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Review

Quantitation of camptothecin and related compounds

M. Palumbo*, C. Sissi, B. Gatto, S. Moro, G. Zagotto

Department of Pharmaceutical Sciences, University of Padova, Via Marzolo, 5, 35131 Padova, Italy

Abstract

Camptothecin and congeners represent a clinically very useful class of anticancer agents. Proper identification and quantitation of the original compounds and their metabolites in biological fluids is fundamental to assess drug metabolism and distribution in animals and in man. In this paper we will review the recent literature available on the methods used for separation and quantitative determination of the camptothecin family of drugs. Complications arise from the fact that they are chemically labile, and the pharmacologically active lactone structure can undergo ring opening at physiological conditions. In addition, a number of metabolic changes usually occur, producing a variety of active or inactive metabolites. Hence, the conditions of extraction, pre-treatment and quantitative analysis are to be carefully calibrated in order to provide meaningful results.

Keywords: Reviews; Camptothecin; Topotecan; Irinotecan; Lurtotecan; SN-38

Contents

1. Introduction

The discovery that the (*S*) enantiomer of camptothecin (CPT), a compound isolated from the bark, leaves and fruit of *Camptotheca acuminata*, can kill cells through the selective poisoning of the human enzyme topoisomerase I bound to its substrate DNA [1], was an important breakthrough for its development as an anticancer agent. The intact lactone form of CPT, first isolated over 30 years ago [2], can bind non-covalently to the complex formed by

*Corresponding author.
E-mail address: manlio.palumbo@unipd.it (M. Palumbo).

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topoisomerase I and DNA, inhibiting the resealing of the broken DNA backbone. This intrinsic ability of CPT to trap this complex has been clearly linked to its antitumor activity, especially for tumors over-expressing topoisomerase I, such as colorectal and cervical cancer [3,4]. Nanomolar concentrations of the drug are in fact sufficient to cause DNA damage in vivo, which becomes irreversible following its collision with DNA processing machineries, such as the proceeding replication forks during the S phase of the cell cycle. This collision event produces irreparable damage in the DNA [5], and finally results in cell death [6,7].

CPT, despite its remarkable side-effects, has become such a promising antitumor agent, that extensive research, considering both pharmacokinetics and pharmacodynamics, has led to the successful development of new closely related compounds. Among them, irinotecan (CPT-11) and topotecan (TPT) have been approved for the treatment of metastatic colorectal cancer and refractory ovarian cancer respectively, and their clinical pharmacology has been thoroughly discussed in other recent reviews [3,8]. New potent and water soluble derivatives have been synthesized and are now in clinical studies, while other potent drug candidates are at the preclinical stage as second generation camptothecins [9]. Indeed, functionalization at position 7, 9 and 10 is compatible with increased activity, as shown by the 9-amino-20-(*S*)-camptothecin, CPT-11, TPT and other amino-containing moieties [10]. Position 11 can be made part of an additional five or six-member ring including position 10, yielding compounds more active than CPT, like lurtotecan (GI147211), which is currently in clinical trials [3]. Position 7 and 9 can be closed in a ring too, offering therefore new opportunities for derivatization to obtain water soluble compounds, as exemplified by many hexacyclic derivatives with good in vitro and in vivo activity [11], and by the excellent cytotoxicity exhibited by compound DX-8951 [12], which is also in clinical trials and is active in some pancreatic tumor cells lines [13]. While the sixth ring can be all-carbon or a heterocycle, the simultaneous presence of an amino group at position 10 of the CPT ring increases potency. Position 11 is still somewhat sensitive to steric hindrance, since the greatest activity has been shown by compounds substituted with a small and

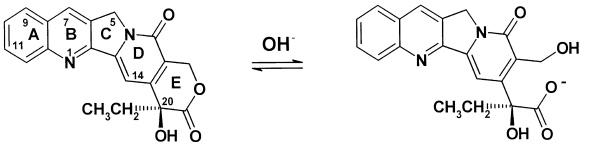


Fig. 1. Ring-opening equilibrium of CPT.

electron-withdrawing group, consistently with the structure-activity relationship (SAR) of CPT itself [14].

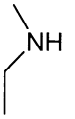
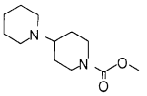
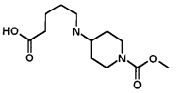
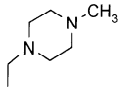
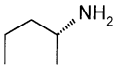
To draw proper quantitative structure–activity relationships and monitor the drug effects in different cell lines and, subsequently, in patients, it is of fundamental relevance to devise safe, flexible, rapid and sensitive analytical methods for the quantitative determination of the original compounds and their (active or inactive) metabolites. The issue of determination of CPT and analogs in biological fluids has been recently reviewed [15]. It is important to recall that the lactone ring structure is not stable at physiological conditions and tends to undergo reversible conversion between a pharmacologically active lactone form and its inactive, lactone ring-opened, carboxylate form (Fig. 1). 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. Due to chemical instability of the drug and to the fact that the lactone forms of these drugs are able to diffuse across cell membranes, including those of the red blood cells, it is very important to operate a rapid extraction and processing of the CPT derivative, along with appropriate sample pre-treatment procedures.

This review will discuss the methods and conditions currently utilized for the separation and quantitative evaluation of CPT and related analogs in clinical trials as well as in preclinical development. Their metabolic products in living organisms will also be considered. The chemical structure of the compounds examined is reported in Table 1.

2. Camptothecin (CPT)

CPT is not an optimal drug, as it exhibits very limited water solubility, in addition to severe toxicity

Table 1
Substitution pattern of pharmacologically relevant CPT analogs

Compound	Substituent position			
	7	9	10	11
Camptothecin (CPT)	H	H	H	H
HCPT	H	H	OH	H
9-AC	H	NH_2	H	H
Topotecan (TPT)	H		OH	H
N-desmethyl-TPT	H		OH	H
N-bisdesmethyl-TPT	H		OH	H
Irinotecan (CPT-11)	$-CH_2CH_3$	H		H
SN-38	$-CH_2CH_3$	H	OH	H
APC	$-CH_2CH_3$	H		H
Lurtotecan (GI147211)		H		
Exatecan (DX8951)			CH_3	F

and erratic absorption. As a matter of fact, its investigation for anticancer drug use was discontinued in the 1970s. However, the following developments associated with the discovery that it targets topoisomerase I, brought CPT back to the clinical setting for further testing. Of course the problems connected to drug pharmacokinetics render it necessary to devise adequate methods for detection and quantitative analysis.

2.1. Analytical separation procedures

Analytical procedures consisted in general in HPLC separation with fluorescence detection.

A sensitive HPLC assay was developed to evaluate the lactone forms of CPT in human plasma as well as its total (lactone plus carboxylate form) concentration [16]. These forms were extracted using different extraction procedures. Total CPT was obtained combining plasma with methanol to ensure deproteinization and acidifying with HCl the clear supernatant before HPLC analysis. The acidification step induces transformation of the carboxylate form into the lactone form. Selective extraction of the lactone was performed by a C_{18} solid-phase column. Washing the column with 20% methanol is effective in completely removing the carboxylate form. Lactone CPT was eluted with 75% methanol and 25 m*M*

KH_2PO_4 at pH 2.55. The extracted lactone samples could be stored without requiring immediate HPLC analysis. The two extracts of CPT were quantitated by reversed-phase HPLC with fluorescence detection. The extraction efficiencies were about 100% and 92% for the total and lactone CPT, respectively. The lower limit of quantitation was about 2 ng/ml for the two preparations. The method was reproducible with a mean inter-day and intra-day variability of 6% for total CPT and 4% and 6%, respectively, for lactone CPT. The assay could effectively quantitate lactone and total CPT in patients receiving single dose.

As mentioned before, a key issue is the precise determination of the lactone and hydroxy-acid form of CPT. Indeed, the analytical methods used for extraction and quantitation of the open and closed form could affect the results to a remarkable extent. A versatile method for the simultaneous quantitation of the two forms has been proposed by Warner and Burke [17]. Previous HPLC separations of the lactone and carboxylate forms of CPTs often required mobile phases containing three to four components; ion-pairing reagent to provide adequate retention of the carboxylate form of the drug; buffer to control the ionic strength and pH of the mobile phase; acetonitrile to control the retention of the lactone form and, in some instances, sodium dodecyl sulfate to reduce peak tailing. In this study, the authors have developed a simplified HPLC methodology, according to which the mobile phase employed included only triethylamine acetate (TEAA) buffer (1–3% triethylamine in water (v/v) adjusted to pH 5.5 with glacial acetic acid) and acetonitrile. TEAA is suggested to act as the ion-pairing reagent, as a masking reagent for underivatized silanols and as the major buffer component. As reported in Fig. 2, this mobile phase provides simultaneous separation of both the carboxylate and the lactone form of camptotecin in less than 10 min. In addition to the adequate retention time of the two species, it can control the tailing due to analyte interaction with underivatized silanols. The authors report the successful simultaneous separation of the lactone and carboxylate forms of CPT and four related analogs by altering only the composition of TEAA buffer with respect to acetonitrile. This suggests that the newly proposed mobile phase could be used for rapid and sensitive analysis of the several CPT derivatives presently under investigation.

