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Reversed-phase high-performance liquid chromatography method for the simultaneous quantitation of the lactone and carboxylate forms of the novel natural product anticancer agent 10-hydroxycamptothecin in biological fluids and tissues

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Abstract

Camptothecins are indole alkaloids isolated from a Chinese tree, *Camptotheca acuminata*, and have a wide spectrum of anticancer activity in vitro and in vivo. A novel camptothecin congener 10-hydroxycamptothecin (HCPT) has been shown to be more active and less toxic than camptothecin, and the lactone HCPT is believed to be responsible for its anticancer activity. In the present study, a reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection was developed and validated for the simultaneous analysis of HCPT for lactone form (I) and carboxylate form (II) in plasma, urine and feces and tissues. Biological samples were prepared by a liquid–liquid extraction method using ice-cold methanol–acetonitrile (1:1, v/v). This method was shown to be reproducible and reliable, with intra- and inter-day variations being less than 7%, and accuracy being 94.3%–102.7%. The limits of determination were 2 ng/ml, 2 ng/ml, 2 ng/g, and 10 ng/ml for HCPT forms I and II in rat plasma, urine, feces, and tissues, respectively. The assay was liner over the range 2–2000 ng/ml (r=0.999, P<0.001) with recoveries of greater than 90% for plasma and urine and approximately 70–80% for feces and tissue homogenates through the extraction procedure. This analytic procedure has been successfully applied to a pharmacokinetic study of HCPT in experimental animals and should be useful in the future human studies.

Keywords: 10-Hydroxycamptothecin

1. Introduction

Water-insoluble camptothecins (CPT, Fig. 1) are indole alkaloids produced by a Chinese tree, *Camptotheca acuminata*, and have been shown to be a very promising class of anticancer agents [see Ref. [1], and references therein]. The anticancer activity of CPT was reported as early as 1966 [2]. However, the earlier clinical trials using its highly water-soluble sodium salt, CPT-Na^+, showed minimal anticancer activity and several unpredictable side effects such as myelosuppression, hemorrhagic cystitis, diarrhea, nausea, vomiting, and dermatitis [1]. Later, CPT-Na^+ was shown to be the product of opening the lactone ring of CPT (carboxylate form) and responsible for the decreased therapeutic effects and increased toxicity compared to its lactone form. In the late 1980s and early 1990s, DNA topoisomerase

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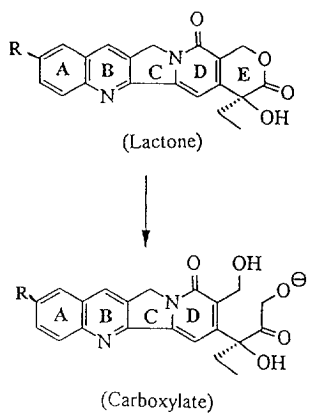

Fig. 1. Chemical structure of camptothecins. CPT: R=H; HCPT: R=OH.

I was demonstrated to be the major target of natural CPT and several semi-synthesized CPT analogues, including irinotecan (or CPT-11) and topotecan. The interest in CPT congeners as anticancer agents has now been renewed with increasing preclinical and clinical studies being conducted. It has clearly been demonstrated that the carboxylate CPT is much less active than the lactone form. Carboxylate CPT binds to human serum albumin with 200-fold higher affinity than lactone CPT, which may facilitate the conversion of lactone CPT to its carboxylate form [3]. Therefore, it is very important to separate and quantitate the two forms of CPT congeners in preclinical and clinical evaluations of the class of anticancer agents.

Compared to water-soluble CPT analogues, fewer studies have been conducted with water-insoluble CPT and its natural analogues. Recently, water-insoluble CPT analogues have been shown to have a wide spectrum of anticancer activity with more potent activity than water-soluble CPT analogues [1]. Several clinical trials using natural CPT have been initiated. One of the natural CPT analogues, 10-hydroxycamptothecin (HCPT), has been shown to have a strong antitumor activity against gastric carcinoma, hepatoma, leukemia, and tumor of head and neck in clinical trials, and, more importantly, HCPT is more potent and less toxic in experimental animals and in human trials compared to CPT [4]. HCPT has now been entering human clinical evaluations, mainly in China. Although the mechanisms responsible for the HCPT anticancer activity are not fully understood, this drug has been demonstrated to be a DNA topoisomerase I inhibitor with specific S-phase cell killing effect [5].

