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Development and validation of a reverse-phase HPLC with fluorescence detector method for simultaneous determination of CZ48 and its active metabolite camptothecin in mouse plasma

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ABSTRACT

A simple and sensitive high-performance liquid chromatography (HPLC) assay for the analysis of CZ48, a potent anticancer candidate, and its active metabolite camptothecin (CPT) in mouse plasma was developed and validated. CZ44 was used as an internal standard (IS). The samples were injected onto a C18 Synergi Polar-RP column (4 μm, 150 mm × 4.60 mm) maintained at 30 °C. The identification of peaks showed high specificity. Shimadzu RF-10AXL fluorescence detector was used at the excitation and emission of 380 and 418 nm, respectively. The mean recoveries were 81.41 ± 0.035%, 86.00 ± 0.053% and 82.21 ± 0.020% for CZ48 and 76.01 ± 0.028%, 77.04 ± 0.042% and 85.93 ± 0.023% for CPT at three concentrations of 10, 100 and 900 ng/ml, respectively. The calibration curve was linear (r^2 = 0.9999) over CZ48 and CPT concentrations ranging from 5 to 1000 ng/ml and 10–1000 ng/ml (n = 6), respectively. The method had an accuracy of >95% and intra- and inter-day precision (RE%) of <1.2% and <2.2% for CZ48 and CPT, respectively, at three different concentrations (10, 100 and 900 ng/ml). The lower limit of quantification (LLOQ) using 0.1 ml mouse plasma was 10 ng/ml for CZ48 and 5 ng/ml for CPT. Stability studies showed that CZ48 and CPT were stable in mouse plasma after 4 h incubation at room temperature or after 1 month storage at −80 °C with three freeze/thaw cycles. The method reported is simple, reliable, precise and accurate and confirmed by the determination of plasma samples in the mice after oral administration of CZ48.

1. Introduction

Camptothecin (CPT) is a well-established topoisomerase 1 inhibitor against a broad spectrum of cancers [1,2]. The pharmacology and antitumor activity of the camptothecin analogues were reviewed by Garcia-Carbonero and Supko [3]. Several additional camptothecin analogues are in various stages of clinical development, including 9-aminocamptothecin, 9-nitrocamptothecin, 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(*S*)-camptothecin, exatecan mesylate, and karenitecin. However, poor aqueous solubility, instability, and toxic effects to normal tissues have limited CPT clinical development. With the continued study on the camptothecin analogues in the laboratory, the interesting findings revealed that the intact lactone ring of camptothecins is a crucial structure for their anticancer activity [4–6] and also for cytotoxicity [7]. Unfortunately, the lactone ring is unstable *in vivo* under physiological conditions at pH 7.4, and readily opens to yield the ring-opened carboxylate form [8], which has less antitumor activity as compared with the ring-closed lactone form [9]. To circumvent problems of rapid lactone hydrolysis, CZ48, a C20-propionate ester of camptothecin, was synthesized as a derivative resistant to lactone hydrolysis [10]. CZ48 has demonstrated strong anticancer activity against human tumor xenografts in nude mice with an exceptional lack of toxicity. CZ48 acts as a prodrug and exerts its anticancer activity by CE mediated hydrolysis to the active metabolite CPT *in vivo* [11].

To generate the preclinical pharmacokinetic data of CZ48 and CPT requires the development of an analytical method for CZ48 and CPT in biological matrices. Several methods have been reported for the extraction and detection of CPT and its analogues in biological fluids, such as high-performance liquid chromatography (HPLC) with fluorescence detection [12–17] and mass spectrometry [18–20]. However, to date, no simple HPLC method has been reported for CZ48 quantification at therapeutic concentrations in human plasma. The objective of the present investigation is

Abbreviations: CPT, camptothecin; CZ48, C20-propionate ester of camptothecin; C_{max}, maximum plasma drug concentration following a single dose; T_{max}, time to C_{max}; K_e, the elimination rate constants; AUC_{0-t}, area under the plasma drug concentration–time curve from time zero to the time t following a single dose; $AUC_{0-\infty}$, area under the plasma drug concentration–time curve from time zero to infinite time following a single dose; $t_{1/2\beta}$, elimination half-life for the terminal phase; MRT, mean residence time.

