Anti-Cancer Drugs 2000, **11,** pp. 315–324

Review paper

Determination of camptothecin analogs in biological matrices by high-performance liquid chromatography

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Several analogs of the topoisomerase I inhibitor camptothecin (CPT) have been introduced in clinical practice in the last decade. All CPT analogs are sensitive to a pH-dependent reversible conversion between a pharmacologically active lactone form and its inactive, lactone ring-opened, carboxylate form. The reversible conversion is also dependent on the, sometimes species-dependent, protein binding properties of the two forms, resulting in different lactone to carboxylate plasma ratios for the various analogs. Pharmacokinetic analysis of the CPT analogs is helpful in understanding the pharmacodynamic outcome of drug treatment, in clinical as well preclinical studies. Measurement of these analogs is habitually complicated by the chemical instability of the lactone moiety and necessitates a rapid centrifugation of the blood sample, preferably at the bedside of the patient, to collect the plasma supernatant. Since the lactone forms of these drugs are able to diffuse across cell membranes, including those of the red blood cells, rapid collection and processing is even necessary in the case where only the total concentrations of the CPT analogs are to be measured. Sample pretreatment procedures of the CPT analogs topotecan, irinotecan, 9-aminocamptothecin and lurtotecan are summarized and discussed in this review. [© 2000 Lippincott Williams & Wilkins.]

Key words: 9-Aminocamptothecin, HPLC, irinotecan, lurtotecan, SN-38, topotecan.

Introduction

The naturally occurring lactone form of camptothecin (CPT; Figure 1) is a poorly water-soluble inhibitor of DNA synthesis, by reversibly stabilizing the cleavable complex between topoisomerase I and DNA. This results in single-strand DNA breaks and thus termination of DNA replication, subsequently followed by cell death.[1–4] Since CPT itself was water-insoluble, the drug was formulated as the water-soluble sodium salt (NSC 100880). Unfortunately, due to this formulation, the delicate balance between the lactone and carboxylate forms was shifted toward the latter at neutral pH. Poor response rates in conjunction with severe toxicities were observed in early clinical trials with this agent and the sodium salt of CPT was shown to yield only 10% of the activity of CPT against mouse leukemia while no anti-tumor activity was found in xenograft models.[1] During the last decade, various types of more or less water-soluble analogs of CPT, such as topotecan (TPT), irinotecan (CPT-11), 9-aminocamp-tothecin (9-AC) and lurtotecan (LRT), have been introduced into clinical practice (Figure 1).

CPT and its analogs share a pH-dependent reversible conversion between the lactone and carboxylate form (Figure 1), from which the intact lactone form is able to diffuse across cell membranes, while the pharmacologically inactive ring-opened carboxylate is trapped into the extracellular compartments, i.e. cell growth medium in the case of *in vitro* experiments and plasma water in *in vivo* studies.[5] The percentages of CPT analogs present in the lactone form at equilibrium in phosphate-buffered saline are in the same order for the different CPT analogs, with values of 17.0 ± 2.0, 15.3 ± 0.8, 13.0 ± 2.0, 15.3 ± 1.7 and $19.0 \pm 1.0\%$ for CPT, TPT, CPT-11, SN-38 (active metabolite of CPT-11) and 9-AC respectively. Addition of human serum albumin (HSA) at a concentration of 40 mg/ml shifts the percentage of lactone at equilibrium for CPT and 9-AC below 2%, while for CPT-11 and SN-38 the percentage increased to, respectively, 24.0 ± 1.0 and $34.8 \pm 1.7\%$. No change has been observed for topotecan in the presence of HSA, with $17.1 \pm 0.4\%$

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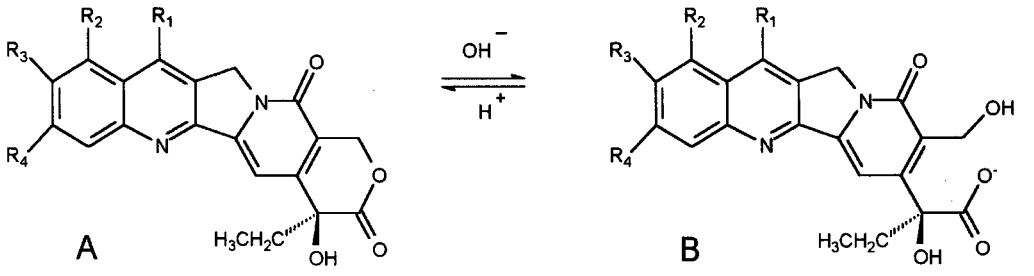