In our preclinical pharmacologic studies of HCPT, in comparison with CPT, we paid more attention to isolate and quantitate carboxylate and lactone forms of HCPT as well as its major metabolites in biological fluids and tissues. To this end, we developed the procedure to extract and analyze the biological samples with a reversed-phased HPLC system. The methods have been successfully used in the pharmacokinetic studies of HCPT in experimental animals. We believe that this procedure will be useful in the future human studies with this novel anticancer drug.

2. Experimental

2.1. Materials

Camptothecin and 10-hydroxycamptothecin were obtained from Midwest (Beijing, China) and the purity for each drug was determined by LC–MS to be greater than 98%. Tetrabutylammonium dihydrogen phosphate (TBAP) was purchased from Waters (Milford, MA, USA) as a ready-for-use solution PIC-A (WAT 08510). All other reagents were of analytical grade, and double-distilled water was used throughout. All solutions used in HPLC analysis were filtered and degassed using a 0.2-μm membrane filter (Gelman Sciences, Ann Arbor, MI, USA) with a filtration system (Millipore, MA, USA).

2.2. Chromatography

The chromatographic system comprised of an HP 1050 HPLC (Hewlett Packard, Palo Alto, CA, USA) with computer-controlled solvent delivery system and an FD 300 Dual Monoch Romator fluorescence detector with an omniscribe recorder. The analytical column (250×4.6 mm I.D.) was reversed-phase C_{18} (Jones Chromatography, Littleton, CO, USA) coupled with inline guard column (RP-18, 5 μm, Lichrospher 100, EM Sciences, Gibbstown, NJ, USA). The mobile phase was 0.075 *M* ammonium acetate buffer (pH 6.4)–acetonitrile (70:30, v/v) to

which PIC-A solution (25 ml/l) was added. The final concentration of TBAP was 5 m*M*. The column was eluted at the flow-rate of 0.8 ml/min, and eluent was monitored spectrofluorometrically with the excitation wavelength at 363 nm and the emission wavelength at 550 nm for HCPT.

2.3. Preparation of standards

Stock solutions of CPT and HCPT (5 mg/ml) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until the use for serial dilution in preparation of standard curves. Standards of both lactone and carboxylate forms of CPT and HCPT were prepared by a modification of the methods described by Rivory and Robert [6], which was used for preparation of CPT standards in their studies. The lactone forms were prepared in acetonitrile–0.01 *M* citric acid (pH 3) (50:50, v/v), and the carboxylate forms were prepared in acetonitrile–0.01 *M* sodium tetraborate (pH 9) (50:50, v/v). CPT and HCPT standards in biological samples (plasma, urine, and feces and tissue homogenates) were prepared by addition of appropriate standard solution to the biological samples in a 100-μl final volume. In brief, the stock solutions of both lactone and carboxylate forms of standard HCPT (3000 ng/ml) were added to plasma, urine, feces and tissue homogenates in final concentrations of 2, 20, 50, 100, 1000, and 2000 ng/ml. These samples were rapidly vortexed and the proteins were precipitated by addition of 100 μl of cold methanol–acetonitrile (1:1, v/v) (−20°C). Following vortex-mixing for 10 s and centrifugation at 9000 *g* for 5 min, the clear supernatant was removed, and filtered prior to injection onto the HPLC column. In the present study, a standard curve for each form (20–2000 ng/ml) was run on a daily basis.

2.4. Preparation of biological samples

Biological samples were obtained at various times from rats following an intravenous administration of HCPT at a dose of 10 mg/kg body weight. Blood samples were centrifuged immediately and the resulting plasma (100 μl) was removed, mixed with 100 μl cold methanol–acetonitrile (1:1) for 20 s, and then centrifuged at 9000 *g* for 5 min. The supernatant was stored at −20°C until analysis. Urine samples (100 μl) collected at scheduled intervals were also added to 100 μl cold methanol–acetonitrile, vortexed and centrifuged at 9000 *g* for 5 min. The supernatant was diluted by HPLC mobile phase buffer prior to injection onto the column. Feces samples were added to physiological saline (0.9% NaCl; 1:5, w/v) overnight, homogenized, and centrifuged at 9000 *g* for 30 min, and the resulting supernatant was used for further analysis. Various tissue samples were homogenized in physiological saline (0.9% NaCl; 1:5, w/v, pH 7.4), and the resulting homogenates (200 μl) were incubated with 200 μl of extraction buffer containing 10% SDS, 5 *M* NaCl, 1 *M* Tris (pH 7.6), 0.5 *M* EDTA and proteinase K (2 mg/ml) at 37 °C for 2 h and then extracted using the methods for plasma sample preparation. Bone marrow samples were obtained from flushing the femurs with ice-cold physiological saline. The harvested bone marrow cells were suspended in 1 ml of physiological saline and lysed by sonicating 5 times for periods of 10 s and then extracted using the methods for plasma sample preparation.