The same type of determination was set up for analyzing for lactone and total 9-amino camptothecin (9-AC) in human plasma. In one instance a method that eliminates the need of solid-phase extractions is described [18]. For 9-AC lactone, the sample preparation consisted in a liquid–liquid extraction with acetonitrile-*n*-butyl chloride (1:4, v/v). The supernatant is evaporated and added to a methanol–perchloric acid–water (1:3:1500 v/v) solution before HPLC injection. The sample preparation for 9-AC total (lactone plus carboxylate) was a simple deproteinization with 5% perchloric acid–methanol (1:1, v/v), which results in the conversion of the carboxylate into the lactone form. Different mobile phases were used for the two assays (Fig. 3): for the lactone form a mixture of methanol–water (40:60 v/v) adjusted to pH 2.20 by addition of perchloric acid, for the total 9-AC a different methanol–water mixture (32.5:67.5 v/v) adjusted to pH 2.10 using perchloric acid. The lower limits of quantitation were 50 pg/ml and 100 pg/ml for 9-AC lactone and 9-AC total, respectively. The within-run precisions at four tested concentrations were ≤6.3% for 9-AC lactone and ≤5.3% for 9-AC total. The between-run precisions were ≤8.9% and ≤5.6%, respectively. The proposed methodology enables pharmacological analysis of 9-AC in a bioavailability and oral phase I. Other authors determined lactone and total 9-AC in micro-volume of human plasma [19]. The analytical methodology involves a protein precipitation step with cold methanol (−30°C) as sample pretreatment procedure. The methanolic extract is used for the determination of total 9-AC. The intact lactone form of 9-AC is separated from the hydroxycarboxylate form in the methanolic plasma extract by solid-phase extraction using a C_{18} column washed with methanol–water mixtures. After evaporation to dryness (nitrogen, 40°C) the extract can be stored at −70°C for at least 3 weeks. The drug is analyzed by reversed-phase liquid chromatography, using methanol–water eluent (pH 2.2) (30:60 w/w) and fluorescence detection (excitation and emission wavelengths set at 370 and 450 nm, respectively). The emission vs. concentration plot is linear over a concentration range 0.2–100 ng/ml with a detection limit and a limit of quantitation of 0.05 (lactone form) and 0.2

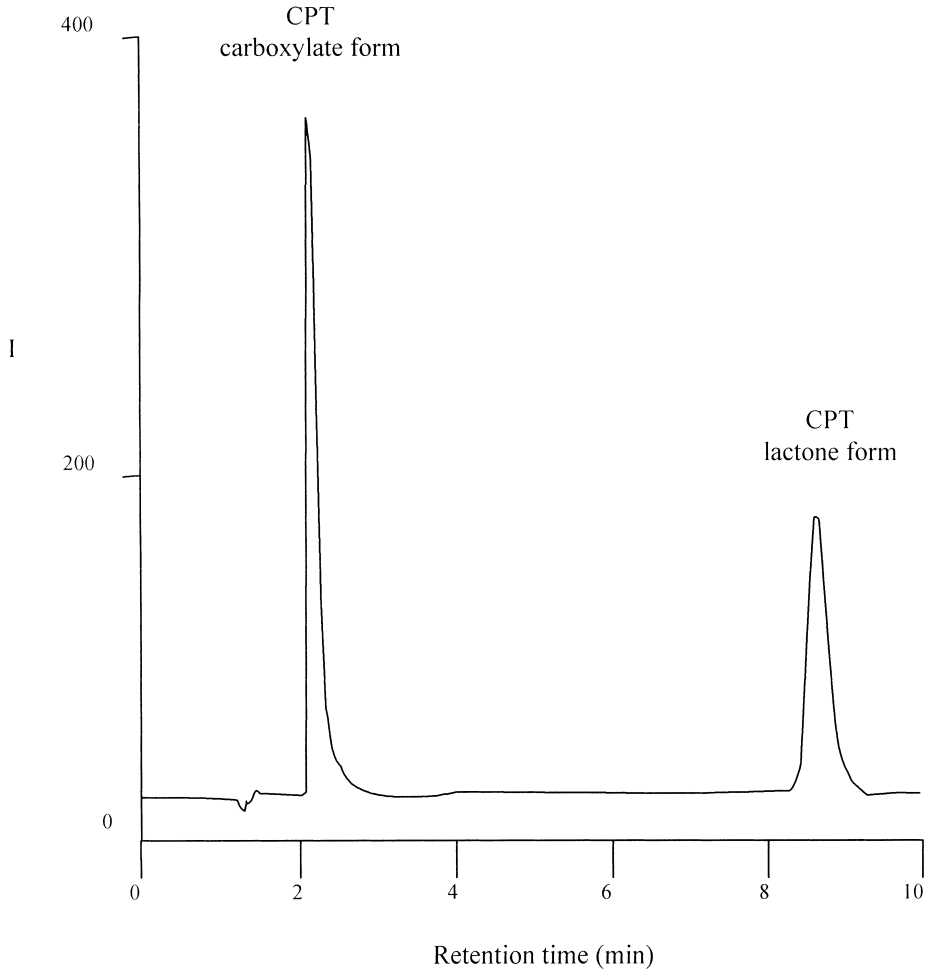


Fig. 2. HPLC separation of the carboxylate/lactone forms of CPT. Column: Waters NovaPak-C_{18} 4 μm particle-size reversed-phase, 150×3.9 mm I.D. column. Mobile phase: 23:77 (v/v) acetonitrile–TEAA buffer (1% v/v), pH 5.5. Flow-rate 1 ml/min. Detection: fluorescence. Excitation 305–395 nm, emission 430–470 nm.

ng/ml (total), starting from a 100 ml plasma sample. The proposed method has been implemented in a phase I clinical trial for pharmacokinetic evaluation of this potential new drug.

Studies on the liver metabolism and biliary excretion of CPT were performed using an isolated perfused rat liver system [20]. CPT was added to the perfusion medium at a concentration of 20 μ*M*, and bile and perfusate samples were collected for 90 min. CPT (lactone and carboxylate) and three novel metabolites (M1–M3) were identified by mass spectroscopy and quantified by reversed-phase HPLC. Kinetic parameters of CPT and its biotransformation products were then estimated in bile and effluent perfusate. Biliary secretion of CPT and its three metabolites reached a peak secretion of 37.6±16.3, 0.94±0.29, 0.19±0.023 and 0.302±0.076 nmol/g liver/min, respectively, after 20 min. The total amount of CPT and M1–M3 excreted into bile during 90 min of perfusion was 63±15.4%, 1.8±0.37%, 0.43±0.06%, and 0.72±0.15% of CPT cleared from the perfusate during 90 min, respectively. In the perfusate, only one metabolite (M3) could be detected. Analysis of the biliary metabolites by

