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High-performance liquid chromatographic analysis for the determination of a novel polymer-bound camptothecin derivative (MAG-camptothecin) and free camptothecin in human plasma

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Abstract

A selective HPLC assay is described for the determination of free and total (free plus polymer-bound) camptothecin (CPT) in human plasma after administration of the anti-tumor drug MAG-CPT (polymer bound camptothecin). Total CPT levels were determined after hydrolysis and free CPT was extracted from acidified plasma using Oasis solid-phase extraction material. Extracts were analyzed on a Zorbax SB-C_8 analytical column, using a mixture of acetonitrile–25 m*M* phosphate buffer (pH 4.0) as the eluent. Detection was performed fluorimetrically. Concentrations of polymer-bound CPT were calculated by subtraction of free from total CPT. The lower limits of quantitation of the methods were 100 ng/ml for total and 1.0 ng/ml for free CPT using 50 μl and 250 μl plasma, respectively. Special attention was paid to the stability of the analytes. The presented method was successfully applied in a clinical pharmacokinetic study in our institute. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Camptothecin; MAG-camptothecin

1. Introduction

Camtothecin (CPT) and analogues are a class of anti-cancer drugs which are capable of selectively inhibiting the topoisomerase I enzyme (TOPO I) [1]. CPT has showed activity against a variety of tumor types both in pre-clinical and early phase I clinical studies [2,3]. However, development of CPT into clinical use is hampered by its unfavorable chemical–physical properties and toxicity profile. CPT is extremely insoluble in water and has a chemically unstable E-ring (lactone function). This lactone

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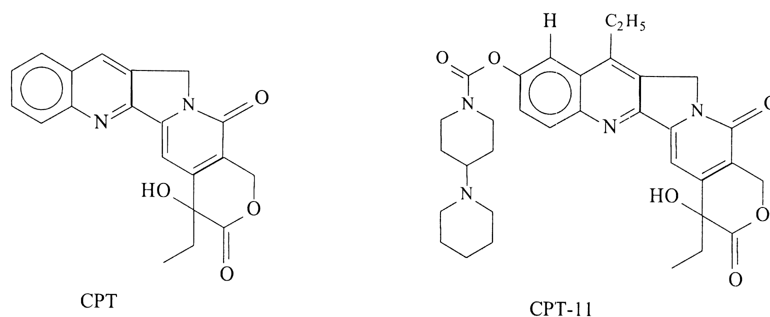

Fig. 1. Chemical structures of CPT and CPT-11 (internal standard).

function is essential for anti-tumor activity while in plasma the pH favors the carboxylate form which has no clinical activity [3,4]. A new approach to overcome these drawbacks is to conjugate CPT (Fig. 1) to a polymer backbone (MAG-CPT, Fig. 2). Polymer drug conjugates are a new and experimental class of drug delivery systems which are increasingly investigated. By conjugating CPT covalently to a polymer backbone, pharmacologic, toxicologic and pharmacokinetic properties of CPT are dramatically changed in vivo [5]. Solubility problems are overcome by this conjugate while the active form of CPT is protected by this linkage from conversion to its inactive form. Furthermore, the release of free CPT into plasma is now dependent on the rate of pH and in lesser case enzyme mediated esterolytic cleavage of MAG-CPT [6]. In pre-clinical studies with polymer bound CPT with similar structures a prolonged circulation of free drug in plasma could be demonstrated [7]. As the activity of camptothecin is as-