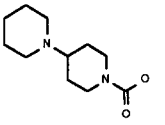
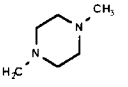
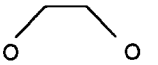
Compound	R_1	R_2	R_3	R_4
Camptothecin	H	H	H	H
Topotecan	H	$CH_2N(CH_3)_2$	OH	H
Irinotecan	CH_2CH_3	H		H
SN-38	CH_2CH_3	H	OH	H
9-Aminocamptothecin	H	NH_2	H	H
Lurtotecan		H		

Figure 1. Chemical structures of the lactone (A) and ring-opened carboxylate (B) forms of CPT and analogs in clinical development.

in the lactone form at equilibrium. This phenomenon is caused by a preferential binding of the carboxylate forms of CPT and 9-AC to HSA, resulting in a shift of the equilibrium towards the carboxylate. In contrast, for TPT, CPT-11 and SN-38, the substituents at the R_1 and R_2 positions (Figure 1), hinder the binding of the carboxylate forms to HSA, and so stabilize the lactone form.[6] No data are available for LRT, from which we expect a stabilized lactone moiety, by substitution at the R_1 position. However, the binding to serum

albumin has been shown to be clearly species dependent. In the case of 9-AC, which demonstrated high antitumor activity in preclinical mouse xenograft models,[7] the lactone moiety is stabilized by murine serum albumin (MSA) but not by HSA, with $35.0 \pm 6.2\%$ in the pharmacologically active lactone form in the presence of MSA and only $0.63 \pm 0.10\%$ in the presence of HSA.[5] Pharmacokinetic analyses of the CPTs are thus important in clinical as well as preclinical studies and are complicated by the chemical instability of the lactone moiety.

To ensure adequate measurements of the pharmacologically active lactone forms of the CPT analogs in kinetic studies, blood samples have to be processed directly after sampling at the site of the patient, either by (i) direct analysis of the samples, (ii) direct extraction of the lactone form from the plasma or (iii) stabilizing the lactone to carboxylate ratio. Stabilization of the lactone to carboxylate ratio is preferable since this is the less laborious approach. In general, separations of the topoisomerase I inhibitors and endogenous compounds were performed by reversed-phase high-performance liquid chromatography (HPLC) methods, coupled with fluorescence detection. In this review we summarize the methods for sample treatment and detection of each CPT analog in biological matrices, and the lower limit of quantitation (LLQ) or lower limit of detection (LLD) for each assay. The LLQ is of great importance for accurate pharmacokinetic analysis and is defined as the lowest concentration of the CPT which can be measured accurately and precisely. While the LLD, which is unreliable regarding accuracy and precision, is defined as the lowest detectable concentration that can be distinguished from the background noise.[8]

Sample treatment of CPT analogs for HPLC measurements

Topotecan

TPT (Hycamtin®, SKF 104864, NSC 609699, (*S*)-9-dimethylaminomethyl-10-hydroxycamptothecin; Figure 1) is a semisynthetic water-soluble CPT analog, prepared by synthetic modification of 10-hydroxycamptothecin.[1] The i.v. formulation of TPT has been registered for the treatment of ovarian cancer in Europe and the USA.[4] Determination of the lactone, carboxylate and total (i.e. lactone plus carboxylate forms) concentrations of TPT in human plasma has been described in several publications. The plasma sample pretreatment in these published methods is based on a simple methanolic protein precipitation step immediately after the collection of the plasma according to the method of Beijnen *et al.*[9] The ratios of the lactone to carboxylate concentrations in the methanolic extracts were found to be stable for at least 4 and 15 months when stored at a minimum of $-70^{\circ}C$.[10,11]

The first assay, published by Beijnen *et al.*,[9] described the simultaneous determination of the lactone and carboxylate form of TPT with a LLD for both compounds of 0.2 ng/ml. A good baseline separation between the lactone form and endogenous material was achieved; however, in blank plasma samples an interfering peak for the carboxylate form was found. In order to get reliable results the chromatograms were reprocessed with subtraction of each corresponding blank chromatogram.