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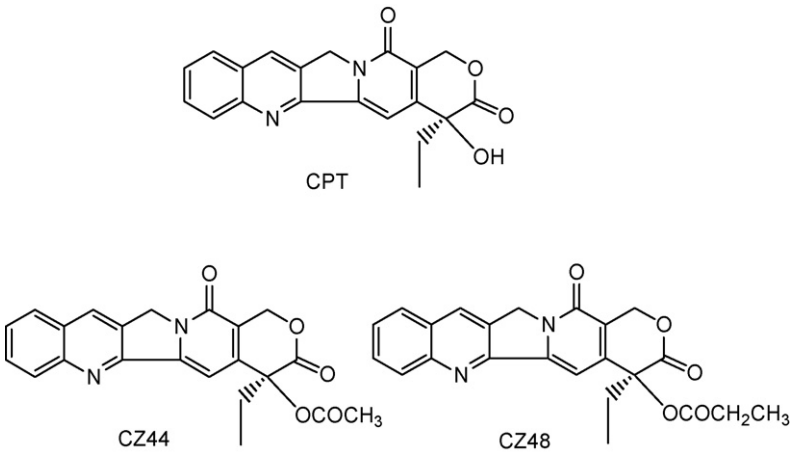

Fig. 1. Chemical structures of (A) CZ48, (B) CPT, and (C) CZ44.

to establish a fully validated HPLC method with a quantification limit sufficiently low to support pharmacokinetic and bioequivalence studies of CZ48 and its major metabolite CPT. The method reported in this paper is a simple, accurate HPLC method to quantify the plasma concentration of CZ48 and CPT with fluorescence detection using liquid–liquid extraction (LLE). This method is fully validated as per FDA guidelines [21]. Additionally, it provides information about the stability of CZ48 and CPT both in plasma and during sample processing (autosampler), which is a clear advantage for determining a large number of plasma samples for pharmacokinetic and bioequivalence studies *in vivo*. CZ44, camptothecin-20-*O*-acetate, was used as an internal standard (IS) when measuring CZ48 concentration by HPLC. The chemical structures of CPT, CZ48 and CZ44 are shown in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetic acid, dimethyl sulfoxide (DMSO), acetonitrile, dichloromethane and diethyl ether were obtained from Sigma–Aldrich (St. Louis, MO, USA). Chromatographic-grade water was produced by a Millipore Milli-Q system (Billerica, MA, USA). CZ48 and CZ44 were synthesized in-house by using the reported procedures [10]. Camptothecin (with a purity of 99%) was purchased from Harbin Foran High-Tech Development Co. (Harbin, Heilongjiang, China).

2.2. Instruments and analysis conditions

The conditions for HPLC analysis of CZ48 were established for the first time. The SHIMADZU Prominence HPLC with LC-20AT Solvent Delivery System was equipped with a SIL-20A auto sampler, and a Shimadzu RF-10AXL fluorescence detector (Columbia, MA, USA). The reverse-phase chromatography was performed on an analytical Phenomenex Synergi Polar-RP C18 column (4 μm, 150 mm × 4.60 mm, Torrence, CA, USA) protected with a Chrom Tech Security Guard TM cartridge system (Apple Valley, MN, USA). The optimized method used a binary gradient mobile phase with 0.1% acetic acid water as mobile phase A (pH 3.0) and acetonitrile as mobile phase B. A flow rate of 1.2 ml/min was used with a 20-μl injection volume. The time program of the gradient was listed in Table 1. Each injection was followed by a 3–5 min equilibrium time before the next injection. The eluted peaks were monitored at excitation and emission wavelengths of 380 and 418 nm, respectively. Shimadzu *LC solution* software was used for system control and data monitoring.

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

Stock solutions of CZ48 and CPT were prepared in DMSO and diluted to 200 μg/ml with acetonitrile. The stock solution of the IS was prepared in DMSO at a concentration of 200 μg/ml and diluted to 400 ng/ml with acetonitrile. Calibration curves were prepared by spiking 100 μl of the appropriate standard solution in 100 μl of blank mouse plasma. Effective concentrations in plasma samples were 10, 20, 50, 100, 200, 500 and 1000 ng/ml for CZ48 and 5, 10, 20, 50, 100, 200, 500 and 1000 ng/ml for CPT. The QC samples were pooled, at concentrations of 10, 100 and 900 ng/ml, as a single batch at each concentration, and then divided into aliquots that were stored in the freezer at −70 °C until analysis. The spiked plasma samples (standards and QCs) were then processed following the LLE procedure on each analytical batch along with the unknown samples.

