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Short communication

Determination of thalidomide by high performance liquid chromatography: Plasma pharmacokinetic studies in the rat

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Abstract

A sensitive and simple high performance liquid chromatography (HPLC) method was developed and validated for the determination of thalidomide in rat plasma. Chromatography was accomplished with a reversed-phase Hypersil C18 column. Mobile phase consisted of acetonitrile-10 mM ammonium acetate buffer (pH 5.50) (28:72, v/v), at a flow rate of 0.8 ml/min. Thalidomide was monitored by ultraviolet detector at 220 nm and it gave a linear response as a function of concentration over 0.02–50 μM. The limit of quantitation in rat plasma was 0.50 ng (0.02 μM plasma concentration) with an aliquot of 20 μl. Results from a 3-day validation study indicated that this method allows for simple and rapid quantitation of thalidomide with excellent accuracy and reliability. Using this validated assay, the effect of coadministered irinotecan (CPT-11) on the plasma pharmacokinetics of thalidomide in rats was determined. Coadministration of CPT-11 (intravenously, 60 mg/kg) increased the maximum plasma concentration (C_{max}) and area under the plasma concentration–time curve ($AUC_{0–10\,h}$) of thalidomide by 32.29 and 11.66%, respectively, as compared to the control, but none of the effect of CPT-11 was of statistical significance ($P > 0.05$). Concomitant CPT-11 also caused a 10.04% decrease in plasma clearance (CL) and 14.51% decrease in volume of distribution (V_d) ($P > 0.05$). These results suggest that coadministered CPT-11 did not significantly alter the plasma pharmacokinetics of thalidomide in rats. Further studies are warranted to explore the pharmacokinetic and pharmacodynamic interactions between CPT-11 and thalidomide.

Keywords: HPLC; Thalidomide; Pharmacokinetics

1. Introduction

In recent years, there is an increased use of oral thalidomide for the management of a variety of autoimmune-related diseases (e.g. erythema nodusum leprosum and Behcet's syndrome) and cancer such as relapsed multiple myeloma and renal carcinoma [1,2]. However, the modes of action, optimal dosing and chemotherapy regimen of thalidomide have not been identified. Anti-angiogenesis, induction of cytokines (in particular tumor necrosis factor-α) and immuno-modulating effects are considered to be the major rationale for the use of thalidomide as an anticancer agent [1,2]. Thalidomide is often used in combination with other cytotoxic agents aimed at producing additive/synergistic activity and alleviating toxicity. Animal studies indicated that thalidomide was able to potentiate the activity of a number of anti-cancer agents including melphalan [3], paclitaxel [4], and 5,6-dimethylxanthenone-4-acetic acid [5]. In clinical settings, thalidomide has been combined with an array of anti-cancer agents including carboplatin [6], cyclophosphamide [7,8], paclitaxel [6], and CPT-11 (irinotecan) [9].

Interestingly, coadministered thalidomide ameliorated the gastrointestinal toxicity and enhanced the anti-tumor activity of CPT-11 in colorectal cancer patients [9]. This was also accompanied by reduced conversion of CPT-11 to its cyto-

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toxic metabolite, SN-38, and increased SN-38 detoxification via glucuronidation [10]. CPT-11 is a DNA topoisomerase I inhibitor used as a first-line therapy in combination with 5-fluorouracil in the management of advanced colorectal cancer [11,12], but its clinical application is limited by severe and unpredictable delayed-onset diarrhea [13,14]. Both pharmacokinetic and pharmacodynamic mechanisms have been implicated for these thalidomide-drug interactions.

In the pharmacokinetic studies of thalidomide, chromatographic methods have been developed to quantify thalidomide in various biological matrices [1,15–18]. However, they often need complicated and time-consuming sample treatments [19,20] and analytical procedures [18], which may lead to marked hydrolysis and chiral inversion of thalidomide in these samples. Appropriate sample handling is necessary to reduce the large variability in pharmacokinetic studies of thalidomide in humans [21]. Although a few enantiomer-selective HPLC methods have been set up to separate enantiomers of thalidomide [22], chiral separation was not considered in many studies [23,24]. Thalidomide is a derivative of glutamic acid, containing two amide rings and a single chiral center [21]. The inter-conversion between the enantiomers of thalidomide is very rapid at physiological pH in aqueous medium and biological matrices such as plasma, undergoing rapid spontaneous hydrolysis [25]. Here, we developed a simple and sensitive HPLC method for the determination of thalidomide in rat plasma and applied it to the pharmacokinetic studies of combination of CPT-11 with thalidomide. Thalidomide may modulate the metabolism and transport of CPT-11 and thus alter its pharmacokinetics in vivo.