One of the assays described by Rosing *et al.*[12] is among the most sensitive with an LLQ of 0.05 ng/ml (Table 1) for the lactone and the total form of TPT. The total concentration of TPT was measured in a second analysis, where the samples were acidified with perchloric acid, which results in the conversion of the carboxylate form in the lactone form, followed by the determination of the lactone form. The amount of the carboxylate form was calculated as the difference between the total and lactone concentration. These authors have also described the impact of column temperature for the assay of TPT in rat and dog plasma.[13] The sample treatment is based on the same method, while the column must be thermostated at 19–21°C to obtain sufficient baseline separations between peaks of endogenous compounds in rat and dog plasma and of TPT. The LLQs were established at 0.10 ng/ml for the lactone and lactone plus carboxylate concentrations in rat plasma, and at 0.20 ng/ml for the concentrations of TPT in dog plasma (Table 1).

The method by Loos *et al.*[10] describes the simultaneous determination of the lactone and the carboxylate forms of TPT with sufficient separation between chromatographic peaks of endogenous materials, and of the carboxylate and lactone forms of TPT, with the LLQ established at 0.10 ng/ml for both TPT forms (Table 1). In this paper, a method for the determination of total TPT in urine is also described. Total TPT, with an LLQ of 10 ng/ml, is measured after acidification of the urine sample with orthophosphoric acid, resulting in the conversion of the carboxylate form into the lactone form.

Warner *et al.*[14] describe non-validated HPLC methods for the simultaneous determination of the lactone and carboxylate forms of several camptothecin analogs in phosphate-buffered saline and for topotecan also in human plasma. The only concentration tested is 2.5 ng/ml for both TPT forms, with a broad peak for the lactone form. Therefore, their application for

Table 1. HPLC methods with corresponding LLQ values for the analysis of TPT

Reference	Year	Matrix	Sample treatment	Detection		LLQ (ng/ml)		
				Ex (nm)	Em (nm)	Carbox	Lactone	Total
12	1995	HP	PP	361	527	–	0.05	–
			PP/AC	361	527	–	–	0.05
13	1996	RP	PP	361	527	–	0.10	–
			PP/AC	361	527	–	–	0.10
		DP	PP	361	527	–	0.20	–
			PP/AC	361	527	–	–	0.20
10	1996	HP	PP	381	525	0.10	0.10	–
		HU	AC	381	525	–	–	10
20	1997	HP	PP	390	520	0.25	0.50	–
				350–470	510–650	0.50	0.75	–
11[a]	1999	HP	PP	380	527	–	0.1	–
			PP/AC	380	527	–	–	0.1
		HU	AC	380	527	–	–	25
		HF[b]	E	380	527	–	–	0.3

Ex = excitation wavelength, Em = emission wavelength, HP = human plasma, RP = rat plasma, DP = dog plasma, HU = human urine, HF = human feces, PP = protein precipitation, AC = acidification, E = extraction with acetronitrile:ammonium acetate.
[a]Simultaneous determination of *N*-desmethyltopotecan.
[b]Concentration in μg/g feces.

simultaneous TPT lactone and carboxylate measurements in human plasma is not suitable for pharmacokinetic analysis in clinical trials after low i.v. dosages or oral administrations of TPT where low concentrations of the two forms of topotecan were expected.[15–19] Another publication of the same group[20] described an improved sensitivity of simultaneous determination of the lactone and carboxylate forms of topotecan in human plasma in comparison with already existing methods (Table 1). However, the LLQs for the lactone and carboxylate form were, respectively, 0.50 and 0.25 ng/ml using a tunable fluorescence detector with excitation and emission wavelengths of 390 and 520 nm, respectively. Using a filter detector, with an excitation filter of 350–470 nm and an emission filter of 510–650 nm, the LLQ values were, respectively, 0.50 and 0.75 ng/ml, which are still much higher than described earlier.[10,12]