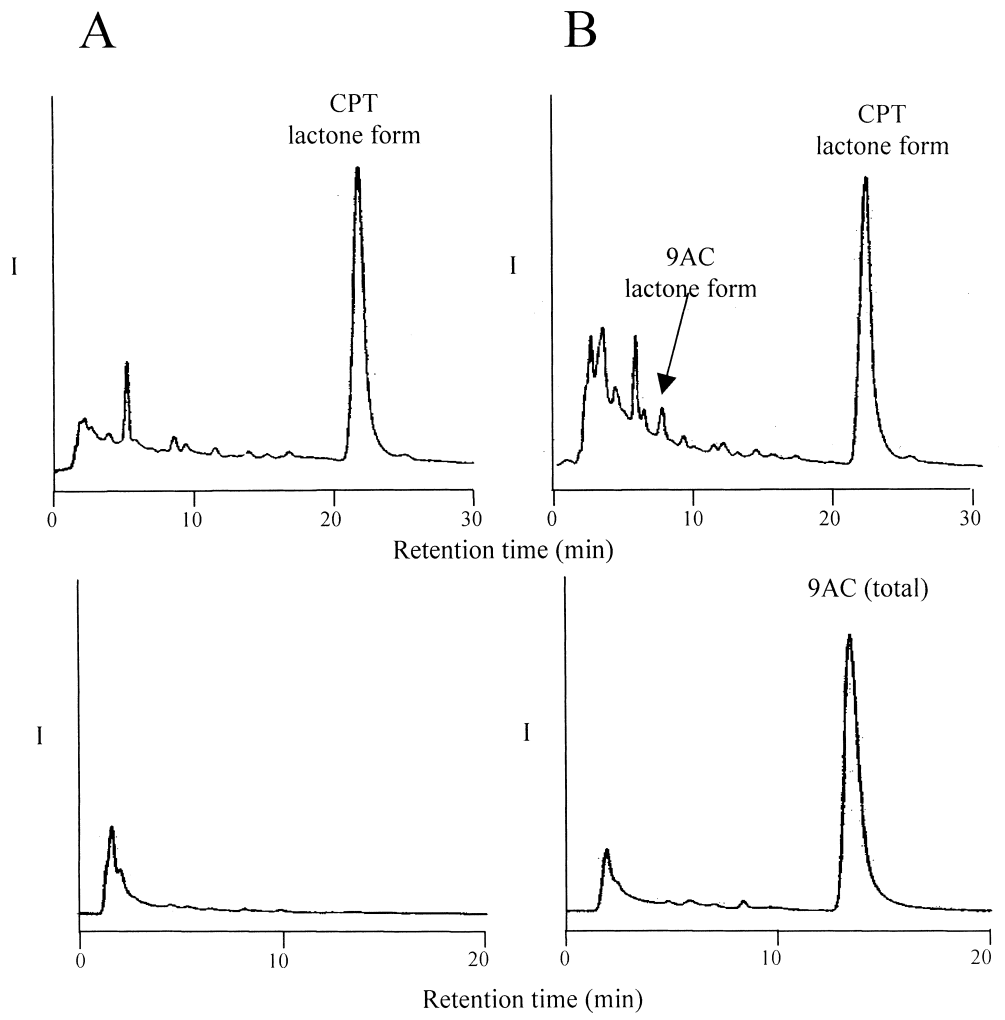


Fig. 3. HPLC separation of 9-AC and CPT as the lactone form (A and B) or total (lactone and carboxylate forms) (C and D). (A) Human plasma containing 250 pg/ml of CPT. (B) Human plasma containing 250 pg/ml of CPT and 171 pg/ml of 9-AC. (C) Ten-fold diluted human plasma. (D) Ten-fold diluted human plasma containing 89 264 pg/ml of 9-AC. Column: Inertsil ODS-80A 5 μm particle-size, 150×4.6 mm I.D. column thermostatted at 40°C. Mobile phase (A and B): 40:60 (v/v) methanol–water, pH 2.2 by perchloric acid. Flow-rate 1 ml/min. Mobile phase (C and D): 32.5:67.5 (v/v) methanol–water, pH 2.1 by perchloric acid. Flow-rate 1 ml/min. Detection: fluorescence. Excitation 370 nm, emission 450 nm, bandwidth 40 nm.

mass spectrometry supported the formation of dihydroxy-CPT derivatives (M1 and M2), whereas M3 appears to be a monohydroxy-analog.

Finally, to determine biliary excretion and concentrations in blood and brain of unbound CPT, a flow-through microdialysis probe was constructed for sampling [21,22]. The shunt linear probe was connected from the bile duct, between the liver side to the duodenum to avoid obstruction of the bile duct or bile salt waste. Microdialysis probes were also implanted into the jugular vein and striatum of rats for unbound drug sampling and determination. CPT was then administered from the femoral vein, and microdialysates were collected from blood and brain of both sites. For automatic analysis of microdialysate, an on-line injector was connected to a microbore high-performance liquid chromatographic (HPLC) column with fluorescence detection (excita-

tion and emission wavelengths set at 360 and 440 nm, respectively). Samples were eluted with a mobile phase containing methanol–100 m*M* monosodium phosphoric acid (35.65, v/v, pH 2.5, adjusted with orthophosphoric acid). The limit of quantification was 1 ng/ml for CPT. Pharmacokinetic parameters in blood and brain [22] were calculated from the corrected data for dialysate concentrations of CPT versus time. CPT was found to rapidly enter the extracellular fluid of brain striatum at 10 min following drug administration.

3. Topotecan (TPT)

Topotecan (TPT), a soluble semi-synthetic derivative of camptothecin, is a specific inhibitor of topoisomerase I and is endowed with potent antiproliferative effects in vitro and in vivo on tumoral cell lines as well as on endothelial cells. Moreover, TPT is able to interfere with the development of blood vessels in many in vivo experimental models.

3.1. Analytical separation procedures

Generally, separation procedures consisted in high-performance liquid chromatography.

An HPLC method for determination of TPT as lactone or total (lactone plus carboxylate forms) in human plasma has been recently presented [23]. Linear responses in analyte standard peak area were observed over the concentration range 0.05–10 ng/ml using 100 μl plasma samples. For lactone determination, the instability of the drug in the biological matrix required to obtain the plasma fraction within 5 min after blood sampling by centrifugation, immediately followed by protein precipitation with cold methanol (−30°C). Stability studies have indicated that TPT is stable in these methanolic extracts for at least 4.5 months at −30°C and 2 months at −70°C. For the total determination of the lactone plus lactone ring-opened forms of the drug, plasma samples were deproteinated with methanol and, subsequently, acidified with 7% (v/v) perchloric acid. Plasma samples for the measurement of total levels of the lactone and the ring-opened forms of TPT were stable for at least 4.5 months when stored at −30°C. After centrifugation, the supernatants were analyzed by HPLC using a C_{18} reversed-phase column and methanol–0.1 *M* hexane-1-sulfonic acid in methanol–0.01 *M* *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) in distilled water pH 6.0 (25:10:65, v/v) as the mobile phase. TEMED was used to reduce tailing and to buffer the eluent at pH 6.0 as the rate of conversion of carboxylate–lactone is relatively low at these conditions. Additionally, at pH 6.0 the basic side chain of topotecan gives ion pairs with hexane sulfonic acid in the eluent resulting in an increased retention and better peak shape. Detection was again performed fluorimetrically with an excitation wavelength of 361 nm and an emission wavelength of 527 nm with an 40 nm bandwidth. Within-run and between-run precision was always less than 12% in the concentration range of interest (0.05–10.0 ng/ml). The limit of quantitation was 0.05 ng/ml. Accuracy measurements ranged between 87.6 and 113.5%. This method was used to evaluate the effects of column temperature in the chromatographic determination of TPT in rat and dog plasma [24]. The analytical procedure (sample pre-treatment, column, mobile phase) was essentially the same, whereas the column was thermostatted between 12 and 25°C. The better resolution between an interfering endogenous compound in rat and dog plasma and TPT was obtained at 19–21°C. However, this endogenous peak was absent in human plasma.

A similar protocol has been used to compare TPT in human plasma and saliva [25]. The sample pretreatment and the supernatant analysis were as reported in [23] but the proportions of mobile phase components was 28:10:62, v/v/v. The assay showed linearity in the tested range of 0.1–75 ng/ml. The limit of quantitation was 0.05 ng/ml. Precision was in the range 0.4–17% (limit of quantitation). Accuracy ranged from 85 to 109%. Extraction recovery from plasma or parotid saliva averaged 90%. The stability of TPT as its lactone form in plasma, blood, and methanolic extracts was tested under various conditions. Particularly interesting was the higher stability of the lactone form in the whole blood.

As for CPT, the quantitation of the lactone and open form of TPT is quite important for pharmacological and structure–activity relationship studies. Filter and tunable fluorescence detection can be employed to detect the drug [26]. Limits of detection in plasma of 0.10 ng/ml for carboxylate and 0.26

ng/ml for lactone have been obtained using a tunable fluorescence detector. A filter fluorescence detector produced limits of detection of 0.15 ng/ml for carboxylate and 0.30 ng/ml for lactone. Reproducible quantitation using a tunable fluorescence detector ranged from 0.25 to 250 ng/ml for carboxylate and from 0.50 to 250 ng/ml for lactone. The filter detector, which had not been previously studied, provided reproducible detection from 0.50 to 250 ng/ml for carboxylate and from 0.75 to 250 ng/ml for lactone.