2. Experimental

2.1. Chemicals and reagents

Thalidomide was provided by Celgene Co. (MA, USA). CPT-11 was kindly supplied by Pharmacia (Kalamazoo, MI, USA). Ammonium acetate was purchased from Merck (NJ, USA). Phenacetin (used as internal standard, IS), carboplatin, cyclophosphamide, paclitaxel, diclofenac, vincristine, vinblastine, 5-fluorouracil, D-lactic acid, and D-sorbitol were obtained from Sigma Chemical (St. Louis, MO, USA). An injectable formulation of CPT-11 was prepared by dissolving CPT-11 (20 mg/ml), D-sorbitol (45 mg/ml), and D-lactic acid (0.9 mg/ml) in Milli-Q water heated to 70–90 °C for 5–10 min. The pH of this clear solution was adjusted to 3.5 by 1 M HCl. The resulting solution was sterile-filtered through a 0.22 μm membrane (Millipore, MA, USA) and stored at 4 °C protected from light until use [26]. The vehicle solution was prepared and sterilized in the same way without CPT-11. Methanol and acetonitrile were of HPLC-grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore). All other reagents were of analytical grades, which are commercially available.

2.2. Equipment and chromatographic conditions

A Shimadzu HPLC system was used to quantify thalidomide in rat plasma. The HPLC system consisted of a Shimadzu SCL-10A$_{VP}$ system controller, a LC-10AT$_{VP}$ pump, a DGU-14A degasser, a SPD-M10A$_{VP}$ diode array detector, and a SIL-10AD$_{VP}$ auto-injector. Data were monitored and analyzed using CLASS VP software. Separation of compounds was carried out at ambient temperature on a Hypersil ODS column (200 mm × 4.6 mm i.d., 5 μm) from Agilent Technologies, preceded by a Phenomenex C18 guard column. The mobile phase (delivered at a flow rate of 0.8 ml/min) consisting of acetonitrile-10 mM ammonium acetate buffer (pH 5.50) (28/72, v/v) was filtered through a 0.45-μm membrane and degassed before use. The eluted peaks were monitored at 220 nm.

2.3. Standard samples

Stock solutions (1 mM) of thalidomide were freshly prepared in dimethyl sulfoxide (DMSO). Working solutions (0.2–500 μM) of thalidomide were then prepared by diluting stock solutions with methanol. All stock and working solutions were freshly prepared. IS stock solution (2.79 mM) was prepared by dissolving 5.00 mg phenacetin in 10 ml methanol and stored at −20 °C.

Plasma standards were prepared by adding 10 μl of the appropriate working solution and 100 μl blank plasma into a tube containing 10 μl IS and mixed by voltexing for 10 s. Typical standard samples contained thalidomide ranging from 0.02 to 50 μM. Plasma proteins were then precipitated and thalidomide was kept stable by the addition of 200 μl ice-cold acetonitrile/methanol (1:1, v/v) containing 2% (v/v) acetic acid [21]. After rigid vortex-mixing for 1 min, the mixtures were centrifuged at 1500 × *g* for 10 min. An aliquot of 20 μl supernatant was injected onto the column for analysis. Similar procedures were performed for plasma samples collected from kinetic studies except that 10 μl of the appropriate working solution was replaced by the same volume of methanol.

2.4. Calibration curves

The calibration curves over concentration range of 0.02–50 μM were constructed by plotting the peak area ratio of the analyte over IS versus the concentrations spiked. Nine concentration points (0.02, 0.05, 0.1, 0.25, 1, 2.5, 10, 25, and 50 μM) were used to obtain the linearity and independent calibration curve was constructed during each run of experiment. The equations were calculated using linear regression. The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis. Concentrations in unknown samples were obtained from the resulting peak area ratios and the regression equa-

tion of the calibration curve using back calculation. The limit of quantification was defined as the lowest drug concentration that could be determined with a coefficient of variation (C.V.) ≤20% and a recovery of 100 ± 20% on a day-to-day basis.