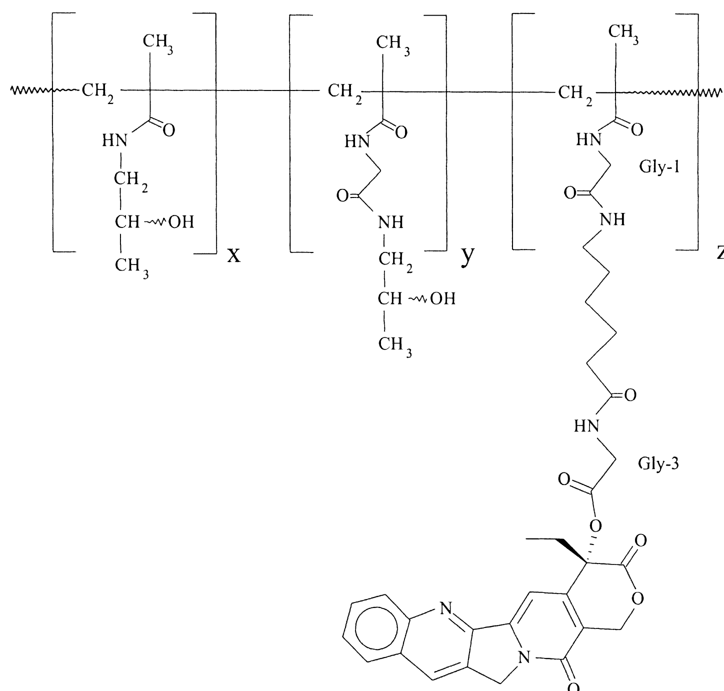

Fig. 2. Chemical structure of MAG-CPT.

sumed to be exposure time-dependent rather than concentration-dependent, prolonged exposure could increase anti-tumor activity [8]. An other major benefit of polymer conjugated drugs is the so-called ''enhanced permeability and retention effect'' (EPR effect) [9] which causes an improved bio-distribution by polymer-bound drugs accumulating specifically in tumor-tissue. In pre-clinical experiments decreased toxicity of normal tissue and improved anti-tumor efficacy probably due to a prolonged intra-tumor retention could be demonstrated [7]. To study the pharmacokinetics of MAG-CPT polymer bound drug as well as free drug should be considered. Fraier et al. [10] developed a selective high-performance liquid chromatography (HPLC) assay for the determination of free and polymer bound CPT in dog plasma. In this report, a selective HPLC method to quantify the levels of free and total (free plus polymer-bound) CPT loading in human plasma is described, which is useful for clinical pharmacokinetic research. The stability of the analytes has been investigated under various conditions. The presented method has been applied successfully in a clinical phase I study with the drug in our hospital.

2. Experimental

2.1. Materials

MAG-CPT (PNU 166148, CPT amount 10.14%) and CPT-11 (Camptosar, purity 91.38%) were provided by Pharmacia & Upjohn (Milan, Italy). CPT (camptothecin, purity 96.1%) was obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Acetonitrile (gradient grade, HPLC-S) was obtained from Biosolve (Amsterdam, The Netherlands). Methanol (HPLC grade, ChromAR) was purchased from Promochem (Wesel, Germany). Disodium tetraborate decahydrate (analytical grade), sodium hydroxide (analytical grade), potassium dihydrogenphosphate (analytical grade), phosphoric acid (85%, analytical grade), and hydrochloric acid (analytical grade) were obtained from Merck (Darmstadt, Germany). Double-distilled water was provided by the Slotervaart Hospital (Amsterdam, The Netherlands). Drug-free heparinized human plasma was obtained from the Central laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Equipment

For the determination of total levels of CPT (free plus bound to the polymeric carrier) and free CPT-levels in human plasma two separate HPLC–fluorescence methods were validated. The chromatographic system consisted of a solvent delivery system type P100 or P1000 and an automatic sample injection device Model SP8880 (Thermo Quest, Fremont, CA, USA) and a Series 200 96 well autosampler (Perkin-Elmer, Norwalk, CT, USA). The autosampler flushing-solvent consisted of water–acetonitrile (30:70, v/v). Separation of the compounds was performed on a Zorbax SB-C_8 column (5 mm, 150×4.6 mm) (Atas) and the column temperature was controlled with a column heater (Jones Chromatography, Lakewood, CO, USA). To protect the analytical column, a guard column (3×10 mm, Chrompack, Middelburg, The Netherlands) packed with reversed-phase material was used. Detection was performed fluorimetrically using an FP-920 Intelligent Fluorescence detector (Jasco International, Tokyo, Japan). Retention times and peak areas were analyzed with a PC1000 data system (Thermo Quest).

2.3. Preparation of stock and working solutions

A stock solution of MAG-CPT of 5000 μg CPT equivalent/ml was prepared by dissolving an accurately weighed amount of MAG-CPT in 25 m*M* phosphoric acid–methanol (50:50, v/v). The stock solution of MAG-CPT was diluted with 25 m*M* phosphoric acid in methanol to give working solutions of 3750, 2500, 500, 125, 25, and 5 μg/ml. Stock solutions of CPT-11 (Fig. 1) and CPT of 100 μg/ml were prepared by dissolving an accurately weighed amount of CPT-11 or CPT in 10 m*M* phosphoric acid in methanol. The stock solution of CPT-11 was diluted with 10 m*M* phosphoric acid in methanol to give a working solution of 1 μg/ml. The stock solution of CPT was diluted with 10 m*M* phosphoric acid in methanol to give working solutions of 10, 1 and 0.1 μg/ml. Stock and working solutions for the preparation of the quality control

(QC) samples of CPT and MAG-CPT were independently prepared. All stock and working solutions were stored at 4°C.