Recently, an assay has been published for the simultaneous determination of TPT and *N*-desmethyltopotecan, one of the known metabolites of TPT, in human plasma, urine and feces.[11] For the determination of drug levels in plasma, two assays were developed, one for the determination of the lactone concentration and one for the determination of the total concentrations of TPT and its metabolite. The LLQ for the lactone as well as the total concentration of TPT and *N*-desmethyltopotecan was established at 0.1 ng/ml. In urine and feces only total levels of TPT and its metabolite were measured. The sample pretreatment for urine samples involved a dilution step in methanol, followed by acidification with phosphoric acid, resulting in LLQ values of 25 and 2.5 ng/ml of TPT and *N*-desmethyltopotecan, respectively. Fecal samples were homogenized in distilled water, followed by a double extraction with a mixture of acetonitrile and ammonium acetate, pH 4. The LLQ for total topotecan in feces was 0.3 μg/g, while the LLQ for *N*-desmethyltopotecan was established at 0.03 μg/g feces.

Irinotecan

CPT-11 (7-ethyl-10-{4-(piperidino)-1-piperidino}-carbonyloxycamptothecin; Figure 1) is a semisynthetic water-soluble analog of CPT, with limited intrinsic cytotoxic activity. In biological systems, CPT-11 is converted by carboxylesterases into its 100- to 1000-fold more active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin; Figure 1). The drug has been marketed in the USA and Europe for the treatment of 5-fluorouracil-refractory (metastatic) colorectal cancer.[1,4] Over recent years, several HPLC methods have been reported for the determination of CPT-11 and its pharmacologically active metabolite SN-38 in plasma. The analysis of these compounds is rather complicated because of the existence of chromatographic peaks of

other CPT-11 metabolites and the poor peak shapes. The peak shapes were optimized by using the cationic ion-pairing reagent tetrabutylammonium phosphate (TBAP) and analogs, which also enables the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 by increasing the retention times of the carboxylate forms on the analytical columns.

The first assay for the determination of total concentrations of CPT-11 and SN-38 in human plasma was described by Barilero *et al.*,[21] with a LLD for both compounds of 1.0 ng/ml. In the described method, a good separation was achieved between the total drug in the lactone form for both CPT-11 and SN-38 after acidification and solid-phase extraction of the plasma sample.

Rivory *et al.*[22] developed a HPLC method for the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 in human plasma. The plasma clean-up step involved a protein precipitation with a mixture of ice-cold methanol:acetonitrile (1:1, v/v). Adequate separation was achieved and the LLQs were established at, respectively, 10 and 2 ng/ml for both forms of CPT-11 and SN-38 (Table 2). The addition of mobile phase prior to injection to the protein-free supernatant was found to be an essential step in the assay. Omission of this buffer resulted in a complex of unresolved peaks. Two other metabolites were found under the chromatographic conditions, one of which has been identified as the β-glucuronide form of SN-38. A second method for the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 in human plasma has been developed and validated by Herben *et al.*[23] The sample pretreatment was based on the same principle as described above, with the LLQs established at 1.0 ng/ml for CPT-11 lactone and carboxylate, and at 0.5 ng/ml for the lactone and carboxylate forms of SN-38 (Table 2). A minor disadvantage of the latter published assay is the rather long overall run time of 20 min, which does not allow the analysis of large numbers of samples, since the protein-free extracts have to be injected directly after the addition of mobile phase.

Sumiyoshi *et al.*[24] developed a method for the simultaneous determination of total concentrations of CPT-11 and SN-38 in human plasma. The sample clean-up consisted of precipitation of plasma proteins with methanol. Subsequently, the samples were evaporated and reconstituted in acidified (pH 2) mobile phase. The LLQs were established at 30 ng/ml for CPT-11 and 1 ng/ml for SN-38 (Table 2). The rather high LLQ of CPT-11 is due to the selected excitation and emission wavelengths of 380 and 556 nm, respectively, to obtain maximum sensitivity for the determination of the pharmacologically active metabolite SN-38. No other metabolites of CPT-11 than SN-38 were reported in this publication.

A method for the simultaneous determination of the carboxylate and lactone forms of SN-38 has been described by Kaneda *et al.*[25] The described method is performed in rat plasma, with LLQs of 5 ng/ml for both forms of SN-38 (Table 2). The sample preparation consists of a protein precipitation with cold (-80°C) methanol followed by addition of aquous zinc sulfate (10%, w/v), followed by centrifugation and direct injection into the HPLC system.