2.5. Method validation

The recovery of HCPT from plasma, urine and feces at three concentrations (20, 100, 1000 ng/ml) were determined by comparing the peak heights of pure standards with those of extracted samples to which the same amount of standards were added. Six replicates were run with four concentrations of HCPT ranging from 20–1000 ng/ml for both lactone and carboxylate forms to determine the intra-day reproducibility. Six replicates with four different concentration (20, 50, 100, 1000 ng/ml) were analyzed four times (4 days) in a week to determine the inter-day reproducibility. The precision of the method at each concentration was calculated as the relative standard deviation (R.S.D.). The accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the spiked concentration. The detection limit was defined as the peak signal of HCPT equal to three times the average noise level.

2.6. Single dose pharmacokinetic study

Male Sprague–Dawley rats weighing 280±10 g each (Harlan, Indianapolis, IN, USA) were administered HCPT dissolved in DMSO at an i.v. bolus dose of 10 mg/kg body weight. Blood samples were drawn at various times, and urine and feces samples were collected using a rat metabolic cage for each animal. At the end of experiment, animals were killed by pentobarbital sodium overdose. Various tissues including liver, kidney, and bone marrow were removed and used for further analyses.

3. Results and discussion

3.1. Separation of HCPT from CPT

HCPT used in the present study is extracted from a Chinese tree, *Camptotheca acuminata*, with minimal other CPTs being impurity (less than 2%). LC–MS analyses indicated that the HCPT used in the present study included 1.5% CPT (data not shown). Because HCPT, with its substitute hydroxy group on the 10-position, has different water solubility compared with CPT, the cationic ion-pairing reagent TBAP made it possible to completely separate HCPT from CPT under the present chromatographic conditions (Fig. 2). Like all known active derivatives of CPT, the lactone form of HCPT is reversibly hydrolyzed to less active (or inactive) carboxylate form. The intact lactone moiety of HCPT is important to its anticancer activity. The carboxylate form of HCPT is only minimally active on topoisomerase I. Therefore, it is essential to quantitate both species in biological samples for proper interpretation of pharmacokinetic-pharmacodynamic data of this class of drugs [7]. The method developed in the present study meets the needs of simultaneous determination of lactone and carboxylate forms of HCPT. A representative chromatogram of lactone forms and carboxylate forms of HCPT and CPT is shown in Fig. 2. In the HPLC analysis, standard CPT and HCPT were mixed prior to injection. The camptothecins show native fluorescence that can be utilized for the detection in HPLC. On-line scanning experiments provided the optimal excitation (363 nm) and emission (550 nm) wavelengths in the mobile phase for HCPT. Under these conditions, it is 10 times more sensitive for detection of HCPT than for CPT.

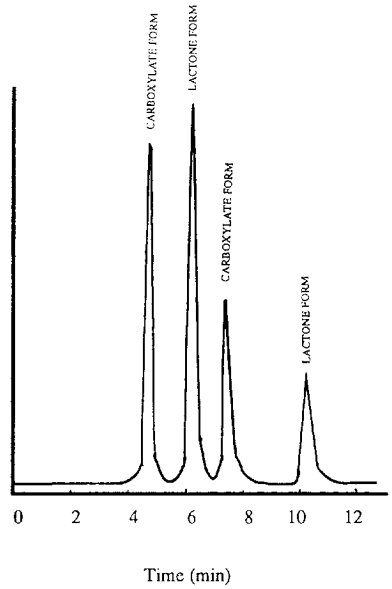

Fig. 2. Chromatogram of HCPT and CPT. Chromatographic conditions: mobile phase, 0.075 *M* NH_4AC–acetonitrile–PIC A (70%/30%/0.005 *M*); flow-rate, 0.8 ml/min; column, 250×4.6 mm I.D., RP-18 coupled with inline guard column. Fluorescence detection wavelengths were set at 363 nm (excitation) and 550 nm (emission). Retention times: carboxylate HCPT, 4.5 min; lactone HCPT, 6.2 min; carboxylate CPT, 7.5 min; lactone CPT, 10.3 min. Concentrations used: lactone and carboxylate HCPT, 0.5 μg/ml; lactone and carboxylate CPT, 5 μg/ml.