2.4. Preparation of calibration standards, QC samples and recovery samples

Calibration standards of MAG-CPT in the range of 100 to 100 000 ng/ml CPT were prepared by adding 20 μl of the stock or working solution to 980 μl of drug-free plasma in polypropylene Eppendorf cups. Aliquots of 50 μl of the calibration standards were stored at −70°C. QC samples of MAG-CPT were prepared in volumetric flasks of 2.0 ml by spiking aliqots of of MAG-CPT stock or working solution to drug-free plasma. QC samples were obtained with concentrations between 100 and 250 000 ng/ml CPT. QC samples were divided in aliquots of 50 μl and stored at −70°C.

Calibration standards of CPT were prepared in volumetric flasks of 10.0 ml by spiking 50 to 250 μl of CPT stock or working solution to drug-free plasma. Calibration standards with concentrations between 1 and 1000 ng/ml CPT were obtained. A volume of 750 μl of 8.5% (v/v) phosphoric acid was then added to 250-μl aliquots of the calibration standards and the samples were stored at −70°C after vortex mixing. CPT QC samples were spiked with prepared stock and working solutions to obtain samples in drug-free plasma with the following concentrations: 1, 2.5, 250, 750, and 2500 ng/ml. A volume of 750 ml of 8.5% (v/v) phosphoric acid was then added to 250-μl aliquots of the calibration standards and the samples were stored at −70°C after vortex mixing. To calculate the recovery over the validated range for both assays, recovery samples of MAG-CPT and CPT were spiked with working solutions to obtain CPT concentrations in the eluent corresponding with the concentrations in the final plasma extracts.

2.5. Sample pretreatment

2.5.1. Total levels of CPT (free plus polymer-bound)

Total levels of CPT were determined after the alkaline mediated hydrolysis of MAG-CPT. Plasma proteins were precipitated by addition of 450 μl of methanol to 50 μl of plasma. After vortex mixing the samples were centrifuged for 3 min at 10 000 *g*. A 40-μl volume of supernatant was pipetted into a 96-well microtiter plate and 170 μl of borate buffer (0.05 *M*, pH 9.8) was added. After vortex mixing the samples were incubated for 24 h at 50°C. After addition of 1350 μl of 8.5% (v/v) phosphoric acid and 40 μl of CPT-11 working solution (1 μg/ml) the samples were mixed and injected into the HPLC system.

2.5.2. Free CPT

During sample preparation, samples and solutions were constantly kept in ice-water. Free CPT was extracted from acidified plasma using 96-well extraction plates (OASIS-HLB, 30 mg). The wells were conditioned with acetonitrile (1 ml) and 8.5% (v/v) phosphoric acid (1 ml). Acidified plasma samples [250 μl of plasma plus 750 μl of 8.5% (v/v) phosphoric acid] were loaded and the wells were washed with distilled water (2×1.5 ml). Elution was performed using a solution consisting of acetonitrile–25 m*M* phosphate buffer, pH 4.0 (60:40, v/v, 0.5 ml). After addition of 0.5 ml of 25 m*M* phosphate buffer, pH 4.0, the samples were mixed and injected into the HPLC system.

2.6. Chromatography

Chromatographic analysis was performed at 30°C. The mobile phase consisted of a mixture of 25 m*M* phosphate buffer, pH 4.0–acetonitrile (90.3:9.7, v/v). The eluent was degassed with helium during 15 min before use. The flow-rate was 1.0 ml/min. Detection was performed fluorimetrically with the excitation wavelength at 380 nm and the emission wavelength at 440 nm. Injection volumes between 5 and 50 μl were applied. CPT eluted at a retention time of 6.3 min and CPT-11 after 3.3 min (Fig. 3).