Table 2. HPLC methods with corresponding LLQ values for the analysis of CPT-11 and SN-38

Reference	Year	Matrix	Sample treatment	Detection		LLQ CPT-11 (ng/ml)			LLQ SN-38 (ng/ml)			Metabolites (no.)
				Ex (nm)	Em (nm)	Carbox	Lactone	Total	Carbox	Lactone	Total	
22	1994	HP	PP	355	515	10	10	–	2	2	–	1
24	1995	HP	PP/AC	380	556	–	–	30	–	–	1	–
25	1997	RP	PP	380	540	–	–	–	5	5	–	–
26	1997	HP	LL	355	515	–	0.5	–	–	0.5	–	–
			PP/AC	355	515	–	–	2.0	–	–	2.0	1
23	1998	HP	PP	375/385[a]	460/525[a]	1.0	1.0	–	0.5	0.5	–	–
27	1998	HU/HF	PP/AC	355	515	–	–	200	–	–	100	2
29	1998	HP	AC/SP	380	532	–	–	–	–	–	0.004	–
30	1999	HP	PP/AC/LL	380	556	–	–	–	–	–	0.005	–
31	1998	DP/RP	PP	362/375[a]	425/560[a]	4.8	5.9	–	1.6	2.4	–	–
32	1999	RP	AC/SP	373/380[a]	420/540[a]	–	–	5	–	–	5	1
33	1999	HS	PP	MS	–	–	–	10	–	–	–	–
			LL	MS	–	–	–	–	–	–	0.5	–

Ex = excitation wavelength, Em = emission wavelength, HP = human plasma, RP = rat plasma, DP = dog plasma, HU = human urine, HF = human feces, HS = human serum, PP = protein precipitation, AC = acidification, SP = solid-phase extraction, LL = liquid–liquid extraction, metabolites = number of identified metabolites (other than SN-38), MS = detection using mass spectrometry.
[a]Setting for CPT-11/SN-38, respectively.

The most sensitive assays available thus far for the simultaneous determination of lactone and total levels of CPT-11 and SN-38 have been developed and validated by De Bruijn *et al.*[26] with a LLQ for the lactone of 0.5 ng/ml for CPT-11 and SN-38 (Table 2). The plasma sample clean-up for the lactone measurement consisted of a single liquid–liquid extraction technique with acetonitrile:*n*-butylchloride (1:4, v/v). The measurement for the determination of the total forms was carried out in a second analysis with LLQs of 2.0 ng/ml for both compounds (Table 2). The plasma samples were acidified and deproteinized with a mixture of perchloric acid and methanol, which resulted in the conversion of the carboxylate forms into the lactone forms, followed by determination of the lactone form. Six other peaks were found in the plasma samples of patients in the assay for the determination of the total forms of CPT-11 and SN-38. Two of them disappeared after incubation of a plasma sample with β-glucuronidase, while the concentration of SN-38 increased, which is indicative for the presence of a β-glucuronide conjugate (SN-38G) of SN-38 in plasma samples of cancer patients treated with CPT-11. This method was subsequently modified to allow analysis of other metabolites in plasma, urine and feces samples as well.[27] Two metabolites of CPT-11 were analyzed and validated in human plasma, known as SN-38G and 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxy-camptothecin (also referred to as APC). The described method for the determination of both metabolites in human plasma was based on the assay described by De Bruijn *et al.*[26] with a slightly modified mobile phase and the plasma extract was necessarily diluted 2-fold with mobile phase prior to chromatography, because of the unusual chromatographic behavior of APC and SN-38G. The LLQ was established at 10 ng/ml APC and SN-38G. The change in mobile phase as compared to the earlier described method resulted in poor accuracy and precision for CPT-11 and SN-38, due to severe tailing bands, particularly below 100 ng/ml. CPT-11 and SN-38 measurements in plasma samples were carried out by re-injection of the plasma supernatant using the earlier described method.[26] Urine and homogenized fecal samples were diluted (1:1, v/v) in blank plasma and further processed as described for human plasma samples, followed by a 10-fold dilution in mobile phase. The LLQs were established at 100 ng/ml for SN-38 and SN-38G, and at 200 ng/ml for CPT-11 and APC (Table 2). The method was also validated for a second major oxidative metabolite of CPT-11, i.e. NPC, in human plasma samples with similar validation characteristics.[28]