The major metabolites of TPT are the *N*-desmethyl and bis-*N*-desmethyl derivatives. The isolation and structural confirmation of *N*-desmethyl-TPT has been carried out during pharmacokinetics studies [27]. While performing the analysis of plasma and urine samples collected in those studies, an additional peak eluting just after TPT was observed. Approximately 100 ng of this potential metabolite was isolated from human urine using a solid-phase extraction procedure and purification by HPLC. Analysis of the isolated material showed it to be approximately 95% pure. Mass spectrometry data along with the chromatographic retention data and fluorescence data (in comparison with synthetic reference standard) indicate that the metabolite is *N*-desmethyl-TPT. During treatment, the maximum plasma metabolite concentration (lactone plus the ring-opened hydroxycarboxylate form) was about 0.7% of the maximum total TPT concentration. The average amount of metabolite excreted in urine during the treatment was 1–4% of the delivered dose. A sensitive method has been developed and validated for the simultaneous determination of TPT and its *N*-desmethyl metabolite in human plasma, urine and feces [28]. Both compounds are reversibly hydrolyzed to their hydroxycarboxylate forms at physiologic pH. Separate HPLC systems have been devised for the determination of lactone and total (lactone plus hydroxycarboxylate forms) concentrations in plasma. The instability of the analytes in plasma required immediate protein precipitation with ice-cold methanol. The lactone forms of the analytes were stable in the methanol extracts for at least 15 months when stored at −70°C. For the determination of the total levels, the plasma was extracted with methanol at room temperature and then acidified with 25 m*M* phosphoric acid (1:1, v/v). The sample pre-treatment procedure for urine included 25-fold dilution in methanol followed by the acidification step (25 m*M* phosphoric acid (1:1, v/v)) while the fecal samples were first homogenized in distilled water (1+2 w/v) and then extracted twice with an acetonitrile–ammonium acetate (pH 4.0) mixture. Separation was optimized for lactone or total TPT analysis. To determine lactone form, the C_{18} reversed-phase column, thermostatted at 50°C, was eluted with 75 m*M* potassium dihydrogen phosphate in water containing 0.2% (v/v) triethylamine (pH 6.0 with 4 *M* hydrochloric acid) and methanol 872.5:27.5, v/v). Total TPT was eluted from a longer column (150×4.6 I.D.) kept at 34°C, using 10 m*M* citric acid–20 m*M* phosphate buffer pH 3.0–methanol (75:25, v/v). Detection was performed fluorometrically at excitation wavelength of 380 nm and emission wavelength of 527 nm. An example is reported in Fig. 4. Within-run and between-run precisions were less than 10% and average accuracies were between 90 and 110%. The methods were utilized in a mass balance study to determine the disposition and routes of elimination of TPT and *N*-desmethyl-TPT in patients. In addition to the mono-desmethyl metabolite, a bis-desmethyl-TPT has been identified [29] during the biotransformation of TPT in the isolated perfused rat liver. Enzymatic hydrolysis of the metabolites with beta-glucuronidase and mass spectroscopy revealed the existence of glucuronidated TPT as well as unconjugated and glucuronidated bis-desmethyl-TPT. Biliary secretion of glucuronidated *N*-bis-desmethyl-TPT was fast reaching a maximum already after 15 min (30.6±15.1 pmol/g liver/min), whereas secretion of TPT, TPT glucuronide and *N*-bis-desmethyl-TPT was delayed (maximum at 30 min: 431±19, 6.4±2.1 and 12.7±2.7 pmol/g liver/min, respectively). The amount of *N*-bis-desmethyl-TPT and *N*-bis-desmethyl-TPT glucuronide excreted into bile during perfusion was about 0.1% of TPT cleared from the perfusate, respectively. Hence, the overall metabolism of the novel TPT metabolites in the rat liver is poorly significant.

4. Irinotecan (CPT-11) and SN-38

Another important derivative of CPT, primarily designed to improve biopharmaceutical properties is Irinotecan, also known as CPT-11. It is important to

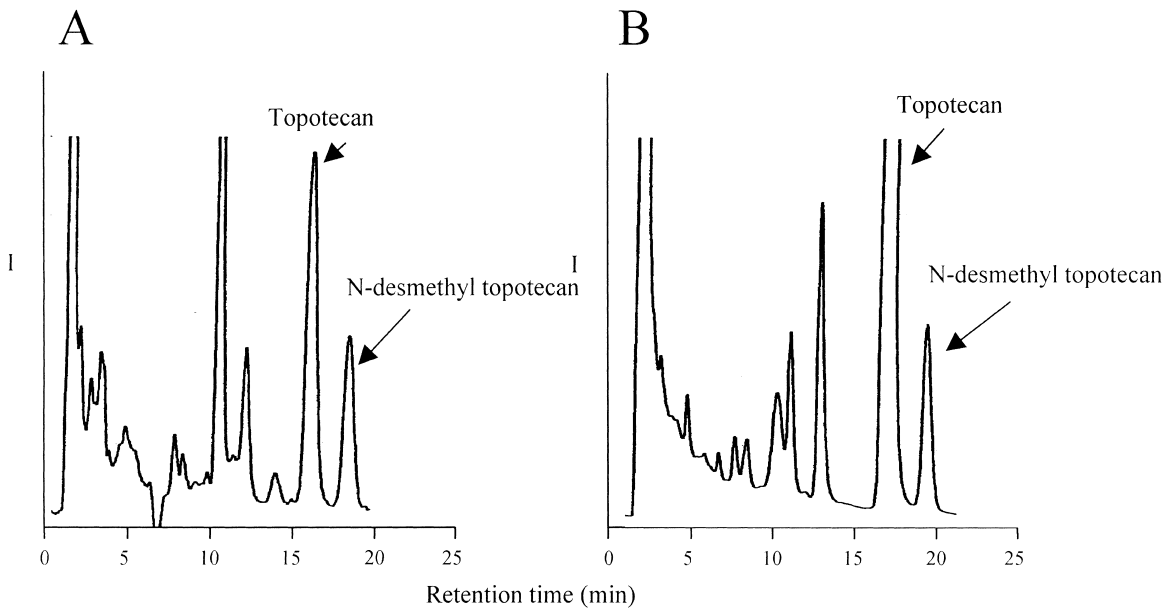


Fig. 4. HPLC separation of TPT and *N*-desmethyl-TPT. (A) Human urine sample containing 1857 ng/ml of topotecan and 128 ng/ml of *N*-desmethyl-TPT. (B) Human fecal sample containing 9.16 μg/g of TPT and 0.786 μg/g of *N*-desmethyl-TPT. Column: Zorbax SB-C_{18} 3.5 μm particle-size, 150×4.6 mm I.D. column thermostatted at 34°C. Mobile phase: 25:75 (v/v) methanol–10 m*M* citric acid, 20 m*M* phosphate buffer pH 3.0. Flow-rate 1 ml/min. Detection: fluorescence. Excitation 380 nm, emission 527 nm, bandwidth 40 nm.

remind that this drug generates an active metabolite, SN-38, having a hydroxyl group at position 10.

4.1. Analytical separation procedures

Also for these compounds, essentially all literature reports use HPLC separation methods.

A sensitive HPLC method has been developed and validated for the determination of the lactone and carboxylate forms of both CPT-11 and SN-38 in plasma [30]. The instability of the compounds required immediate bedside protein precipitation of plasma samples with an ice-cold mixture of methanol and acetonitrile. These methanolic extracts could be stored at −70°C for at least 3 months without degradation of the analytes. Separation of the lactone and carboxylate forms of CPT-11 and SN-38 was achieved on a Cls reversed-phase column with a mobile phase composed of a mixture of 0.1 *M* ammonium acetate, triethylamine, and acetonitrile (800:1:156, w/v/w) and 5 m*M* tetra-butyl ammonium phosphate. Detection was performed fluorometrically. Within-run and between-run precision was always less than 11% in the concentration ranges 1.0–100 ng/ml and 0.5–25 ng/ml, for CPT-11 and SN-38, respectively.

Another study by Chollet et al. deals with the simultaneous determination of the lactone and carboxylate forms of CPT-11 and SN-38 [31]. In order to avoid any compromise on the wavelength setting, the authors developed chromatographic conditions allowing simple automated wavelength setting changes, which have been prevented using conventional C_{18} columns. This was achieved by means of a Symmetry C_{18} column combined to a gradient elution program using acetonitrile and 75 m*M* ammonium acetate plus 7.5 m*M* tetrabutylammonium bromide at pH 6.4. The developed conditions allowed an elution order suitable for an automated wavelength change to achieve reliable peak integration. CPT-11 and SN-38 derivatives were detected by excitation at 362 and 375 nm, measuring emission at 425 and 560 nm, respectively (Fig. 5). Sample preparation was performed by plasma extraction with cold methanol which has been proven to prevent lactone/carboxylate equilibrium displacement. The developed method allowed the detection of amounts less than 3 pg of each derivative injected on column.