2.7. Method validation

A three-run validation was completed for each assay, producing calibration lines ranging from 100 to 100 000 ng/ml for the determination of the total levels of CPT and 1 to 1000 ng/ml for the determination of the levels of free CPT. The following parameters were determined: linearity, within-run

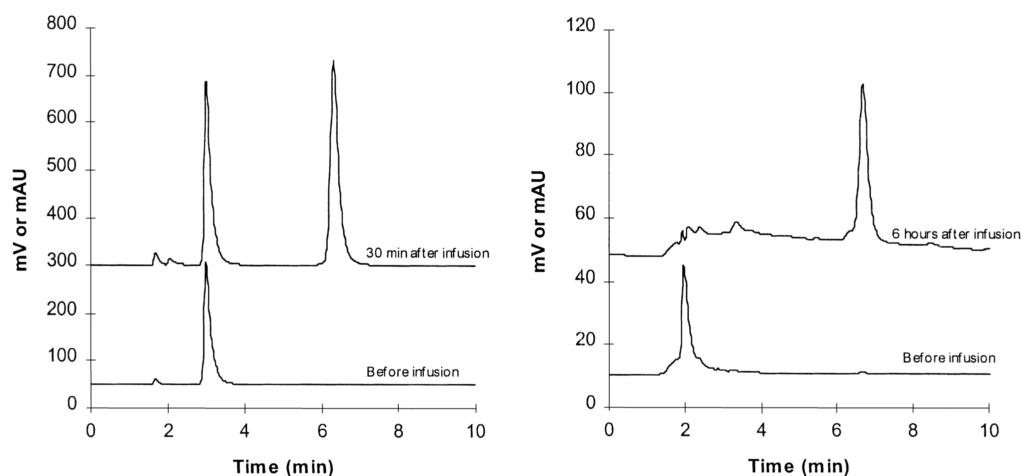

Fig. 3. Typical chromatograms for the analysis of total CPT (left) and free CPT (right): samples were taken from a patient treated with MAG-CPT at a dose level of 34 $mg/m^2/day$. CPT is eluting after approximately 6.3 min and CPT-11 (the internal standard used in the analysis of total CPT) is eluting after approximately 3.3 min.

and between-run precision, accuracy, specificity, selectivity and absolute recovery. To establish the best weighting factor, back-calculated calibration concentrations were calculated and the deviations from nominal concentrations were calculated. The model with the lowest total bias across the range was considered to be the best fit. The accuracy and the precision of the methods were determined by analyzing spiked quality control samples with analyte concentrations around the lower limit of quantitation (LLOQ), in the low, medium and high concentration range of the calibration curve and an additional quality control concentration above the upper limit of quantitation (ULOQ). The last-mentioned samples were measured after dilution with blank plasma (10-fold) to demonstrate parallelism. Each quality control sample was analyzed in a minimum of five replicates together with a calibration curve, in at least three analytical runs except for the quality controls around the LLOQ and above the ULOQ. These samples were assayed in one validation run using five replicates. The accuracy was determined in percent difference between the mean concentration and the nominal concentration. The relative standard deviation (RSD) was used to report the precision. Six batches control human plasma were processed and analyzed to determine whether endogenous plasma constituents co-eluted with CPT and/or the internal standard CPT-11 (determination of total levels of CPT). The overall CPT recovery was determined by comparing the slope of the processed human calibration curve (area versus concentration) to a standard curve prepared in the eluent.

2.8. Stability

2.8.1. Stability in the biomatrix after three freeze–thaw cycles

The stability of MAG-CPT and CPT in the biomatrix was investigated under the following conditions: (1) plasma samples were spiked with MAG-CPT and after three freeze–thaw cycles from −70°C to ambient temperatures, total levels of CPT were measured, (2) acidified plasma samples were spiked with MAG-CPT and after three freeze–thaw cycles from −70°C to ambient temperature or 4°C levels of free CPT were measured, (3) acidified plasma samples were spiked with CPT and after three freeze–thaw cycles from −70°C to approximately 0°C (ice-water bath), levels of free CPT were measured. All samples were spiked at three concentration levels (in the low, mid and high concentration range of the calibration curve) and analyzed in triplicate.

2.8.2. Stability in the biomatrix during sample pretreatment

The stability of MAG-CPT and CPT in the biomatrix during sample pretreatment was investigated by performing the following experiments: (1) plasma samples spiked with MAG-CPT were kept at ambient temperatures and at time zero and at 24 h total levels of CPT were determined, (2) acidified plasma samples spiked with MAG-CPT were kept at approximately 0°C (ice-water bath) and at $t=0$, 15, 30, 60 and 120 min levels of free CPT were determined, (3) acidified plasma samples spiked with CPT were kept at ambient temperatures and at time zero and at 24 h total levels of CPT were determined. All samples were spiked at three concentration levels (in the low, mid and high concentration range of the calibration curve) and analyzed in triplicate.

