in rats. The ratios of AUC_{0-24} to $AUC_{0-\infty}$ were 71.37% for MCPT and 66.02% for HCPT. Both the elimination half-life ($T_{1/2}$) and the mean residence time (MRT) of HCPT were longer than that of MCPT, suggesting a longer residence time for HCPT *in vivo*.

4. Conclusion

10-Methoxycamptothecin (MCPT) and 10-hydroxycamptothecin (HCPT) were first isolated and identified at the same time, but few studies were conducted on MCPT while HCPT have been widely investigated. In this study, HCPT was identified as the major metabolite of MCPT in rat plasma through HPLC/PDA and LC–MS/MS analysis. A sensitive and reliable RP-HPLC method with fluorescence detection was developed and validated for the simultaneous analysis of MCPT and HCPT in rat plasma. A piecewise linear function was used to estimate both lower and higher concentrations. The calibration curves were linear ($r^2 > 0.999$) for MCPT and HCPT within concentrations of 1.25–20 ng/mL and 20–320 ng/mL. The intra- and inter-day assay data variations as well as the stability data showed no significant degradation under current experimental conditions. This method was successfully applied to the quantification of MCPT and HCPT in rat plasma even 24 h after i.v. administration. The mean plasma concentration–time curve and the pharmacokinetic parameters of MCPT and HCPT were calculated. The results showed that MCPT is mainly metabolized to demethylated HCPT in rats (the ratio of $AUC_{0-\infty}$ of HCPT to that of MCPT was 151.47%).

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