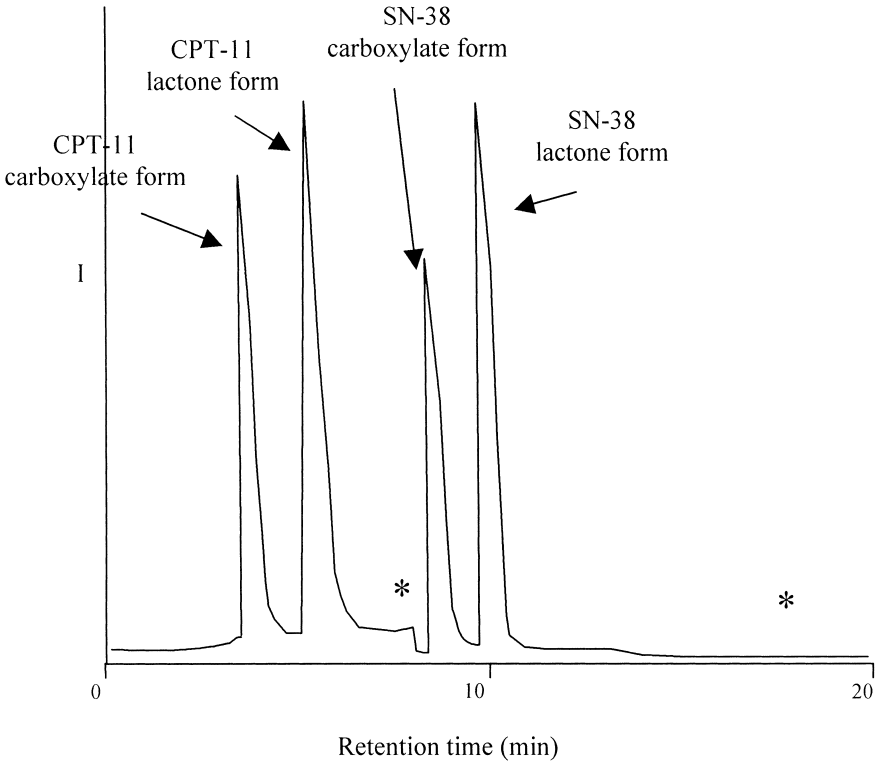


Fig. 5. HPLC separation of carboxylate/lactone forms of CPT-11 and SN-38. Column: Waters Symmetry-C_{18} 4 μm particle-size, 150×3.9 mm I.D. column. Mobile phase: (A) 0.075 *M* ammonium acetate plus 7.5 m*M* tetrabutylammonium bromide, pH 6.4 with glacial acetic acid, (B) acetonitrile. Isocratic B 18% for 3.50 min, linear increment of B from 18 to 29% between 3.50 min and 10.50 min, return to B 18% in 2.00 min. Equilibration time 4.50 min. Flow-rate 1.8 ml/min. Detection: fluorescence. Excitation 362 nm, emission 425 nm from $t=0.00$ min and $t=7.20$ min. Excitation 362 nm, emission 425 nm from $t=7.20$ and $t=17\pm1$ min. *indicates change of wavelength.

The method has been applied to pharmacokinetic and toxicokinetic studies in rat and dog.

Rapid simultaneous determination of CPT-11 and its metabolite SN-38 in plasma is reported also by Escoriaza et al. [32]. Both deproteinization of plasma specimens and addition of the internal standard CPT were achieved by incorporating to samples a solution of CPT (1 μg/ml) in acetonitrile–1 m*M* ortho-phosphoric acid (90:10); this acidified acetonitrile solution, drug-free, was also added to accomplish complete deproteinization: this procedure reduced sample preparation time to a minimum. After de-proteinization, samples were treated with potassium dihydrogenphosphate (0.1 *M*, pH 4.2) and injected into a Nucleosil C_{18} column. Mobile phase consisted of potassium dihydrogenphosphate (0.1 *M*)–acetoni-trile (67:33), at a flow-rate of 1 ml/min. CPT-11, SN-38 and CPT were detected by fluorescence with excitation wavelength set at 228 nm and emission wavelengths of CPT-11, SN-38 and CPT fixed, respectively, at 450, 543 and 433 nm. The limits of quantitation for CPT-11 and SN-38 were 1.0 and 0.5 ng/ml, respectively. This method showed good precision: the within day relative standard deviation (RSD) for CPT-11 (1–10 000 ng/ml) was 5.17% (range 2.15–8.27%) and for SN-38 (0.5–400 ng/ml) was 4.33% (1.32–7.78%); the between-day RSDs for CPT-11 and SN-38, in the previously described ranges, were 6.82% (5.03–10.8%) and 4.94% (2.09–9.30%), respectively. This assay allowed determin-ing plasma pharmacokinetics of CPT-11, SN-38 and its glucuronidated form in one patient.

Simultaneous determination of CPT-11, and its metabolites SN-38 and SN-38 glucuronide in rat plasma was performed with a fully automated on-line solid-phase extraction system, PROSPEKT [33].

Plasma samples were pre-treated with 0.15 *M* phosphoric acid to inactivate carboxylesterase and beta-glucuronidase in rat plasma, and added with the internal standard solution (containing 1 μg/ml CPT) and then loaded on a cartridge-C_{18} analytichem previously activated with methanol, water and 0.01 *M* H_3PO_4 in turn. The cartridge was washed with 0.01 *M* H_3PO_4 to remove the plasma and contaminants.The analytes were then directly eluted from the cartridge to the analytical column with the mobile phase (0.05 *M* KH_2PO_4–acetonitrile (70:30, v/v) containing 4 m*M* sodium 1-decanesulfonate (pH 3.5 with H_3PO_4)). The method was validated for CPT-11 (5–25 000 ng/ml), SN-38 (5–2500 ng/ml) and SN-38 glucuronide (2.5–500 ng/ml). This method is fast as it enabled the determination of many samples within a relatively short time with easy sample preparation. The following advantages were claimed, compared with conventional determination methods: automation of a complicated sample preparation, time-saving by the simultaneous determination of three compounds, the direct determination of SN-38 as the glucuronide, and the small amount of plasma required for the determination.

Monitoring of the lactone forms and lactone plus carboxylate (total) forms of CPT-11and SN-38, in human plasma was performed following selective sample pre-treatment [34]. For the lactone forms, it involved a single solvent extraction with acetonitrile-*n*-butyl chloride (1:4, v/v), whereas the sample clean-up for the total recovery was a simple protein precipitation with aqueous perchloric acid–methanol (1:1, v/v), which results in the conversion of the carboxylate to the lactone forms. Chromatography was carried out on a Hypersil ODS column, with detection performed fluorometrically. The methods have been validated, and stability tests under various conditions have been performed. The lower limits of quantitation are 0.5 and 2.0 ng/ml for the lactone and total forms, respectively. The assays have been used in a single pharmacokinetic experiment in a patient to investigate the applicability of the method in vivo.

Two methods for sensitive determination of CPT-11 and SN-38 have been further presented, which combine liquid chromatography with mass spectrometry detection (LC–ES–MS and LC–MS–MS) [35,36]. In the first case [35], sample pre-treatment for CPT-11 involved a simple protein precipitation with acetonitrile, whereas a liquid–liquid extraction was necessary for SN-38 (diethyl ether–dichloromethane 4:1,v/v). A Symmetry C_{18} reversed-phase column was used for the chromatographic separation, together with a gradient elution of acetonitrile in 5 m*M* ammonium formate buffer (pH 3) as mobile phase. After ionization in the pneumatically assisted electrospray source and in-source collision induced dissociation, acquisition was performed in the selected ion monitoring mode. Orifice voltage (OR), which modifies the absolute and relative abundances of the pseudo-molecular and fragment ions, was optimized for each ion analyzed. As reported in Fig. 6, high OR (70 V) resulted in fragments characteristic of the loss of CO_2 in a much more pronounced fashion for SN-38 compared to CPT-11. Recoveries were 69 and 47% on average. Detection limits were 2.5 and 0.25 ng/ml and quantitation limits 10 and 0.5 ng/ml for CPT-11 and SN-38, respectively. Reproducibility was good and the method was linear from limits of quantitation up to 10 000 ng/ml for CPT-11, and up to 100 ng/ml for SN-38. This method appears to be suitable both for pharmacokinetic studies and routine therapeutic drug monitoring. In the second case [36], conventional LC–MS spectra allowed on-line molecular mass determination of CPT-11 and its main metabolites, whereas structural information was obtained by tandem mass spectrometry (LC–MS–MS). At least 16 metabolites were detected in bile, while eight of them were also detected in urine. Three compounds were identified as the parent drug, 7-ethyl-10-hydroxycamptothecin (SN-38), and SN-38 glucuronide. The major metabolic pathway consists in oxidations of the terminal piperidine ring of the CPT-11 side chain, which eventually results in the formation of a primary amine. Other metabolites result from oxidation of the CPT nucleus. Finally, decarboxylation of the open form of CPT-11 was observed. Several metabolites resulted from combinations of these pathways. The structures of the identified metabolites indicated for the first time a major role of monooxygenases in the elimination of a CPT derivative in humans.