2.8.3. Stability in the final extract

The stability of CPT in the final extract was investigated for both assays during 24 h at ambient temperatures. Samples were assayed at three concentration levels (in the low, mid and high concentration range of the calibration curve) in triplicate.

2.8.4. Long-term stability in the biomatrix

The long-term stability of CPT and MAG-CPT in the biomatrix at −70°C was investigated in plasma samples spiked with MAG-CPT (determination of total levels) and acidified plasma samples spiked with CPT (determination of levels of free CPT). Samples were spiked at three concentration levels (in the low, mid and high concentration range of the calibration curve) and analyzed after 8 and 7 months, respectively. Clinical samples were re-assayed after 1.5 months of storage at −70°C.

2.8.5. Stability during handling of the samples in the clinic

The release of free CPT from the polymer has been studied under conditions which mimic handling of blood samples in the clinic according the following procedure. Whole blood was conditioned at 37°C for 10 min. Standard solutions were added to achieve total CPT concentrations of 75, 25, 2.5 μg/ml plasma. After spiking, the blood samples were chilled on an ice-water bath for 10 min and subsequently centrifuged at 2500 *g* (4°C) for 5 min to separate the plasma. A 250-μl volume of plasma was added to 750 μl 8.5% phosphoric acid (chilled in ice-water). The diluted samples were flash frozen in a CO_2–ethanol bath. Then the plasma samples were thawed and kept on an ice-water bath. Within 15 min the stabilized samples were processed. To determine the amount of free CPT in the working solutions of MAG-CPT an aliquot was injected into the HPLC system together with recovery standards.

2.8.6. Stability in stock and working solutions

Stock and working solutions of MAG-CPT and CPT were kept at approximately 4°C and after 4 to 6 months of storage, the CPT concentrations were determined by comparison of the CPT responses of freshly prepared stock and working solutions. Samples were assayed in triplicate.

2.9. Human experiments

The presented method was used to support a phase I and pharmacokinetic study of MAG-CPT. Patients were treated with a 30 min intravenous infusion of MAG-CPT daily for 3 days every 4 weeks. The starting dose was 17 $mg/m^2/day$ whereafter doses were escalated in subsequent patient cohorts until dose limiting toxicities were observed. On day 1 blood samples (5 ml each), were collected in cooled heparinized tubes pre-infusion, and at 15, 30, 40, 60, and 90 min, and 2.5 and 5.5 h after the start of the infusion. On day 2, samples were collected prior to infusion and at 30 min, and 4.5 and 8.5 h after the start of the infusion. On day 3, samples were collected prior to infusion and at 30 and 50 min, and 3.5, 6.5, 24 and 48 h after the start of the infusion. Additional samples were collected weekly thereafter until start of next cycle. Blood samples were immediately immersed in ice-water at the bed-side. Plasma was obtained by refrigerated centrifugation of the samples (5 min; 3000 *g*, 4°C). For the determination of free CPT, 0.25 ml of plasma was added to 0.75 ml 8.5% phosphoric acid and subsequently mixed on a vortex mixer for 10 s. Plasma and plasma phosphoric acid mixtures were immediately frozen in a dry ice–ethanol bath and stored at −70°C until analysis. Concentrations of polymer-bound CPT were calculated by subtraction of free from total CPT. The

study protocol was approved by the Medical Ethics Committee of the hospital, and all patients gave written informed consent.

3. Results and discussion

3.1. Validation

Two HPLC–fluorescence methods were validated for the determination of total levels of CPT (free plus bound to the polymeric carrier) and free CPT levels in human plasma. For the assay of total levels of CPT, plasma proteins were precipitated with methanol and an aliquot of the methanolic supernatant was brought to pH 9.8 to hydrolyze the polymer-bound CPT. After acidification and addition of the internal standard CPT-11, the sample was injected into the HPLC system. We used CPT-11 (Fig. 1) as an internal standard because it is an structural analog of CPT with a similar chromophore and both are detected fluorimetrically by 380/440 nm. Free CPT was extracted from acidified plasma using Oasis solid-phase extraction (SPE) material in 96-well plates. The plasma had to be acidified on the ward immediately after sampling due to the poor stability of MAG-CPT and therefore no internal standard could be used. For the determination of the total levels the linear regression of the ratio of the area of CPT and the area of CPT-11 peaks, versus the CPT concentration was weighted by $1/x$ (the reciprocal of the CPT concentration). The linear regression for the determination of the free levels of CPT (area of the CPT versus the concentration) was weighted by $1/x^2$. The LLOQs of the methods were 100 ng/ml for the determination of total levels of CPT and 1.0 ng/ml for free CPT using 50 μl and 250 μl plasma volumes, respectively. Concentration ranges were from 100 to 100 000 ng/ml for total CPT and 1 to 1000 ng/ml for free CPT. For the calibration standards the back-calculated concentrations from the response were calculated (see Table 1). The deviation of the nominal concentration for all concentrations were between the −8.82 and 11.83%. The RSD values ranged from 1.16 to 9.41%. The assay performance data for the determination of the total levels and free levels of CPT are shown in Table 2. The accuracy was for all tested concentrations were all within 15% and both the within- and between-run precisions were less than the required 15% [11]. The tested batches of control human plasma contained no co-eluting peaks >20% of the CPT area at the LLOQ. It is recommended to inject several blank samples after the analysis of high CPT concen-