2.5. Method of validation

All validation runs were performed on 3 consecutive days and all samples used for validation were prepared as standard samples. Six different plasma concentrations (0.02, 0.1, 0.5, 2.5, 10, and 50 μM) of thalidomide were investigated for recovery, whereas phenacetin (IS) was measured at the concentration used in sample preparation. The recovery was determined by comparing the peak areas of plasma samples with those replaced by an equal volume of phosphate-buffered saline at pH 7.4 after the same sample handling. Intra-day and inter-day precision and the mean accuracy were determined by repeated analysis ($n = 5$) of thalidomide at six different concentrations on a single day and on 3 consecutive days, respectively.

2.6. Stability of thalidomide in rat plasma

The different pH values of rat plasma (pH 7.4, 7.0, and 6.0) were achieved by adjusting the pH using 0.1 M HCl. Freshly prepared stock thalidomide (5 mM) was made using DMSO and added to 3.3 ml rat plasma to obtain 50 μM thalidomide (DMSO final concentration 1%, v/v). The rat plasma with thalidomide was vortexed for 10 s, incubated at 37 °C over 24 h. At indicated time points (0, 1, 2, 3, 4, 5, 6, 7, 12, and 24 h), an aliquot (100 μl) of the rat plasma was collected in triplicate and processed as standard samples. In addition, the stability of thalidomide in DMSO at 4 °C was determined by monitoring thalidomide concentration every day over 1 week. Thalidomide was determined by HPLC followed by back calculation with the calibration curves.

2.7. Pharmacokinetic studies in rats

Healthy male Sprague–Dawley rats (200–230 g), purchased from the Laboratory Animals Centre, National University of Singapore, Singapore, were used in the kinetic studies and all animal procedures were approved by the Animal Ethical Committee of the National University of Singapore. Rats were randomized to two groups ($n = 5$ for each group) receiving thalidomide (100 mg/kg, i.p.) alone or in combination with CPT-11 (60 mg/kg, i.v.) with free access to food and water before and after the administration of thalidomide. Thalidomide was dissolved in DMSO and injected (1 μl/g body weight) 30 min following CPT-11 administration. Control rats received thalidomide in combination with the vehicle solution for CPT-11 injectable formulation. Blood samples were collected by snipping the tail vein at 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h following drug administration using heparinized tubes. Plasma was obtained by immediate centrifugation at 1500 × g for 10 min at 4 °C. A 100-μl aliquot of the plasma was processed as described above in Section 2.3. The supernatant after protein precipitation was transferred to a clean polypropylene tube and an aliquot (20 μl) of the solution was injected into HPLC for analysis. All samples were put on ice to minimize the degradation of thalidomide before injection. The plasma concentration of thalidomide was then determined by HPLC.

2.8. Pharmacokinetic calculations

Pharmacokinetic parameters were calculated by standard model-independent pharmacokinetic formulae using WinNonlin® program (Scientific Consulting Inc., NC, USA). $AUC_{0–10\,h}$ was calculated using the log trapezoidal rule without extrapolation to infinity, whereas $AUC_{0–\infty}$ was calculated by the following equation:

$$AUC_{(0–\infty)} = AUC_{(0–10\,h)} + \frac{C_{10\,h} \times t_{1/2\beta}}{\ln 2}$$

where $C_{10\,h}$ is the plasma thalidomide concentration at last time point (10 h). The residual area was extrapolated using the elimination constant that was estimated by linear regression analysis of the terminal slope of $\log_e$ plasma concentration–time curve. The elimination half-life $t_{1/2\beta}$ was calculated as $0.693/\beta$, where β is the elimination rate constant calculated from the terminal linear portion of the plasma $\log_e$ concentration–time curve. V_d was estimated as dose/($\beta \times AUC_{0–\infty}$). The total plasma clearance (CL) was calculated as the total administered dose/$AUC_{0–\infty}$ assuming 100% bioavailability by this route.

2.9. Statistical analysis

Data are presented as mean ± S.D. Statistical significance was assessed using a standard unpaired Student's t-test.