Since the terminal disposition half-life of SN-38 in cancer patients treated with CPT-11 could not be estimated accurately in early pharmacokinetic studies, an assay for the determination of SN-38 at lower concentrations was needed. The first very sensitive assay was reported by Rivory *et al.*[29] The plasma sample was acidified prior to solid-phase extraction and the LLQ for the total concentration of SN-38 was established at 10 pM (about 4 pg/ml) (Table 2). However, the recovery of SN-38 was concentration dependent and ranged from 48 up to 92%, and therefore log–log calibration curves with least-squares linear regression were required. CPT-11 did not interfere with the assay. Compared to this method for the determination of SN-38 at low concentrations, a simplified method with comparable sensitivity has been described recently.[30] The method described by Rivory *et al.* consisted of a time-consuming solid-phase extraction and showed concentration-dependent recoveries. In the assay described by de Bruijn *et al.*,[30] protein precipitation followed by a one-step solvent extraction with chloroform was used for sample clean-up. The LLQ was established at 5 pg/ml (Table 2), with standard curves being linear over nearly three orders of magnitude. The use of acetonitrile as organic modifier in the mobile phase instead of methanol resulted in sharpening of the peaks and improved peak symmetry. No interference of CPT-11 was observed in the analytical runs.

Chollet *et al.*[31] described a method for the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 in rat and dog plasma. This is yet another method, in which the lactone and carboxylate forms could be determined simultaneously, after cold (-20°C) methanol protein precipitation. The LLQs in dog and rat plasma were similar, and were validated at 4.8 and 5.9 ng/ml for the carboxylate and lactone forms of CPT-11, respectively, and at 1.6 and 2.4 ng/ml for the carboxylate and lactone form of SN-38, respectively (Table 2).

Kurita *et al.*[32] have also described a method for the determination of total levels of CPT-11 and its metabolites SN-38 and SN-38G in rat plasma. The LLQs were established at 5 ng/ml for CPT-11 and SN-38 in rat plasma (Table 2). The method described determination of the compounds with a fully automated on-line solid-phase extraction system, which may have a potential advantage for processing large numbers of samples simultaneously.

Recently, a non-fluorescence HPLC method has been developed,[33] using electrospray mass spectrometry, for the detection of CPT-11 and SN-38 concentrations in human serum. The sample clean-up for the measurement for CPT-11 involved a protein

precipitation with a LLQ of 10 ng/ml, while the LLQ for SN-38 was validated at 0.5 ng/ml after a liquid–liquid extraction (Table 2). The use of a mass spectrometer as a detector does not increase the sensitivities of the determinations of CPT-11 and SN-38 compared to previously reported methods using an ordinary fluorescence detector.

9-Aminocamptothecin

9-AC (NSC 603071; Figure 1) was the first synthetic analog of CPT with promising antitumor efficacy in *in vivo* models. However, 9-AC was inappropriate for further clinical development, due to its poor water solubility. Eventually, the solubility problems were solved by the development of a colloidal dispersion formulation and 9-AC has since been implemented in numerous clinical trials with the drug given either by bolus or prolonged continuous i.v. infusion schemes or orally.[1,4,34]

Up to now, four analytical methods have been published for the determination of the lactone and the lactone plus carboxylate form of 9-AC in human plasma. In the presence of HSA, the lactone form of 9-AC is rapidly converted to the carboxylate form with the equilibrium at the site of the carboxylate, which also necessitates a rapid processing of the blood samples for pharmacokinetic studies. In all methods, the blood sample was centrifuged directly, although the plasma was processed using totally different methods. The method published by Supko *et al.*[35] for the measurement of the intact lactone of 9-AC requires a rapid deproteinization of the plasma sample with methanol, followed by direct injection of diluted supernatant into the HPLC system. For the measurement of the total drug concentration, the plasma sample was acidified using perchloric acid, followed by methanolic deproteinization. To increase the sensitivity of the assays, an in-line post-column acidification of the eluent to pH 1.8–2.2 was necessary, which results in a LLQ of 5.0 ng/ml for the lactone and total concentration of 9-AC (Table 3).