The issue of the clinical usefulness of salivary monitoring of CPT-11 and SN-38 was assessed by examining the clinical pharmacological profile of both drugs in nine patients with thoracic malignan-

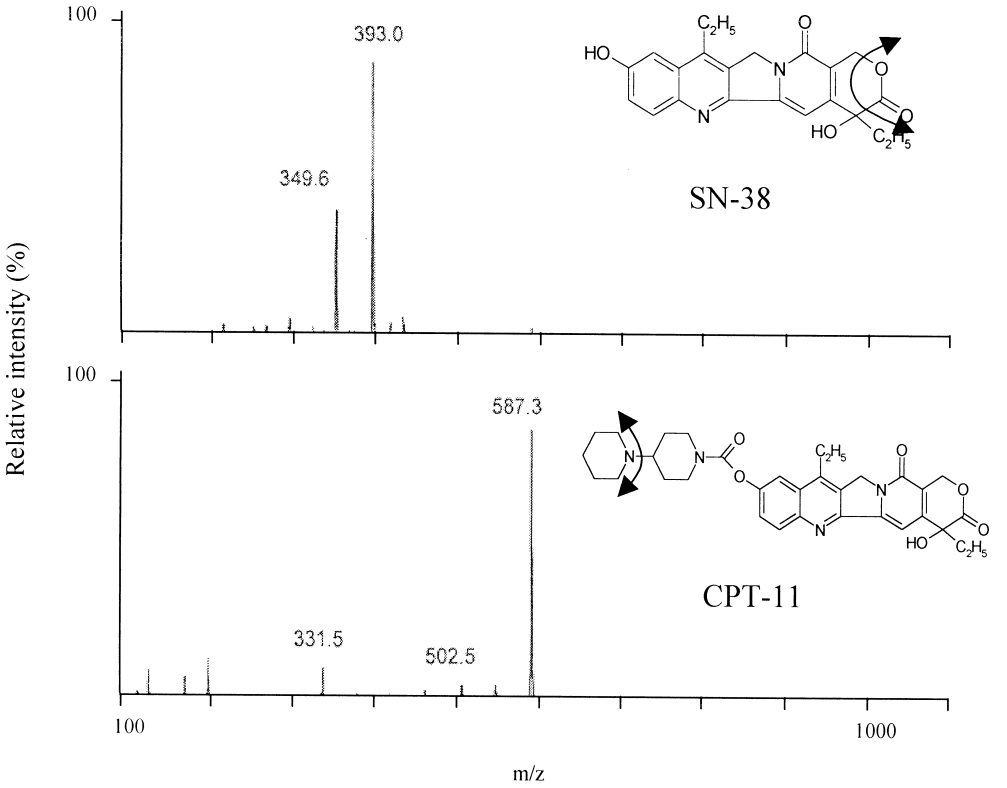


Fig. 6. Mass spectra and proposed fragmentation pattern of CPT-11 and SN-38. Nebulization gas flow 1.55 l/min, ionspray voltage 5000 V, orifice voltage 70 V.

cies who received a given dose of CPT-11 (21 courses) [37]. Both CPT-11 and SN-38 were detectable in saliva, and the concentration–time curves in plasma and saliva showed a very similar pattern. A good correlation was observed between the saliva concentration and the plasma concentration for both CPT-11 and SN-38. Hence, it might be feasible to use saliva instead of plasma for pharmacokinetics/pharmacodynamics studies of CPT-11.

Simultaneous determination of the lactone and carboxylate forms of SN-38 metabolite in rat plasma is reported by Kanedfa et al. [38]. Plasma samples were pre-treated with chilled methanol and zinc sulfate to precipitate protein, and were then directly injected into the HPLC system. Chromatography was carried out with a Puresil C_{18} column, and the mobile phase consisted of 0.1 *M* ammonium acetate buffer (pH 5.5) and acetonitrile (70/30, v/v) containing 20 m*M* of tetra-*n*-pentylammonium bromide. The column effluent was monitored with a spectrofluorometer (excitation wavelength 380 nm, emission wavelength 540 nm). The method was valid for SN-38 lactone (5–2500 ng/ml) and carboxylate (5–1000 ng/ml). In addition, trace amounts of SN-38 in human plasma were detected using solid-phase extraction with a C_{18} column for sample clean-up and concentration following acidification of human plasma with two volumes of 0.1 *M* hydrochloric acid [39]. The elution protocol of the solid-phase extraction step was optimized in term of analyte recovery, baseline stability and sensitivity. Methanol content was adjusted to 40% (v/v). In fact, at lower methanol contents significant fluorescence associated with the sample is revealed, whereas at higher methanol contents the recovery of SN-38 is reduced. Using blank plasma spiked with SN-38, the assay was found to be linear over the concentration range of 10–500 p*M* (3.9–195 pg/ml) with acceptable total and within-day imprecision. The recovery of SN-38 was found to be concentration-dependent,

ranging from 48.3% (10 p*M*) to 91.5% (500 p*M*) whereas that of the internal standard CPT was 96.9% (500 p*M*). The authors claim a sizeable increase in sensitivity over other published methods and consider it to be suitable for the measurement of ''through'' concentrations of SN-38 during the treatment of patients with a weekly regimen of CPT-11.

Quantitation of very low (femtomole) amounts of SN-38 in plasma samples was also described [40]. Sample pre-treatment involved protein precipitation with acetonitrile, followed by a one-step solvent extraction with chloroform, with CPT used as the internal standard. Chromatographic separation was achieved on an analytical column packed with Hypersil ODS material, and isocratic elution with a mixture of acetonitrile–0.1 *M* ammonium acetate containing 10 m*M* tetrabutylammonium sulfate (23:77, v/v), pH 5.3 (hydrochloric acid). The column effluent was monitored fluorometrically setting excitation and emission wavelengths at 380 and 556 nm, respectively. The limit of quantitation of the method presented was at the low femtomole level (similar to 8.4 fmol; equivalent to 5 pg/ml), with the standard curves being linear over nearly three orders of magnitude. Intra-assay precision was $<9\%$, while inter-assay variations were between 2 and 5%. The extraction efficiency was concentration independent and averaged 88.0%. The described method is proposed to be useful to assess the extent of enterohepatic recirculation of SN-38 in cancer patients following intravenous CPT-11 treatment.

A summary of the HPLC conditions used to separate CPT-11 and SN-38 is reported in Table 2.

Another major plasma metabolite of CPT-11,7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin (APC), was isolated from the plasma of patients [41]. Plasma samples (0.5 ml) containing comparatively large amounts of this metabolite were extracted by solid-phase columns and subjected to HPLC and mass spectrometry in parallel to fluorometric detection. Purified fractions were subjected to proton nuclear magnetic resonance, and the structure determined as APC, and further validated by chemical synthesis. In contrast to CPT-11, APC was not hydrolyzed to SN-38 by human liver microsomes or purified human liver carboxylesterase. Furthermore, APC did not inhibit the hydrolysis of CPT-11 in these preparations.

An additional quantitatively important polar metabolite, which was also present in the plasma and urine of patients treated with CPT-11 was subsequently identified as 7-ethyl-10(4-amino-1-piperidino)carbonyloxycamptothecin [42]. This new metabolite could be hydrolyzed to SN-38 by human liver microsomes and purified human liver carboxylesterase. Unlike APC, this new metabolite can therefore contribute directly to the activity and toxicity profile of CPT-11 in vivo.

The determination of CPT-11 and its three metabolites SN-38, SN-38 glucuronide and APC in human plasma, urine and feces was studied by Sparreboom et al. [43]. Sample pre-treatment from the various biological matrices involved a rapid protein precipitation with simultaneous solvent extraction with methanol–5% (w/v) aqueous perchloric acid (1:1, v/v), followed by proper dilution in methanol–0.1 *M* ammonium acetate containing 10 m*M* tetrabutylammonium sulfate (39:0, v/v), pH 5.3 with hydrochloric acid. Separation of the compounds was achieved as reported in [40]. The column effluent was monitored fluorometrically at excitation and emission wavelengths of 355 and 515 nm, respectively, obtaining the HPLC chromatogram reported in Fig. 7. Results from a 4-day validation study suggested that this single-run determination allows for simple, simultaneous and rapid quantitation and identification of all analytes with adequate reliability.

Another CPT-11 metabolite, 4-piperidinopiperidine (4-PP), was also considered [44]. Plasma samples were prepared for analysis following C_{18} solid-phase extraction. Chromatography was performed on a Phenyl column. Selected reaction monitoring with the mass transitions m/z 169.2→84.2 and 139.2→98.1 was used for the detection of 4PP and the internal standard (I.S.) 1-piperidineproprionitrile, respectively. The assay was linear from 14.8 to 591.0 n*M* with absolute recoveries of 4PP (59.1 n*M*) and I.S. (143.7 n*M*) of 85.7 and 86.7%, respectively. The accuracy and imprecision of the method was $\geq 96.8\%$ and $\leq 8.5\%$ over the concentration range studied, respectively. 4PP was detectable in plasma following the administration of 125, 350, 500 and 600 mg/m^2 CPT-11 to patients, with AUC (4PP) correlated with the dose. Plasma concentrations of 4PP declined slowly with a long terminal half-life (33 h). The conclusion was drawn that the con-