3. Results and discussion

Representative chromatograms for thalidomide are shown in Fig. 1. Under the chromatographic conditions used for the analysis, the retention times for thalidomide and IS were 7.6 and 9.5 min, respectively. This method employed simple liquid−liquid extraction [21] procedure as an alternative for solid-phase extraction [27], and resulted in extraction efficiency (recovery) of >90% at concentrations of 0.02–50 μM. No concentration dependence was observed. The liquid–liquid extraction method is basically derived from our published study [21] in which a high (>95%) extraction efficiency was obtained for thalidomide at 0.05–100 μM in cell culture medium. The recovery of the IS, determined at the concentration used, also by comparing with that in phosphate-buffered saline was 90.4 ± 7.9% ($n = 5$). Matrix-specific interfering peaks that required modification of the mobile phase composition were not observed in any cases,

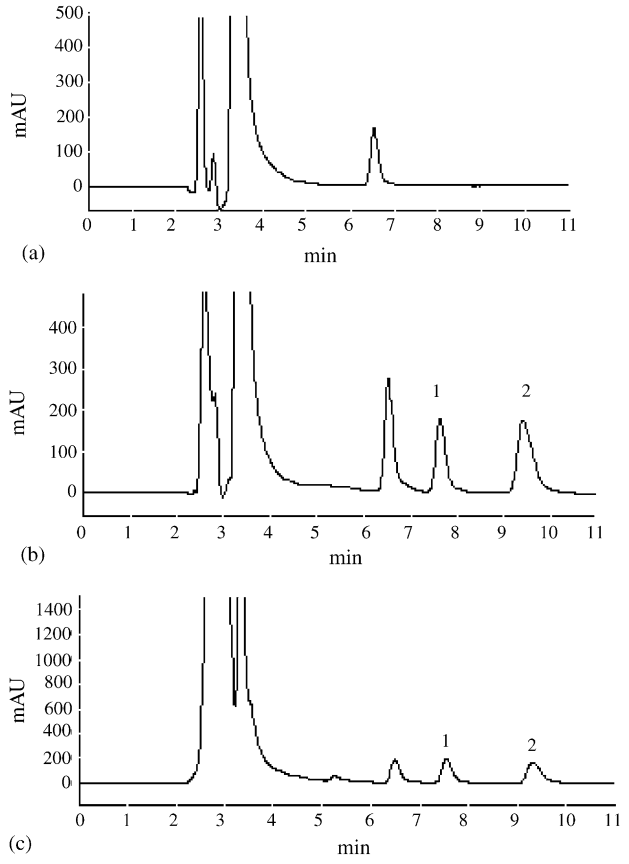

Fig. 1. Representative chromatograms of blank rat plasma (a) plasma spiked with thalidomide and IS (b) and plasma from a rat receiving 60 mg/kg CPT-11 and 100 mg/kg thalidomide (c). Under the chromatographic conditions used for the analysis of thalidomide, the retention times for thalidomide (peak 1) and IS (peak 2) were 7.6 and 9.5 min, respectively.

including in the presence of CPT-11, SN-38, carboplatin, cyclophosphamide, paclitaxel, diclofenac, vincristine, vinblastine, or 5-fluorouracil. All these compounds did not show any UV absorbance at 220 nm and thus generated no interfering peaks for thalidomide.

Appropriate handling of biological samples containing thalidomide is crucial to avoid degradation. The degradation rate of thalidomide is dependent on pH and temperature [23,28,29], and thus the degradation half-life of thalidomide in plasma, blood, or other biological samples at pH 7.4 is similar. As shown in Fig. 2, thalidomide was unstable in rat plasma at pH 7.0 and 7.4 with degradation half-lives of 9.0 and 3.2 h, respectively. Thalidomide degraded rapidly with <13% remaining after 24 h incubation in rat plasma at pH ≥ 7.0. Within 2 h, about 10 and 15% of thalidomide was hydrolyzed at pH 7.0 and 7.4, respectively. However, no significant degradation of thalidomide was observed over 24 h when rat plasma pH value was decreased to 6.0 with about 92% thalidomide remaining. In acetonitrile/methanol (1:1, v/v) containing 2% (v/v) acetic acid (pH 4.5) at 4 °C, thalidomide was stable over 1 week with <5% degradation. Thalidomide was shown to be stable in DMSO over 1 week at 4 °C