Table 1
Back calculated concentrations of free and total CPT

Nominal plasma concentration (ng/ml)	Mean measured concentration (ng/ml)	RSD (%)	Deviation (%)	*n*
Total CPT				
100.03	111.86	2.49	11.83	3
500.15	456.02	3.84	−8.82	3
2500.75	2433.84	3.76	−2.68	3
10 003.00	10 243.01	3.74	2.40	3
50 015.00	47 529.61	1.61	−4.97	3
75 022.50	74 713.53	3.28	−0.41	3
100 030.00	102 683.56	1.73	2.65	3
Free CPT				
1.00	0.99	1.16	−0.67	3
5.00	5.15	4.99	3.07	3
24.99	26.03	4.26	4.15	3
99.96	102.16	9.41	2.20	3
499.82	485.45	4.06	−2.87	3
749.72	742.81	5.01	−0.92	3
999.63	951.09	1.36	−4.86	3

RSD, Relative standard deviation; *n*, number of analytical runs.

Table 2
Assay performance data for the determination methods of free and total CPT

Nominal plasma concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	Within-run precision (%)	Between-run precision (%)	*n*
Total CPT					
103.18	95.26	−7.68	11.92	NA	5
2503.40	2769.08	10.61	8.61	9.18	15*
25 033.50	26 743.05	6.38	2.43	4.86	15*
75 101.20	75 132.04	0.04	4.46	5.98	15*
250 500.00	267 973.48	6.98	6.83	NA	5
Free CPT					
1.00	1.01	1.40	3.73	NA	5
2.50	2.41	−3.47	4.40	7.55	15*
250.34	247.63	−1.08	3.41	5.53	15*
751.02	755.95	0.66	4.21	4.99	15*
2503.41	2778.01	10.97	3.41	NA	5

n, Number of replicates; NA, not applicable. *Number of individual analytical runs was three.

trations to prevent carry-over effects. The mean recovery for the determination of total levels of CPT was 87.38±4.92% and for the assay of the free CPT levels a recovery of 82.97±1.06% was found.

3.2. Stability

A number of stability experiments were performed and the results are summarized in Table 3 (assay for

Table 3
Stability data of MAG-CPT for the assay of total CPT

Stability test	Sample description	Initial nominal concentration (ng/ml)	Recovery (%)	RSD (%)	Number of replicates
3 Freeze–thaw cycles	Plasma spiked with MAG-CPT	2500	101.8	3.78	3
		25 000	92.0	15.85	3
		75 000	103.4	3.30	3
24 h in the biomatrix at ambient temperatures	Plasma spiked with MAG-CPT	2500	87.8	3.39	3
		25 000	99.6	2.68	3
		75 000	98.1	1.74	3
24 h in the final extract at ambient temperatures	Extracted plasma spiked with MAG-CPT	2500	101.8	1.99	3
		25 000	100.3	3.41	3
		75 000	101.0	1.34	3
8 months at −70°C	Plasma spiked with MAG-CPT	2500	105.6	0.56	3
		25 000	95.8	3.79	3
		75 000	98.1	0.85	3
1.5 months at −70°C	Clinical plasma samples	3313	91.3	NA	1
		16 597	113.0	NA	1
		37 212	108.6	NA	1
4 months at 4°C	Stock and working solutions of MAG-CPT	5 000 000	97.1	2.60	3
		500 000	99.9	5.45	3
		25 000	108.9	4.31	3
		5000	106.3	12.8	3

RSD, Relative standard deviation.