2.4. Sample processing

A portion of 100 μl blank mouse plasma, spiked plasma or pharmacokinetics study plasma were transferred to a 2-ml test tube, and then 100 μl of IS working solution (400 ng/ml) was spiked. 200 μl of 1% acetic acid was added to the mixture and vortex mixed for 10 s. To the mixture was added 1 ml diethyl ether. After vortex mixing for 10 s at room temperature, incubating at room temperature for 10 min on a shaker at 10 rpm, and centrifuging at 10,000 × *g* for 15 min, the upper layer was transferred to a clean tube and evaporated to dryness using an evaporator at 40 °C under a stream of nitrogen. Then, the dried extract was reconstituted in 200 μl

Table 1
HPLC mobile phase gradient conditions for analysis of CZ48, CPT and IS

Time (min)	Flow rate (ml/min)	A%	B%	Duration (min)
0.00	1.2	80.0	20.0	0.0
2.00	1.2	80.0	20.0	8.0
10.00	1.2	50.0	50.0	5.0
15.00	1.2	20.0	80.0	1.0
16.00	1.2	0.0	100.0	1.0
17.00	1.2	80.0	20.0	1.0
18.00	1.2	80.0	20.0	0.0

Mobile phase A: water with 0.1% (w/v) acetic acid (pH 3.0) and mobile phase B: acetonitrile.

of water/acetonitrile (50/50, v/v diluent) and a 20-μl aliquot was injected into the chromatographic system.

2.5. Method validation

Plasma samples were quantified using the peak area ratios of CZ48 or CPT to that of the IS. Standard curves in the form of $y = A + Bx$, where y represents the plasma concentration of the analytes and x represents the ratio of the analytes peak area to that of the IS, were calculated using weighted $(1/\chi^2)$ least squares linear regression. To evaluate linearity, plasma calibration curves were prepared and analyzed in duplicate on 5 separate days.

The accuracy and precision were also determined by replicate analyses $(n = 6)$ of QC samples at three concentration levels on