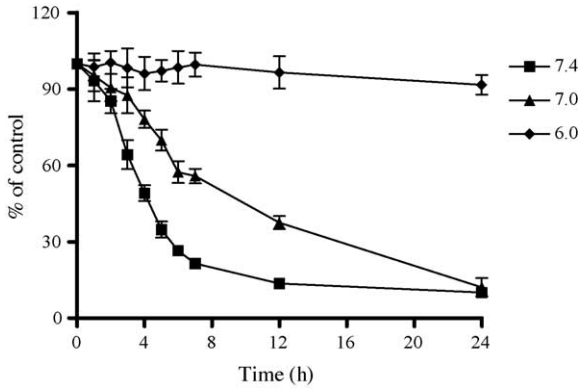

Fig. 2. Stability of thalidomide in rat plasma at pH 6.0, 7.0, and 7.4.

with 92% remaining. Acidifying and quick chilling of the plasma samples by ice-cold organic solvents with 2% acetic acids (to pH 4.5) were conducted in this study to prevent the spontaneous degradation of thalidomide. This would provide sufficient protection to the thalidomide against degradation during analysis and under storage conditions at −20 °C.

Calibration curves were linear over the concentration range of 0.02–50 μM with the equation of $y = (0.0100 \pm 0.0006)x - (0.0031 \pm 0.0003)$, $r^2 = 0.9994$ ($n = 5$), where x is the plasma concentration of thalidomide and y is the area ratio of thalidomide over IS. The limit of quantitation in rat plasma for thalidomide was 0.50 ng (about 0.02 μM in rat plasma) when an aliquot of 20 μl was injected onto HPLC. The validation data in terms of intra-day and inter-day precision and accuracy are represented in Table 1. The differences between the theoretical and the actual concentration and the coefficient of variation were less than 15% at any quality control sample concentrations.

In this study, we developed and validated a simple and sensitive HPLC method for the determination of thalidomide in rat plasma based on our previously published method in which thalidomide was determined in transport buffer for Caco-2 cells [21]. During the development of the present

Table 1
Intra-day ($n = 5$) and inter-day ($n = 3 \times 5$) precision and accuracy of the HPLC determination of thalidomide in rat plasma

Concentration (μM)	No. of samples (n)	C.V. (%)	Accuracy (%)
Intra-day			
0.02	5	4.6	90.5
0.1	5	3.1	102.6
0.5	5	6.3	93.7
2.5	5	3.2	105.2
10	5	3.5	102.3
50	5	6.1	104.5
Inter-day			
0.02	15	3.6	89.0
0.1	15	4.1	105.8
0.5	15	7.2	92.8
2.5	15	2.0	105.5
10	15	7.3	95.1
50	15	2.1	102.2

Table 2
Pharmacokinetic parameters of thalidomide in rats treated with thalidomide alone or in combination with CPT-11 ($n = 5$ for each group)

Parameter	Treatment		% Change	P-value*
	CPT-11 + thalidomide	Thalidomide alone		
Dose/kg	100 mg (258 μmol)	100 mg (258 μmol)	–	–
C_{max} (μM)	25.72 ± 10.00	19.44 ± 7.00	+32.29	0.28
T_{max} (h)	2.00 ± 0.00	2.00 ± 0.00	0	–
$AUC_{0-10\,h}$ (μM h)	149.02 ± 52.58	133.46 ± 39.17	+11.66	0.61
V_d (l/kg)	11.88 ± 3.44	13.89 ± 7.30	−14.51	0.59
β (1/h)	0.19 ± 0.03	0.20 ± 0.06	−3.43	–
$t_{1/2\beta}$	3.71 ± 0.69	3.75 ± 1.00	0.89	0.95
$AUC_{0-\infty}$ (μM h)	186.74 ± 56.56	165.10 ± 37.55	13.10	0.50
CL (l/(h kg))	2.24 ± 0.71	2.49 ± 0.79	−10.04	0.61

* Compared to the controls using unpaired t-test.