A more sensitive assay was developed by Takimoto *et al.*[36] with LLQs of 0.09 and 0.9 ng/ml (Table 3) for the lactone and total concentrations of 9-AC, respectively. Using a solid-phase extraction for the determination of the lactone form immediately after collecting the plasma, which separated the lactone from the carboxylate, the sample could be stored for at least 2 months at -70°C prior to analysis. For the measurement of total 9-AC concentrations, the plasma samples were acidified prior to solid-phase extraction by a 10-fold dilution with phosphoric acid (Table 3).

Another, more convenient and sensitive assay was developed later and does not require a direct sample clean-up step.[37] The lactone to carboxylate ratio of 9-AC was stabilized, for at least 4 months, by immediate freezing of the plasma samples at the site of the patient. After thawing the samples, the lactone form was extracted into an organic phase using liquid–liquid extraction, with a mixture of acetonitrile:*n*-butylchloride (1:4, v/v), while the carboxylate form remains in the water phase. For the determination of the total 9-AC concentrations, the sample clean-up consists of a simultaneous protein precipitation/acidification step with a mixture of methanol and perchloric acid. The LLQs were established at, respectively, 0.05 and 0.10 ng/ml for the lactone and lactone plus carboxylate forms (Table 3).

A sample clean-up procedure involving a direct deproteinization of the plasma at the site of the patient with cold methanol has been reported recently.[38] For the determination of the lactone form, the methanolic extract should be further processed within 48 h after sampling, using a solid-phase extraction procedure,

Table 3. HPLC methods with corresponding LLQ values for the analysis of 9-AC

Reference	Year	Matrix	Treatment	Detection		LLQ (ng/ml)	
				Ex (nm)	Em (nm)	Lactone	Total
35	1992	HP	PP	352	418	5.0	–
			AC/PP	352	418	–	5.0
36	1994	HP	SP	365	440	0.09	–
			AC/SP	365	440	–	0.9
37	1997	HP	LL	370	450	0.05	–
			PP/AC	370	450	–	0.10
38	1998	HP	PP/SP	370	450	0.2	–
			PP/AC	370	450	–	0.2

Ex = excitation wavelength, Em = emission wavelength, HP = human plasma, PP = protein precipitation, AC = acidification, SP = solid-phase extraction, LL = liquid–liquid extraction.

while for the determination of total drug levels the methanolic extract was acidified prior to injection into the HPLC system (Table 3). The LLQs for the lactone and total concentrations of 9-AC in human plasma were established at 0.2 ng/ml for both the lactone as well the total drug levels.

Lurtotecan

LRT (G147211, 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(*S*)-camptothecin; Figure 1) is also a semisynthetic analog of CPT and has recently been formulated as a liposomal preparation with the intent to stabilize the lactone moiety of the compound and so improve the efficacy of LRT.[39]

Only two methods were validated and published for the determination of the drug after administration of non-liposomal LRT. Stafford *et al.*[40] published the first of these, and describe the quantitation of the lactone and carboxylate forms of LRT in dog plasma using solid-phase extraction techniques. For the lactone-only determination the plasma was diluted with a buffer of pH 7.4 and applied on a solid-phase cartridge, followed by a wash step which removes the carboxylate, while the lactone form remains at the cartridge, and was eluted and concentrated before injection into the HPLC system. For measurement of the total concentration of LRT, the plasma was acidified with hydrochloric acid before solid-phase extraction. The excitation and emission wavelengths were set at 378 and 420 nm, respectively. The LLQ was established at 0.05 and 0.10 ng/ml for the lactone and total plasma concentrations, respectively, which was the most sensitive determination of any CPT analog reported at that time.

The second method was developed and published by Selinger *et al.*[41] in which only a method for the determination of the lactone form of LRT is described, using human whole blood as matrix. The advantage of using whole blood instead of plasma is the rapid and simple sample handling at the site of the patient. After drawing the blood sample, it can be kept for a maximum of 30 min in an ice-water bath, before freezing at -70°C. On the day of analysis the blood sample is further processed using a liquid–liquid extraction, with a mixture of acetonitrile:*n*-butylchloride (1:4, v/v), for the measurement of the lactone form. The LLQ has been validated at 0.15 ng/ml, using fluorescence detection as described above.