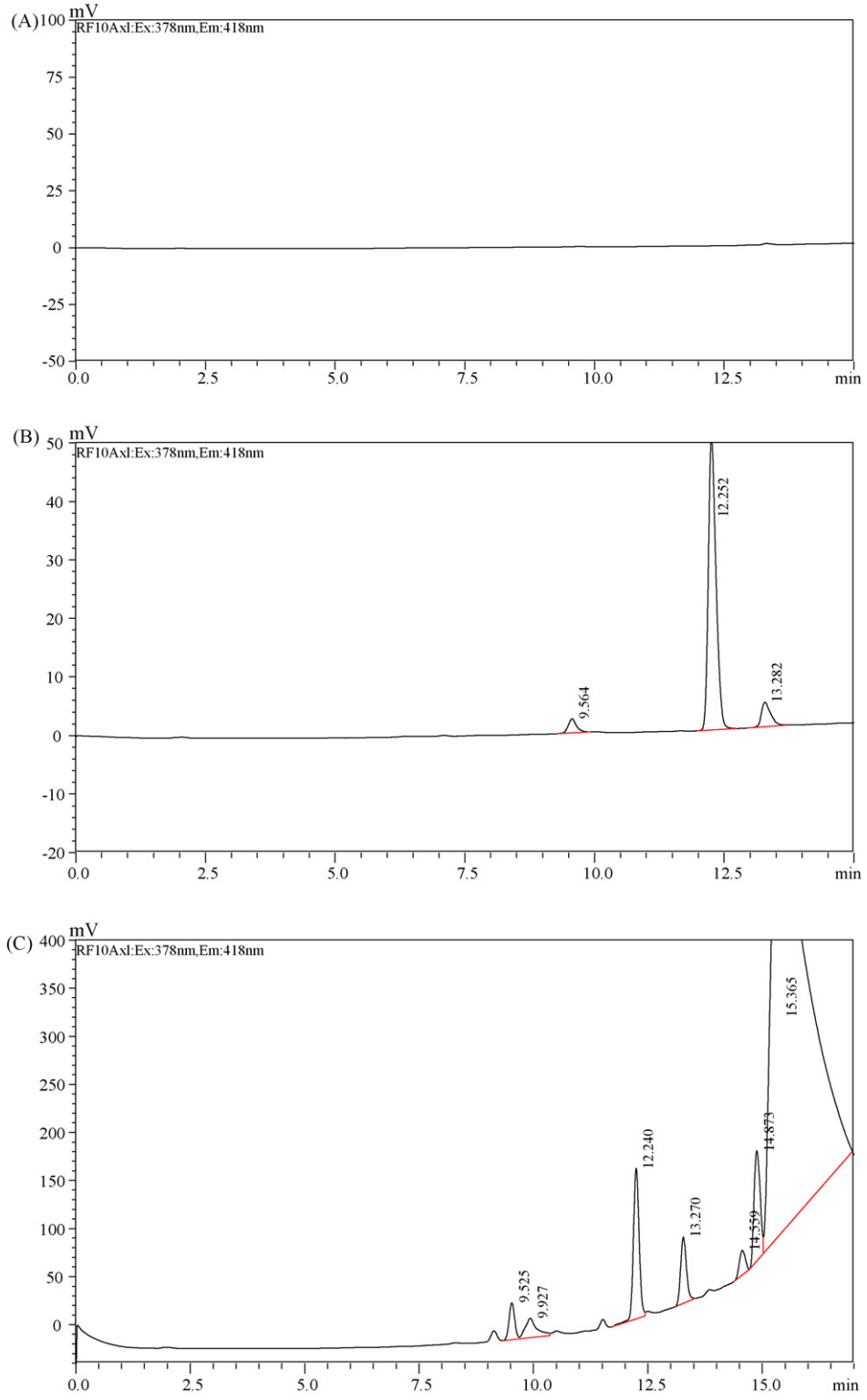

Fig. 2. Representative chromatograms of: (A) blank mouse plasma; (B) blank mouse plasma spiked with CZ48 and CPT at 10 ng/mL, and internal standard (IS) CZ44 (400 ng/mL); (C) mouse plasma sample at 1 h after oral administration of CZ48 at dose of 2 g/kg and spiked with IS.

three different validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision by the relative standard deviation (R.S.D.%). The concentration of each sample was determined using the calibration curve and analyzed on the same day.

The absolute extraction recoveries of CPT and CZ48 at three QC levels were evaluated by assaying the samples as described above and comparing the peak areas of the CZ48 and of the CPT with the IS, and then comparing with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

2.6. Stability

The short-term stability was examined by keeping replicates of the plasma QC samples at room temperature for 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h and refreezing for 12–24 h. Autosampler stability of CZ48 and CPT was tested by analysis of processed and reconstituted plasma QC samples, which were stored in the autosampler tray for 24 h. The stability of CZ48 and CPT in mouse plasma was tested after storage at or below −70 °C for 30 days. The stability of standard solutions was also tested at room temperature for 4 h and after refrigeration (4 °C) for 30 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of CZ48 and CPT after each storage period was related to the initial concentration determined for the samples that were freshly prepared and processed immediately.

2.7. Pharmacokinetic studies in mice

The concentrations of CZ48 and its major metabolite CPT in mouse plasma were determined by using this HPLC method. Groups of 48 healthy null mice were chosen for the pharmacokinetic study. Animal handling and procedures used were according to standard operating procedure approved by the institutional animal care and use committee. An oral formulation of CZ48 was prepared by mixing CZ48 in cottonseed oil (100 mg/ml) with 30 min sonication. After fasting 10 h, each mouse received 2 g/kg CZ48 orally by gavage. Serial blood samples (1 ml) from the heart were collected into sodium heparin-containing tubes before and at 0.5, 1.0, 2.0, 4.0, 5.0, 6.0, 8.0, 12.0 and 24.0 h time points after administration. Plasma was separated by centrifugation at 2000 × *g* for 10 min at 4 °C and stored frozen at −20 °C until analysis.