3.2. Method validation

The method presented in the manuscript provided not only complete separation, with corresponding retention times of 4.5 and 6.2 min for carboxylate and lactone HCPT, but also showed the usefulness of the method in a pharmacokinetic study. The inter- and intra-day variations of retention times (with C.V. being less than 2%) are shown in Table 1.

This method also yielded good recoveries of both lactone (I) and carboxylate (II) of HCPT (Table 2). In the present study, lactone and carboxylate forms of HCPT were added separately to biological samples, i.e., plasma, urine, feces and tissue homogenates, prior to incubation and/or extraction. The

Table 1
Intra-day and inter-day variations of retention time of both forms of HCPT

Form of HCPT	Actual retention time (min)	Intra-day[a]		Inter-day[b]	
		Measured mean (min)	C.V.[c] (%)	Measured mean (min)	C.V.[c] (%)
Carboxylate	4.50	4.51	1.1	4.45	1.5
Lactone	6.20	6.20	1.0	6.25	1.8

[a] $n=10$, the results are the mean of 10 determinations of standard HCPT.
[b] $n=5$, the results are the mean of 5 determinations of standard HCPT.
[c] C.V.=(S.D./mean)×100.

recoveries of both forms of HCPT in rat plasma, urine and feces at concentrations ranging from 20–1000 ng/ml were over 90% for plasma and urine, and about 80% for feces. As illustrated in Table 2, the average recovery of lactone HCPT was between 75% and 91% in tissue samples and lower than that of carboxylate form. Our preliminary data indicated that HCPT, especially the carboxylated form, highly bound to plasma and tissue protein (data not shown). Therefore, the extraction procedure, using EDTA and proteinase K with relatively long-term (2 h) incubation at 37°C, was helpful to increase the recovery of HCPT. However, under these conditions, lactone HCPT is converted to carboxylate form (10–25%), which may be responsible for the lower recovery of lactone HCPT compared to its carboxylate form. These results indicated that, under these conditions, the concentration of lactone HCPT in tissues would be underestimated, although the total amount of HCPT (lactone plus carboxylate forms) might be correct.

Standard curves for both forms of HCPT with concentrations ranging from 20 to 2000 ng/ml were linear in plasma, urine, feces, and tissues. The mean correlation coefficients (r) for daily calibration curves were greater than 0.999, and the regression equation was $Y=200X+9.68$, where Y is the concentration (ng/ml) and X is the peak height. The limits of determination were 2 ng/ml, 2 ng/ml, 2 ng/g, and 10 ng/ml for HCPT forms I and II in rat plasma, urine, feces, and tissues, respectively. The validation data on intra- and inter-day variations are illustrated in Table 3. For plasma, urine and fecal samples, the R.S.D.s were less than 7%, with accuracy being between 94.3–102.7% (detected values vs. spiked concentrations). Similar results were obtained with tissue homogenates (data not shown). It should be noted that, in the present study, standard lactone and carboxylate forms of HCPT were added to fecal and tissue homogenates to study the recovery of both forms of HCPT, so that the concentrations in feces and tissues were expressed as ng/ml of homogenates. However, in pharmacologic studies, the concentrations of HCPT in feces and tissues should be expressed as ng/g of feces or wet tissue (not homogenates), which can be easily calculated based on the volume of homogenizing buffer used and the concentrations in the homogenates.

Table 2
Recovery of two forms of HCPT from rat plasma, urine, feces and tissues

Concentration (ng/ml)	Recovery (%)[a]									
	Plasma		Urine		Feces		Liver		Kidney	
	I[b]	II[b]	I	II	I	II	I	II	I	II
20	92±11	101±14	90±11	94±9	76±14	79±8	75±15	91±10	78±10	94±12
100	94±5	102±4	89±7	95±5	78±7	77±10	84±9	94±5	87±5	101±8
1000	93±4	98±3	92±4	94±4	79±6	79±5	90±8	95±4	91±4	95±6

[a] Recovery is mean±S.D. of six determinations.
[b] I=lactone form of HCPT; II=carboxylate form of HCPT.