A method for the determination of total LRT levels in human plasma and urine after administration of NX211, i.e. liposomal LRT, has recently been developed and validated.[42] The sample clean-up for the determination of total drug levels in plasma involved a deproteinization with 10% (w/v) aqueous perchloric acid:acetonitrile (2:1, v/v), while for the determination of the unchanged drug in urine a single solvent extraction with *n*-butanol:diethyl ether (3:4, v/v) was accomplished after acidification of the urine sample. Fluorescence detection in both assays was performed with excitation and emission wavelengths of, respectively, 378 and 420 nm. The LLQ in plasma was established at 1.0 ng/ml, which is sufficient for pharmacokinetic analysis of patient samples in an ongoing phase I trial. The fluorescence signal of LRT in the urine assay was increased 14-fold prior to detection by post-column exposure of the eluent to UV light, resulting in an LLQ of 0.50 ng/ml in the human urine samples.

Conclusions and perspectives

CPTs form a class of antineoplastic agents demonstrating significant antitumor activity against a broad range of human malignancies, including refractory ovarian and colorectal cancers. In recent years, a substantial number of publications have yielded valuable insight into the mechanisms of action and resistance, clinical pharmacodynamics and considerations of dosage and schedule, and route of drug administration. Many of these studies have been made possible by the development of selective analytical methodologies to specifically monitor the parent drugs and individual biotransformation products, with sufficient sensitivity to detect the compounds at levels achieved after therapeutic dosing.

The pH-dependent instability of the lactone moiety in the core structure of the CPTs necessitates a rapid centrifugation of the blood sample, preferably at the site of the patient, to collect the plasma supernatant. Even when only total concentrations of the CPTs are to be measured, this rapid collection of the plasma is necessary since the lactone forms of these drugs are able to diffuse across cell membranes, including those of the red blood cells, and thus a change in the lactone to carboxylate ratio has an effect on the total drug concentrations in the plasma compartment. To ensure adequate measurements of the lactone concentrations, the plasma samples have to be further processed immediately after centrifugation.

The most laborious methods for the determination of the lactone-only concentrations are those in which each individual plasma sample has to be analyzed or extracted directly after collection of the plasma. Clearly, the most convenient approach at the site of the patient is the one in which the lactone to

carboxylate ratio is stabilized by direct freezing of the plasma or whole blood samples. For the lactone only measurements, the samples were further processed using either solid-phase or liquid–liquid extraction techniques. In both cases, only the lactone form is extracted, while the carboxylate form is eluted during the wash steps in the case of the solid-phase extractions or remains in the water-phase in case of the liquid–liquid extractions. The total concentrations of the CPTs in the directly frozen plasma samples were analyzed after acidification of the samples followed by solid-phase extractions of the total amount of the drugs in the lactone form or by measurements of the compound in the lactone form after injection of supernatants of deproteinized and acidified samples. Another practically convenient approach to stabilize the lactone to carboxylate ratio is by methanolic deproteinization of plasma samples directly at the site of the patient. The methanolic extracts should be stored upon analysis at a minimum of $-70^{\circ}C$ to prevent degradation of the lactone form. The advantage of this stabilization is the possibility of simultaneous measurement of the lactone and carboxylate forms of the CPTs in one single run. However, this approach is not feasible for all CPT analogs, except for TPT and CPT-11, since the separation between the hydrophilic carboxylate forms and endogenous compounds (with similar fluorescence characteristics) in the reversed-phase HPLC methods are not sufficient enough for adequate determination of the carboxylate forms. Moreover, the overall run times have to be as short as possible to enable determination of complete runs of patient samples during the day time, since the lactone to carboxylate ratio is not stable at $4^{\circ}C$,[10] making automated injections overnight infeasible. For methods in which insufficient separation between the carboxylate form and endogenous compounds was achieved, the methanolic extracts were acidified and the total concentrations of the drugs were measured in a second analysis. Since the camptothecins have strong fluorescence characteristics, relatively low concentrations of these compounds could be measured in biological matrices, even after simple protein precipitation extraction procedures without the need of any concentration step.

The new dimension in chemotherapy provided by TPT, CPT-11 and other analogs in the treatment of a variety of (solid) tumors assures growth in the area of CPT-related chemotherapeutic drugs. In general, with the continued application of clinical pharmacokinetic studies, coupled with new approaches in CPT drug design and formulation, more rational and selective chemotherapy should be possible in the future.

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(Received 4 March 2000; revised form accepted 21 March 2000)