Pharmacokinetic parameters were determined from the plasma concentration–time data. The elimination half-life ($t_{1/2}$) was calculated with the non-compartmental model of TOPFIT program on a personal computer. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration point (t = 24.0 h) (AUC_{0-t}) was calculated by the linear trapezoidal method. Extrapolation to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$, where C_t is the last measurable plasma concentration and k_e is the elimination rate constant.

3. Results and discussion

3.1. Chromography

It was demonstrated that the determination of the analyte was not interfered with by endogenous substances in the plasma in chromatographic separation. In a binary gradient mobile phase with 0.1% acetic acid in water and acetonitrile, the plasma assay demonstrated good linearity between 10 and 1000 ng/ml for CZ48, and 5–1000 ng/ml for CPT. Under the present chromatographic conditions, the run time for each sample was 18 min. The retention times were 9.5, 12.1 and 13.23 min for CPT, IS, and CZ48, respectively. Fig. 2B represents chromatograms of CZ48, CPT and CZ44 from mouse plasma after LLE along with a blank plasma sample.

3.2. Preparation of plasma samples

The sample preparation step is critical for accuracy and sensitivity of the assay method. The most widely employed sample preparation methods are currently LLE, protein precipitation (PPT), and solid-phase extraction (SPE). PPT often provides higher recovery compared to LLE, especially for compounds having high polarity. However, an unidentified metabolite of CZ48 in the plasma had an interference with CPT if the PPT method was used in our study. The LLE procedure was thus used for the sample preparation in this investigation. Six organic solvents, *n*-hexane, ethyl acetate, diethyl ether, dichloromethane, isopropanol, *t*-butylmethylether and their mixtures in different combinations and ratios were evaluated. Diethyl ether was found to be optimal. This procedure gave a clean chromatogram for a blank plasma sample and yielded the highest recovery for the analytes from the plasma.

3.3. Method validation

3.3.1. Selectivity and specificity

Selectivity was assessed by comparing the chromatograms of six different batches of blank mouse plasma with the corresponding spiked plasma. Fig. 2 shows the typical chromatograms of a blank (A), a spiked plasma sample with CZ48, CPT and the IS (B), and a plasma sample from a mouse 2 h after an oral administration (C). There was no significant interference from endogenous substances observed at the retention times of the analytes. Typical retention times for CPT, CZ44 and CZ48 were 9.5, 12.1 and

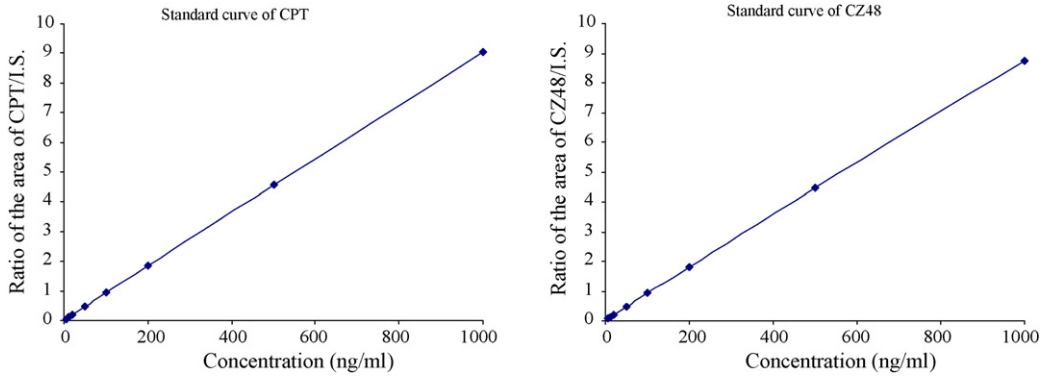

Fig. 3. Linearity curves of CZ48 and CPT.