Table 2
Summary of the HPLC methods for the separation of CPT-11 and SN-38

Source	Sample treatment	Column	Mobile phase	Fluorescence detection conditions		Lower limit quantitation (ng/ml) C/L/T	Ref.
Human plasma	Protein precipitation (methanol/acetonitrile)	Cls reversed-phase	0.1 *M* ammonium acetate–triethylamine–acetonitrile (800:1:156, w/v/w) and 5 m*M* tetrabutylammonium phosphate	CPT-11 SN-38	exc 375 em 460 exc 385 em 525	1/1/0 0.5/0.5/0	[30]
Dog/rat plasma	Protein precipitation (methanol)	Symmetry C_{18}	Gradient: acetonitrile–75 m*M* ammonium acetate plus 7.5 m*M* tetrabutylammonium bromide at pH 6.4	CPT-11 SN-38	exc 362 em 425 exc 375 em 560	4.8/5.9/0 1.6/2.4/0	[31]
Human plasma	Protein precipitation and acidification (acetonitrile–1 m*M* orthophosphoric acid (90:10))	Nucleosil C_{18}	0.1 *M* potassium dihydrogenphosphate–acetonitrile (67:33)	CPT-11 SN-38	exc 228 em 450 exc 228 em 543	0/0/1 0/0/0.5	[32]
Rat plasma	Acidification: (0.15 *M* phosphoric acid) solid-phase extraction: (C_{18})	Symmetry C_{18}	0.05 *M* KH_2PO_4–acetonitrile (70:30, v/v) containing 4 m*M* sodium 1-decanesulfonate (pH 3.5 with H_3PO_4)	CPT-11 SN-38	exc 373 em 420 exc 380 em 540	0/0/5 0/0/5	[33]
Human plasma	Lactone: liquid–liquid extraction (acetonitrile-*n*-butyl chloride (1:4, v/v)) Total: Protein precipitation and acidification (aqueous perchloric acid–methanol (1:1, v/v))	Hypersil ODS	Methanol–0.1 *M* ammonium acetate containing 0.01 *M* tetrabutylammonium sulphate (40/60, v/v for lactone; 35/65, v/v for total)	CPT-11 SN-38	exc 355 em 515 exc 355 em 515	0/0.5/2 0/0.5/2	[34]
Human serum	CPT-11: protein precipitation (acetonitrile) SN-38: liquid–liquid extraction (diethyl ether–dichloromethane 4:1, v/v)	Symmetry C_{18}	Gradient: acetonitrile in 5 m*M* ammonium formate buffer (pH 3)	CPT-11 SN-38	ms ms	0/0/10 0/0/0.5	[35]
Rat plasma	Protein precipitation (methanol and zinc sulfate)	Puresil C_{18}	0.1 *M* ammonium acetate buffer (pH 5.5)–acetonitrile (70/30, v/v) containing 20 m*M* of tetra-*n*-pentylammonium bromide	SN-38	exc 380 em 540	5/5/0	[38]
Human plasma	Acidification: (0.1 *M* hydrochloric acid) Solid phase extraction: (C_{18})	Radial-Pak C_{18}	0.075 *M* ammonium acetate buffer (pH 5.3)–acetonitrile (77:23, v/v)	SN-38	exc 380 em 532	0/0/0.004	[39]
Human plasma	Protein precipitation: (acetonitrile) liquid–liquid extraction: (chloroform)	Hypersil ODS	Acetonitrile–0.1 *M* ammonium acetate containing 10 m*M* tetrabutylammonium sulfate (23:77, v/v), pH 5.3 (hydrochloric acid)	SN-38	exc 380 em 556	0/0/0.005	[40]

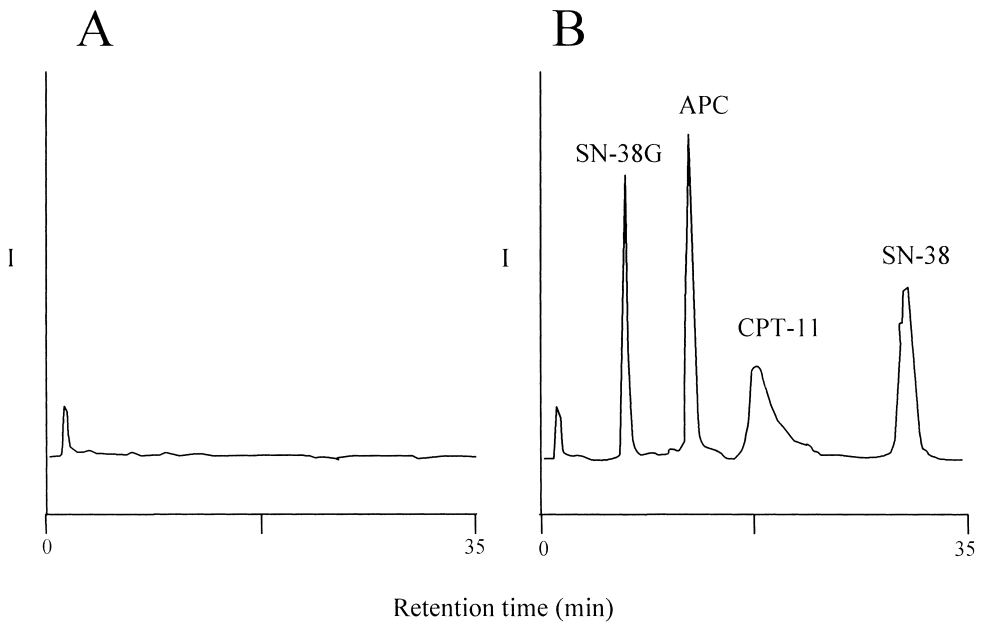


Fig. 7. HPLC separation of CPT-11 and its metabolites. (A) Human plasma. (B) Human plasma containing 200 ng/ml of each compound. Column: packed with Hypersil ODS (5 μm particle-size, 100×4.6 mm I.D.) and thermostatted at 50°C. Mobile phase: 30:70 (v/v) methanol–0.1 *M* ammonium acetate containing 10 m*M* tetrabutylammonium sulphate, pH 5.3 with hydrochloric acid. Flow-rate 1 ml/min. Detection: fluorescence. Excitation 355 nm, emission 515 nm.

centrations of 4PP in plasma were in the sub-micromolar range (<200 n*M*) and substantially lower than those capable of inducing apoptosis of cancer cells.

Besides being metabolically unstable, CPT-11 is also photolabile. An ion-pair high-performance liquid chromatographic method using a polymer-based column bonded octadecyl group was developed for the simultaneous determination of the drug and its three main photodegradation products. The analytes were detected by ultraviolet absorption at 254 nm [45]. Dodds et al. have further investigated the photodegradation of CPT-11 [46]. The drug was exposed to laboratory light for up to 5 days in 0.9% saline solution (pH 8.5). Five significant photodegradation products were observed and a HPLC assay was employed to isolate them from CPT-11 using gradient conditions. The structures were elucidated by nuclear magnetic resonance spectroscopy and tandem mass spectrometry and shown to be the result of extensive modifications of the lactone ring of CPT-11. Three of the compounds were found to belong to the mappicine group of alkaloids. Four of the five identified photodegradation products were observed and quantitated by isocratic HPLC, using fluorescence detection. In general, CPT-11 was found to be unstable under neutral and alkaline conditions for all systems investigated, with the exception of bile. From these results it follows that care should be taken to protect samples, particularly those intended for the isolation and identification of novel metabolites of CPT-11.

5. Lurtotecan (GI147211)

The piperazinyl derivative lurtotecan is also known as GG211 or GI147211. It exhibits antileukemic and antitumor activity. Its determination in human blood involves collection of blood at the clinical site, immediate freezing, and storage at −70°C [47].

5.1. Analytical separation procedures

Again, HPLC methods are largely employed. The lactone form can be extracted from blood at physiological pH with a mixture of *n*-butyl chloride and

acetonitrile (4:1), while the carboxylate is not extracted under these conditions. After evaporation, the extract is injected into an HPLC system with a fluorescence detector (excitation at 378 nm, emission at 420 nm). In this determination, the internal standard 6,7-dimethoxy-4-methylcoumarin is used. The main advantages claimed for this procedure are the separation of lactone and carboxylate by means of extraction, simplified specimen collection at clinical sites and the ability to inject almost all of the extracted material (extraction recovery, 60%) into an HPLC system. The method has been validated over the range 0.15–100 ng/ml with sufficient precision and accuracy (coefficient of variation below 10%) to support pharmacokinetic studies. Under the conditions of this procedure, the drug was stable in human blood at −70°C for at least 93 days, as well as through two additional freeze–thaw cycles.

HPLC has also been used for the analysis of the lactone and carboxylate forms in dog plasma [48]. The method consisted of two solid-phase extraction procedures. The first, referred to as the lactone-only method, utilizes a diol cartridge to separate the two forms and measures the lactone directly. The second method, referred to as the total method, converts the carboxylate form to the lactone through acidification of the plasma sample. Total lactone was then extracted from the sample using a C_{18} cartridge. The carboxylate was quantitated indirectly from the difference of the total and lactone-only. The range of the standard curve for the lactone-only method is 0.096–38.5 n*M* (0.05–20 ng/ml) and for the total method is 0.193–19.3 n*M* (0.1–10 ng/ml). Indeed, the method appears to be very sensitive for a CPT analog.