total CPT) and Table 4 (assay for free CPT). The stability of CPT was only critical when free CPT levels were measured in the presence of MAG-CPT. When acidified samples were assayed for the free levels in the presence of MAG-CPT, a significant increase of the CPT concentration was observed after three freeze–thaw cycles. In practice a increase in concentration after three freeze–thaw cycles is of minor concern as a clinical sample will undergo only one cycle as the complete sample will be processed before analysis. We found no evidence of degradation of CPT in plasma spiked with MAG-CPT or CPT after three freeze–thaw cycles. In order to test the stability in the biomatrix during sample pretreatment plasma samples were spiked with different MAG-CPT concentrations and the acidified samples were kept in an ice-water bath. Under these conditions we observed an increase of the free CPT concentrations in time (see Fig. 4). An increase of approximately 12% was measured after 15 min for all tested concentrations (see also Table 4). Thawed, acidified clinical samples are to be kept in an ice-water bath for utmost 15 min before the extraction. The total in vitro release (handling of samples in the clinic plus the sample pretreatment procedure) was concentration dependent: at a concentration of 2.5 μg/ml MAG-CPT the release of free CPT was 0.17%, at 25 μg/ml 0.06% and at 75 μg/ml a release of 0.07% was found. This might cause an overestimation of measured free CPT as the per-

Table 4
Stability data of MAG-CPT and CPT for the assay of free CPT

Stability test	Sample description	Initial concentration		Recovery (%)	RSD (%)	Number of replicates
		MAG-CPT (ng/ml)	Free CPT (ng/ml)			
3 Freeze–thaw cycles	Acidified plasma spiked with CPT	NA	2.5	96.2	13.78	3
		NA	250	95.7	3.42	3
		NA	750	99.4	6.52	3
3 Freeze–thaw cycles	Acidified plasma spiked with MAG-CPT	2500	2.53	117.0	5.42	3
		25 000	12.95	128.0	3.42	3
		75 000	40.25	140.0	1.77	3
15 min in an ice-water bath	Acidified plasma spiked with MAG-CPT	2500	1.26	112.7	1.47	3
		25 000	11.60	113.1	4.40	3
		75 000	24.95	111.3	1.82	3
24 h in the biomatrix at ambient temperatures	Acidified plasma spiked with CPT	NA	2.5	91.9	4.92	3
		NA	250	100.4	7.81	3
		NA	750	103.3	4.51	3
24 h in the final extract at ambient temperatures	Extracted plasma spiked with CPT	NA	2.5	100.3	4.13	3
		NA	250	100.6	0.70	3
		NA	750	99.6	5.91	3
7 months at −70°C	Acidified plasma spiked with CPT	NA	2.5	93.9	12.91	2
		NA	250	109.2	2.53	3
		NA	750	105.8	4.30	3
1.5 months at −70°C	Acidified plasma; clinical plasma sample	3313	40.24	98.3	NA	1
		16 597	4.93	116.4	NA	1
		37 212	79.55	101.2	NA	1
5 months at 4°C	Stock and working solutions of CPT	NA	100 000	94.7	4.19	3
		NA	10 000	95.1	0.74	3
		NA	1000	96.4	1.91	3
		NA	100	95.9	3.94	3

RSD, Relative standard deviation; NA, not applicable.

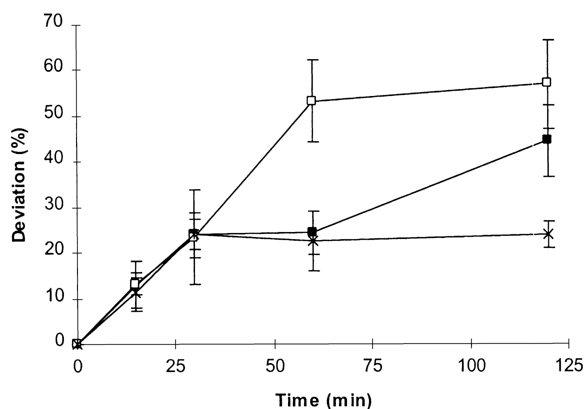

Fig. 4. Stability of MAG-CPT in acidified plasma kept on an ice-water bath (determination of free levels of CPT). Mean percentage deviation from time zero (±SD) of plasma plasma spiked with 2500 ng/ml MAG-CPT (■), plasma spiked with 25 000 ng/ml MAG-CPT (□), plasma spiked with 75 000 ng/ml MAG-CPT (×).