Table 2
Accuracy and precision for the analysis of CZ48 in mice plasma ($n = 18$)

Added, C (ng/ml)	Found, C (ng/ml)	Inter-day, R.S.D. (%)	Intra-day, R.S.D. (%)	Relative error (%)
10	11.1	14.8	6.6	0.9
100	98.8	4.6	5.7	−1.2
900	899.1	1.1	1.0	−0.1

Table 3
Accuracy and precision for the analysis of CPT in mice plasma ($n = 18$)

Added, C (ng/ml)	Found, C (ng/ml)	Inter-day, R.S.D. (%)	Intra-day, R.S.D. (%)	Relative error (%)
10	10.1	7.3	6.3	1.2
100	102.2	4.6	5.7	2.2
900	905.0	2.3	2.2	0.6

Table 4
Stability of CZ48 and CPT plasma samples ($n = 3$)

Experimental condition	Added, C (ng/ml)	CZ48		CPT	
		Found, C (ng/ml) ± S.D.	Relative error (%)	Found, C (ng/ml) ± S.D.	Relative error (%)
Standard solution, 2 h at RT	10	9.5 ± 0.24	−5.5	10.0 ± 0.7	0.4
	100	101.9 ± 1.85	1.9	102.08 ± 1.5	2.1
	900	903.0 ± 7.7	0.3	900.3 ± 4.44	0.0
Standard solution, 30 days at 4 °C	10	9.3 ± 2.2	−8.0	10.0 ± 2.2	−0.2
	100	98.5 ± 0.8	−1.5	100.6 ± 1.0	0.6
	900	887.9 ± 3.9	−1.4	899.7 ± 4.3	−0.0
Autosampler samples, 24 h at 4 °C	10	11.1 ± 1.9	10.1	10.1 ± 0.2	0.8
	100	101.9 ± 1.6	1.9	100.6 ± 0.9	0.6
	900	903.3 ± 3.5	0.4	905.5 ± 4.3	0.6
Plasma sample, 30 days storage at −70 °C	10	9.9 ± 0.2	0.6	10.4 ± 0.7	3.8
	100	101.9 ± 1.8	1.8	102.1 ± 1.5	2.0
	900	897.0 ± 7.7	−0.3	902.3 ± 4.4	0.2
3 freeze–thaw cycles	10	8.9 ± 1.9	−12.1	9.4 ± 1.1	−6.7
	100	97.8 ± 1.6	−2.3	96.0 ± 0.9	−4.2
	900	887.5 ± 3.5	−1.4	893.2 ± 4.3	−0.8

13.23 min, respectively. Meanwhile, the specificity was verified by comparing retention times of CZ48, CPT and the IS CZ44 ($n = 10$). The differences were less than 1%.

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

Visual inspection of the plotted duplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges 10–1000 ng/ml for the CZ48 and 5–1000 ng/ml for CPT. The typical standard curve was $y = 0.0824 + 0.004033x$ for CZ48 and $y = 0.004537 + 0.00501x$ for CPT, where y represents the ratio of the peak area of the analytes to that of the IS and x represents the plasma concentrations of the analytes. Fig. 3 plotted the linear calibration curves for CZ48 and CPT over the concentration range in mouse plasma.

The LLOQ was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±15% and a precision below 15% were obtained. The present method offered LLOQ of 10 and 5 ng/ml in 0.1 ml plasma samples for CZ48 and CPT, respectively. Under the present LLOQ, the CZ48 and CPT concentrations in plasma samples can be determined even 24 h after administration. Thus, this method established is sensitive enough to investigate the pharmacokinetic behaviors of CZ48 and CPT and the relationship between dose and pharmacological effect in mice.

3.3.3. Precision and accuracy

Tables 2 and 3 summarize the results. The intra- and inter-day precision and accuracy for CZ48 and CPT were evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this assay, for each QC level of CZ48, the intra-day precision was 6.6% or less, and the inter-day precision was 14.8% or less. The accuracy was within ±1.2%. For each QC level of CPT, the intra-day precision was 6.3% or less, and the inter-day precision was 7.3% or less. The accuracy was within ±2.2%. The results above demonstrate that the values are within the acceptable range and the method is accurate and precise.

3.3.4. Recovery and stability

The recoveries determined at three concentrations (10, 100 and 900 ng/ml) for CZ48, were 81.41 ± 0.035%, 86.00 ± 0.053% and 82.21 ± 0.020% ($n = 3$), respectively, and for CPT were 76.01 ± 0.028%, 77.04 ± 0.042% and 85.93 ± 0.023% ($n = 3$), respectively.