HPLC method, there were concerns about the extraction of thalidomide from rat plasma, stability of thalidomide in rat plasma, sensitivity and selectivity, in a sense that plasma is a much more complicated matrix than transport buffer. To simplify the method development, we employed the same sample extraction and processing procedure, mobile phase, and measures for stabilizing thalidomide as those published previously [21]. The analytical column dimension in this study was 200 mm × 4.6 mm instead of 150 mm × 4.6 mm as employed previously by us for thalidomide in transport buffer. Thus, about 2.3 min longer retention time for thalidomide was observed in the current study. Despite this, our developed method still has shorter retention times of thalidomide and IS than those reported previously by other investigators [25,30]. The short retention time makes it feasible for the determination of a labile compound like thalidomide, which undergoes rapid hydrolytic degradation under physiological pH. The LOQ value (0.50 ng, about 0.02 μM) in this study was slightly lower than that (0.625 ng, namely 0.025 μM) reported in our previous study [21]. However, this value was much lower than those (4.44 and 2.0 ng, respectively) reported by Torano et al. [31] and Heney et al. [30]. Lower LOQ may be important for kinetic studies of thalidomide, as the low concentration range over 0.05–0.5 μM can be encountered when plasma is sampled over 24 h. However, a drawback of our HLPC method reported here was lack of chiral separation. In vivo study indicates that *R*- and *S*-thalidomide had faster oral absorption than racemate when given separately [23].

We applied this method to the study of plasma pharmacokinetics of thalidomide in rat. Fig. 3 shows representative plasma concentration–time profiles in rats receiving thalidomide alone and in combination with CPT-11. Coadministration of CPT-11 (60 mg/kg, i.v.) increased the C_{max} and plasma $AUC_{0-10\,h}$ of thalidomide by 32.29 and 11.66%, respectively, as compared to the control, but none of the effect of CPT-11 was of statistical significance ($P > 0.05$) (Table 2). Concomitant CPT-11 also caused a 10.04% decrease in plasma CL and 14.51% decrease in V_d ($P > 0.05$). These results indicate that coadministered CPT-11 did not alter the plasma pharmacokinetic of thalidomide in rats. This is not surprising, as spontaneous hydrolysis is the major elimination pathway of thalidomide, while cytochrome P450 (CYP2C)-mediated metabolism [32] plays only a minor role in its elimination. The V_d of thalidomide was insignificantly altered in rats receiving combination therapy compared to those receiving thalidomide alone; this is further supported by our recent in vitro studies where CPT-11 and its active metabolite, SN-38, did not influence the binding of thalidomide to rat plasma [33]. Although CPT-11 and SN-38 are 80 and 99% [34] bound in blood, respectively, thalidomide is not extensively bound to blood or plasma components, with 55–66% [29] of plasma protein binding observed.

The study at our laboratory has demonstrated that coadministered thalidomide significantly increases the AUC of CPT-11 and decreased the AUC of SN-38 [33]. This may partially explain the finding that coadministered thalidomide reduced the gastrointestinal toxicity of CPT-11. The reason for the reduced plasma SN-38 levels by thalidomide is unknown, but inhibition of carboxylesterases and modulation of glucuronidation of SN-38 by thalidomide and its metabolites are likely. In addition to pharmacokinetic mechanism, pharmacodynamic component may also play an important role in the protective effect of thalidomide on CPT-11-induced diarrhea. Studies are ongoing at our laboratory to examine the

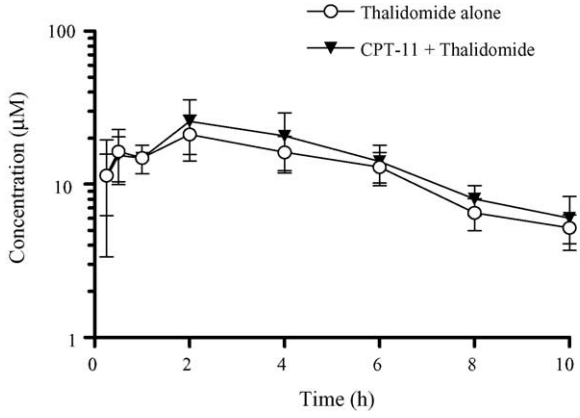

Fig. 3. Plasma concentration–time profiles for thalidomide in rats treated with thalidomide alone or in combination with CPT-11.

detailed pharmacokinetic and pharmacodynamic interactions between thalidomide and CPT-11 in rats.

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