Table 3
Intra-day and inter-day variations of HPLC analysis of HCPT in rat plasma, urine and feces

Concentration (ng/ml)	Plasma				Urine				Feces			
	I[a]		II[a]		I		II		I		II	
	R.S.D.[b] (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
Intra-day[c]												
20	4.7	97.3	5.2	100.7	4.1	98.6	3.8	97.5	5.6	97.6	4.5	94.3
50	2.7	98.6	2.9	98.4	2.8	97.8	2.1	98.2	2.7	95.8	3.1	97.6
100	2.2	98.1	2.5	97.1	2.1	98.7	2.7	96.5	2.4	97.7	2.2	98.4
1000	1.9	99.7	2.2	97.8	1.4	99.4	1.9	100.2	2.2	98.7	2.3	96.7
Inter-day[d]												
20	5.9	101.1	6.4	102.7	4.7	97.5	4.8	99.7	6.7	98.5	6.0	98.7
50	4.7	97.1	4.3	99.7	3.8	98.7	4.1	98.2	4.7	95.6	4.9	99.5
100	4.6	99.4	3.3	98.4	4.1	98.9	4.7	97.5	4.9	96.8	4.1	97.8
1000	4.0	98.9	3.8	99.1	4.4	98.8	4.4	96.7	3.2	97.8	3.7	97.6

[a] I=Lactone form of HCPT; II=Carboxylate form of HCPT.
[b] R.S.D. represents relative standard deviation.
[c] $n=6$, the results are the mean of six determinations of triplicate samples in a single day.
[d] $n=4$, the results are the mean of four determinations of triplicate samples in 1 week.