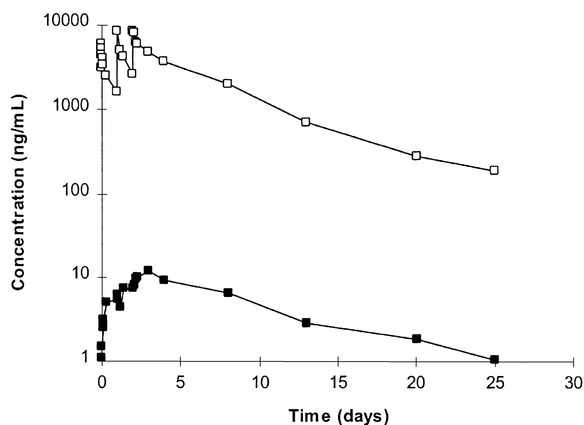

Fig. 6. Plasma concentration–time curve of a patient treated with MAG-CPT at the lowest dose level (17 mg/m^2/day). Represented are plasma concentrations of free CPT (■) and plasma concentrations of bound CPT (=total CPT−free CPT) (□).

centage of free CPT levels in real clinical samples are between 0.02 and 2.9% of the measured total CPT. We found no evidence of degradation of CPT in plasma or in the final extract after 24 h at ambient temperature. Spiked plasma samples with MAG-CPT (determination of total CPT) and acidified spiked plasma samples with CPT (determination of free CPT) were stable after 8 and 7 months storage at −70°C, respectively. Clinical plasma samples showed no significant degradation of MAG-CPT up to 1.5 month of storage at −70°C. In Fig. 5 levels of free CPT and total CPT in plasma of a patient treated with MAG-CPT measured at time zero and after 1.5 months are shown. Stock and working solutions were found to be stable up to 4 months when stored at approximately 4°C.

3.3. Human experiments

The presented method was successfully applied in a phase I and pharmacokinetic study performed in our institute. In Fig. 6 the plasma concentration–time curves of bound and free CPT of a patient treated at the lowest dose-level (17 mg/m^2/day) in this study are shown. All samples up to 25 days were within limits of quantitation. This demonstrates the ap-

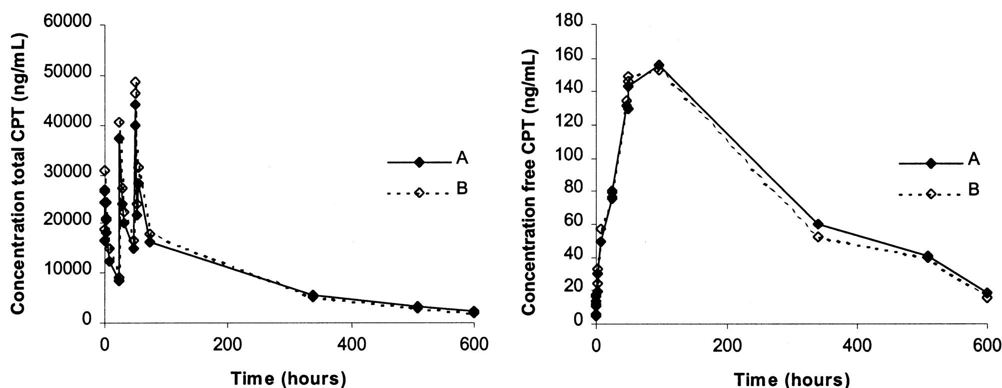

Fig. 5. Stability in plasma of clinical samples. Plasma samples were taken from a patient treated with MAG-CPT at a dose-level of 68 mg/m^2/day. Total CPT (left graph) and free CPT (right graph) were determined at time zero (A) and after 1.5 months (B).

plicability of the assay for complete pharmacokinetic evaluation of the drug.

4. Conclusions

We present a sensitive and validated assay for the quantification of total (bound and free) CPT and free CPT in human plasma and demonstrated the applicability for pharmacokinetic evaluation of the new drug MAG-CPT in patients. Polymer-bound CPT concentrations are calculated as total levels minus free levels. For the determination of free CPT, it is of major importance that the collected blood samples are chilled, centrifuged, stabilized appropriately and timely (within 15 min) to avoid further ex vivo release of CPT from its polymeric carrier. Detailed attention has been paid to the stability of the analytes, during handling of the clinical samples as well during storage and analysis. This is of great importance, since release of relatively small amounts of CPT from the polymer may result in a significant overestimation of the free fraction present in the plasma of patients.

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