All stability determinations were carried out also using 10, 100 and 900 ng/ml CZ48 and CPT plasma samples. No significant degradation occurred under all experimental conditions. Stability data are shown in Table 4.

3.4. Application of the method to a pharmacokinetic study

The mean plasma concentration–time curves of CZ48 and CPT are shown in Fig. 4. The main pharmacokinetic parameters of CZ48 and CPT in 48 mice were also calculated (Table 5). After oral administration, T_{max} of CZ48 and CPT were found to be 2.0 and 1.0 h, respectively. C_{max} values of CZ48 and CPT were 284.86 ± 85.55 ng/ml and 42.28 ± 6.72 ng/ml, respectively. Plasma

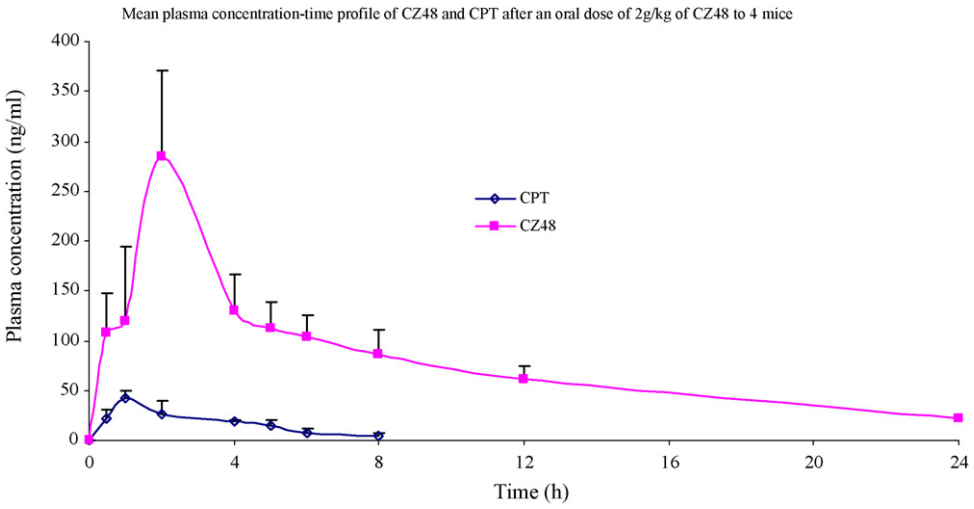

Fig. 4. Mean concentration vs. time profiles over 24 h of CZ48 and CPT in mouse plasma from four subjects each receiving a single dose of 2 g/kg CZ48.

Table 5
The pharmacokinetic parameters of CZ48 and its major metabolite CPT after oral administration with 2000 mg/kg of CZ48

Parameters	CZ48	CPT
AUC (0–8) (ng h/ml)	–	136.4 ± 26.9
AUC (0–24) (ng h/ml)	1927.7 ± 113.9	–
AUC (0–∞) (ng h/ml)	2233.4 ± 396.1	146.7 ± 34.8
C_{max} (ng/ml)	284.9 ± 85.6	42.3 ± 6.7
T_{max} (h)	2.0 ± 0.0	1.0 ± 0.0
$t_{1/2}$ (h)	8.7 ± 4.2	1.9 ± 0.6
K_e (1/h)	0.1 ± 0.0	0.4 ± 0.1
MRT (h)	11.6 ± 4.3	3.4 ± 0.9

concentrations declined with $t_{1/2}$ of 8.70 ± 4.18 h for CZ48 and 1.87 ± 0.63 h for CPT. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 1927.66 ± 113.92 ng h/ml and 2233.44 ± 396.05 ng h/ml for CZ48, and 136.43 ± 26.94 ng h/ml and 146.70 ± 34.76 ng h/ml for CPT, respectively.

4. Conclusion

A simple, sensitive, accurate and precise HPLC method was developed employing LLE for sample preparation. This method was convenient for the quantification of CZ48 and its major active metabolite CPT in mouse plasma samples and fully validated according to the FDA and ICH guidelines set forth for bioanalytical method validation. We also successfully applied this method to the determination of pharmacokinetic profiles of CZ48 and its metabolite CPT in null mice with a LLOQ of 10 and 5 ng/ml, respectively. This method will be useful for clinical pharmacokinetic studies in humans.

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