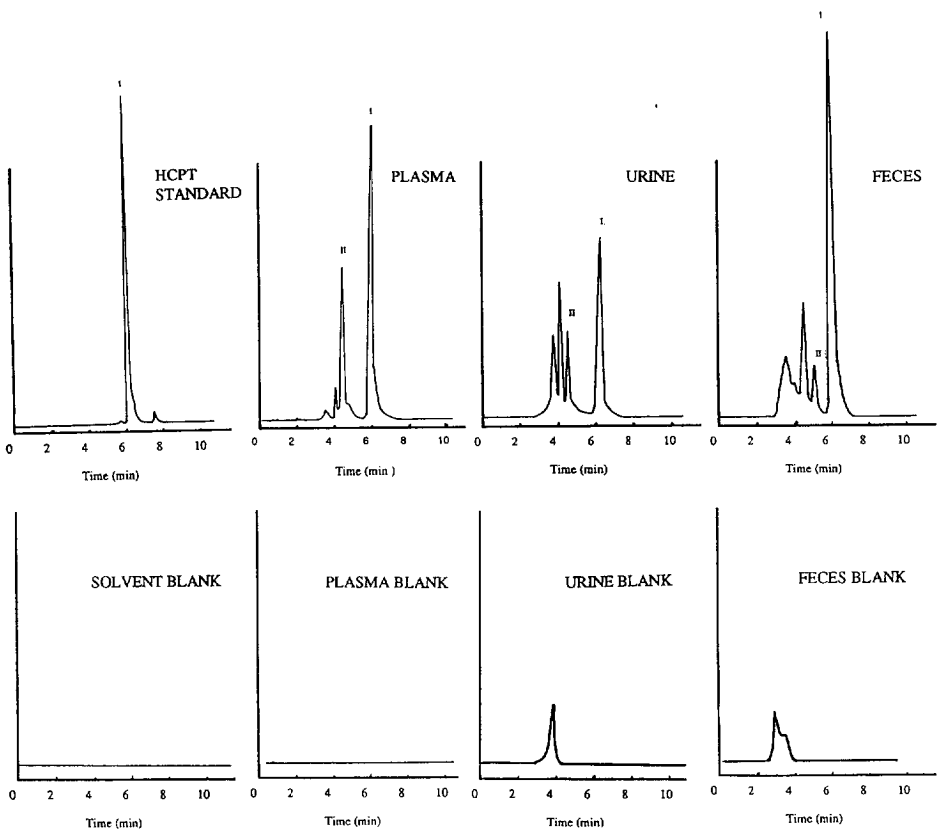

Fig. 3. HPLC analysis of plasma, urine and fecal extracts. Samples were obtained from rats after i.v. administration of HCPT at a dose of 10 mg/kg. Peak I: lactone form; peak II: carboxylate form. Plasma sample was taken at 10 min after dosing, with lactone and carboxylate forms being 1.14 and 0.25 μg/ml, respectively. Urine sample was collected 0–12 h after dosing, with lactone and carboxylate forms being 1.12 and 0.80 μg/ml, respectively. Fecal sample was collected 0–24 h after dosing, with lactone and carboxylate forms being 7.28 and 0.50 μg/ml, respectively. The fecal concentrations for lactone and carboxylate forms were calculated to be 36.40 and 2.50 μg/g, respectively.

3.3. Application in pharmacokinetic study

The method described has been successfully applied to the quantitation of HCPT in rat plasma, urine, feces, and tissues. The representative chromatograms of plasma, urine and feces are presented in Fig. 3, along with the chromatograms of blank samples from untreated animals. In the early stage after i.v. administration, HCPT in the plasma samples was mainly in lactone form following an immediate treatment and analysis and could be stable at −20°C for at least 1 week after protein precipitation, but it was easily converted to its carboxylate form in presence of plasma protein [8]. This could be partly explained by the binding to serum albumin, because albumin has long been noted for its preferential binding of small, hydrophobic molecules that

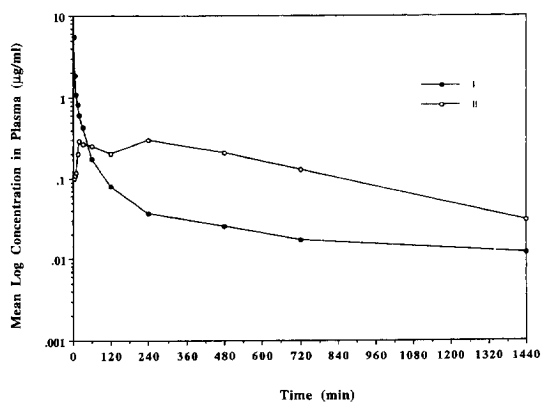

Fig. 4. Plasma concentration vs. time curves of HCPT lactone and carboxylate forms. Samples were obtained from rats after i.v. administration of HCPT at a dose of 10 mg/kg. Values for each time point are the mean of plasma concentrations from at least three animals. I (closed circle)= lactone form; II (open circle)= carboxylate form.

carry a negative charge [9], and HCPT is also a hydrophobic molecule carrying a negative charge at physiological pH. Our plasma pharmacokinetic studies also demonstrated that the carboxylate form of HCPT increased as the lactone form decreased over the experimental period (Fig. 4). The ratio of lactone/carboxylate forms was shown to be 1:1 at 1 h, 1:2 at 2 h, 1:9 at 4 h and thereafter following administration.

Representative chromatograms of liver, kidney, and bone marrow samples are shown in Fig. 5, along with the chromatograms of blank samples from untreated animals. As indicated above, the lactone form of HCPT in those samples might be underestimated, due to the partial conversion of lactone to carboxylate form. Although the concentration of lactone form of HCPT was lower than that of its carboxylate form in liver and kidney, it was higher in bone marrow. The mechanisms responsible for the differences are not determined. However, the tissue-dependent pharmacokinetics of HCPT may be important to its therapeutic and toxic effects.

Under the present chromatographic conditions, no significant non-specific peaks were detectable in

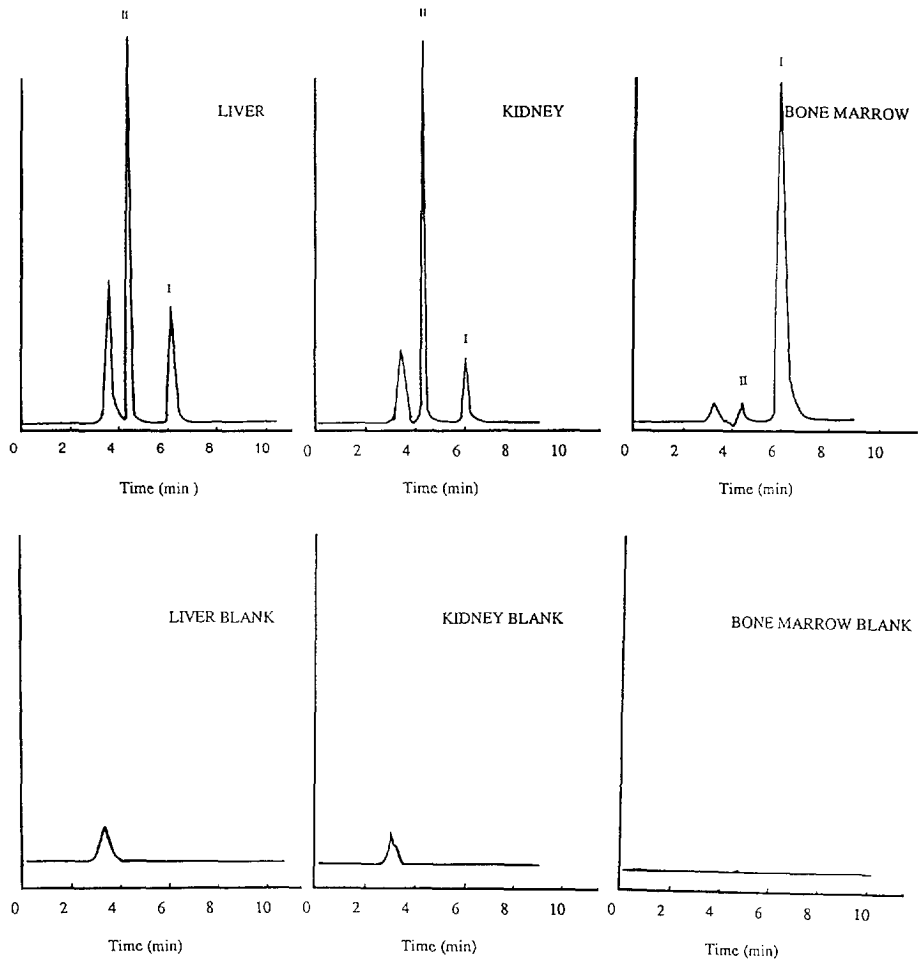

Fig. 5. HPLC analysis of HCPT in rat liver, kidney and bone marrow. Samples were obtained from rats after i.v. administration of HCPT at a dose of 10 mg/kg. Peak I: lactone form; peak II: carboxylate form. Liver sample was taken 6 h after dosing, with lactone and carboxylate forms being 0.27 and 2.21 μg/ml of homogenates, respectively. Kidney sample was collected 6 h after dosing, with lactone and carboxylate forms being 0.10 and 2.80 μg/ml, respectively. Bone marrow sample was collected 12 h after dosing, with lactone and carboxylate forms being 0.88 and 0.25 μg/ml, respectively.

blank samples of plasma and bone marrow, but a small endogenous (non-specific) peak was detected in blank samples of urine, feces, liver, and kidney, with the retention time being 3.5 min (Figs. 3 and 5). Additional peaks in samples of plasma, urine and tissues from treated animals were detected earlier than HCPT peaks on the chromatograms. These are likely to represent the glucuronide conjugates of HCPT in the lactone and carboxylate forms (data not shown). Further identification of these peaks is under way. As seen in Figs. 3 and 5, the noise (background) with fluorescence detection was very low so that the sensitivity was increased compared with UV detection. In our preliminary HPLC studies, the eluent was also monitored by UV detection at 245 and 265 nm, with multiple non-specific peaks being detected (data not shown).

With the increasing interest in water-insoluble and water-soluble CPTs as novel anticancer agents, it is important to develop sensitive, accurate, and precise analytical methods for the preclinical and clinical evaluations of these compounds [1,6,7,10]. This report represents the first study in separation of the lactone and carboxylate forms of HCPT and its metabolites in biological samples. Our simultaneous assay for both forms of HCPT has been implemented to determine the preclinical pharmacokinetics of HCPT and demonstrated to be a rapid (<10 min) and reproducible assay requiring a small sample volume. Therefore, this method will be useful in the future clinical and mechanistic studies of the novel anticancer drug.

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