Total drug determination in liposomal preparations has been recently described [49]. An example of separation is reported in Fig. 8.Sample pre-treatment involved deproteinization with 10% (w/v) aqueous perchloric acid–acetonitrile (2:1, v/v), and chromatographic separations were achieved on an Inertsil–ODS analytical column. The lower limit of quantitation (LLQ) was established at 1.00 ng/μl in plasma and at 100 ng/ml in urine. The within-run and between-run precisions were <7.5%. Drug concentrations in urine of <100 ng/ml were determined by a modified procedure comprising a single solvent extraction with *n*-butanol-diethyl ether (3:4, v/v). In

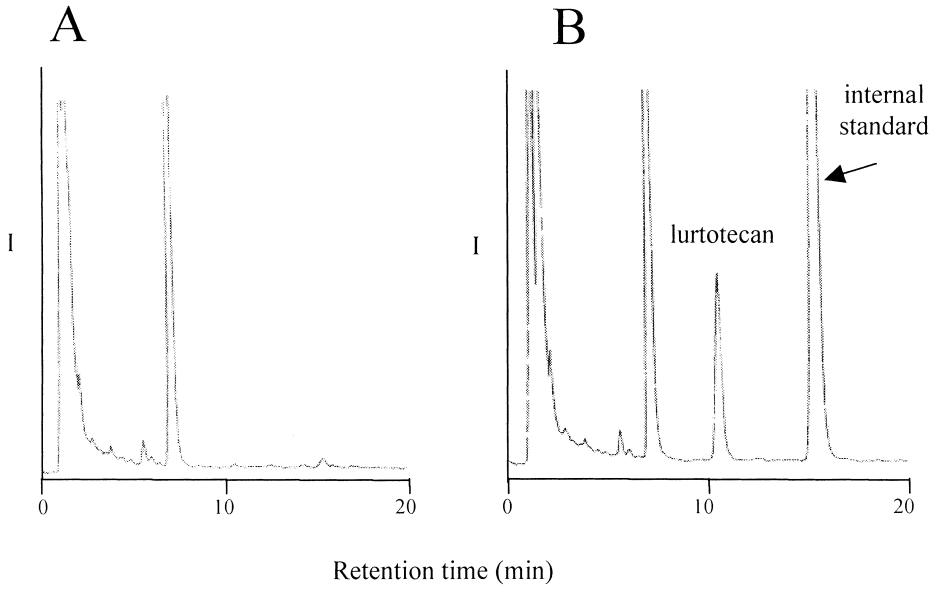


Fig. 8. HPLC separation of lurtotecan. (A) Human plasma. (B) Human plasma containing 10 ng/ml of lurtotecan. The internal standard is 6,7-dimethoxy-4-methylcoumarin. Column: packed with Inertsil ODS-80A (5 μm particle-size, 150×4.6 mm I.D.) and thermostatted at 60°C. Mobile phase:10:72.5:17.5 (v/v/v): 0.1.0 *M* ammonium acetate (pH 5.5)–water–methanol, pH 5.5 with acetic acid. Flow-rate 1.25 ml/min. Detection: fluorescence. Excitation 378 nm, emission 420 nm, bandwidth 30 nm.

this assay, the fluorescence signal of lurtotecan was increased 14-fold prior to detection by post-column exposure to UV light (254 nm) in a photochemical reaction unit. The LLQ of this assay was 0.500 ng/ml and the within-run and between-run precisions were reported to be <10%.

6. Separation of other CPT-related compounds

Liquid chromatographic methods were principally utilized for effective separation. The active congener 10-hydroxy-CPT (HCPT) was determined quantitatively by HPLC with fluorescence detection [50] both in the lactone form and carboxylate form in plasma, urine, feces and tissues, as shown in Fig. 9. 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%–103%. The limits of determination were 2 ng/ml, 2 ng/ml, 2 ng/g, and 10 ng/ml for both forms in rat plasma, urine, feces, and tissues, respectively. The assay was linear over the range 2–2000 ng/ml with recoveries of greater than 90% for plasma and urine and approximately 70–80% for feces and tissue homogenates through the extraction procedure. This analytical procedure could be applied to pharmacokinetic studies in experimental animals and in humans.

A CPT–polymer conjugate (MAG–camptothecin) was evaluated in dog plasma using a high throughput, selective and sensitive HPLC method [51]. Free CPT (intact lactone plus carboxylate) was extracted from acidified plasma using solid-phase extraction in 96-well plates. For the assay of the total CPT, plasma proteins were first precipitated with methanol in a 96-well plate containing a 10-μm melt blown polypropylene membrane. The methanolic supernatant was separated and collected into a second 96-well plate by simply applying vacuum to the plate. After hydrolysis at pH 9.8 for 18 h and re-acidification, samples were injected directly from the collection plate onto the HPLC system. MAG–CPT concentration was then calculated by subtraction of free from total CPT. The LLOQs of the method were 1.17 ng/ml for free CPT and 103.10 ng/ml (as CPT equivalent) for MAG–CPT using 0.1 and 0.05 ml of plasma, respectively. The stability of MAG–CPT in

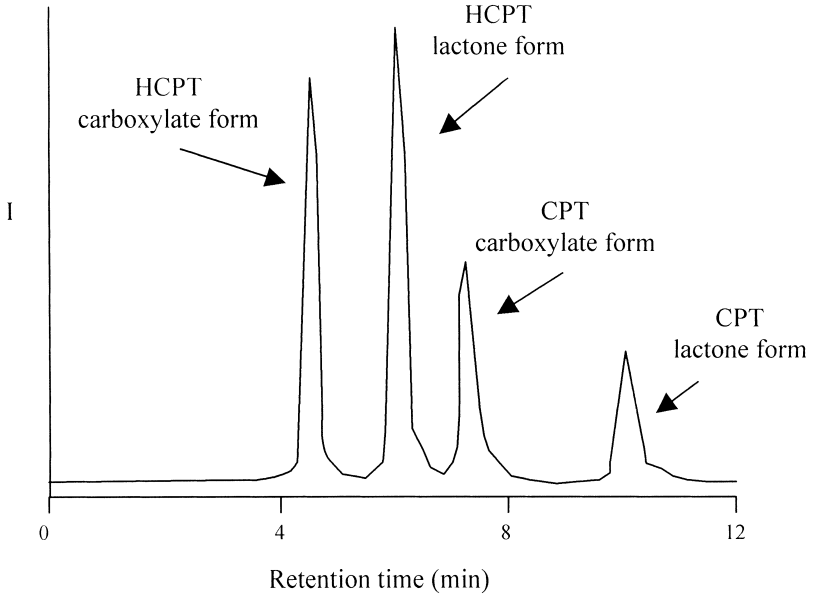


Fig. 9. HPLC separation of HCPT and CPT-11. Column: reversed-phase C_{18} (250×4.6 mm I.D.) and thermostatted at 50°C. Mobile phase: 30:70 (v/v) acetonitrile–0.075 *M* ammonium acetate buffer (pH 6.4) containing 5 m*M* tetrabutylammonium dihydrogen phosphate. Flow-rate 0.8 ml/min. Detection: fluorescence. Excitation 363 nm, emission 550 nm.

plasma alone and after its stabilization could be evaluated, along with the suitability of the method for in vivo sampling.

Another CPT analog, which became recently available is the fluorinated compound DX-8951 (Exatecan). A sensitive HPLC method for the determination of the lactone form and the lactone plus hydroxy-acid forms of this derivative in human plasma using fluorescence detection has been reported [52]. The assay method consisted of two analytical procedures. In procedure I, the lactone form was collected by the stepwise separation on a C_{18} cartridge (Fig. 10). In procedure II, the lactone plus hydroxy-acid forms were collected using another batch of the plasma sample by co-elution of the two forms from a C_{18} cartridge with acidic solution. The hydroxy-acid form of DX-8951 was quantitated from the difference of the lactone plus hydroxy-acid forms and the lactone form. Thereafter, these pre-treated samples were assayed by HPLC under the same HPLC conditions with a spectrofluorometer and a reversed-phase ODS column. The mobile phase was acetonitrile–0.05 *M* potassium dihydrogen phosphate (pH 3) (18:82, v/v) at a flow-rate of 1.0 ml/min. The analytical method for the assay of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 in plasma was validated over the range 0.2–50 ng/ml. A similar procedure has been developed and validated for the determination of lactone and total drug (lactone plus hydroxy-acid) of DX-8951 in mouse plasma [53]. The limits of quantitation of lactone and total drug were 3 ng/ml in plasma and a linear range of determination was observed over the concentration of 3 to 500 ng/ml.

Since ring opening of the lactone moiety in CPT inhibits drug activity, CPT derivatives exhibiting a stabilized E ring have been finally prepared [54]. Spectroscopic evidence is presented, which demonstrates that at physiological pH 7.4, 20-*O*-acyl derivatives of CPT are substantially more stable in the lactone form than the 20-OH parent. HPLC analysis showed that the lactone ring of a 20-*O*-ether derivative of CPT underwent endocyclic ring opening at pH≥8.5, while the lactone ring of 20-*O*-acyl CPT derivatives remained unaffected. These results might be useful for the development of new more stable active agents.

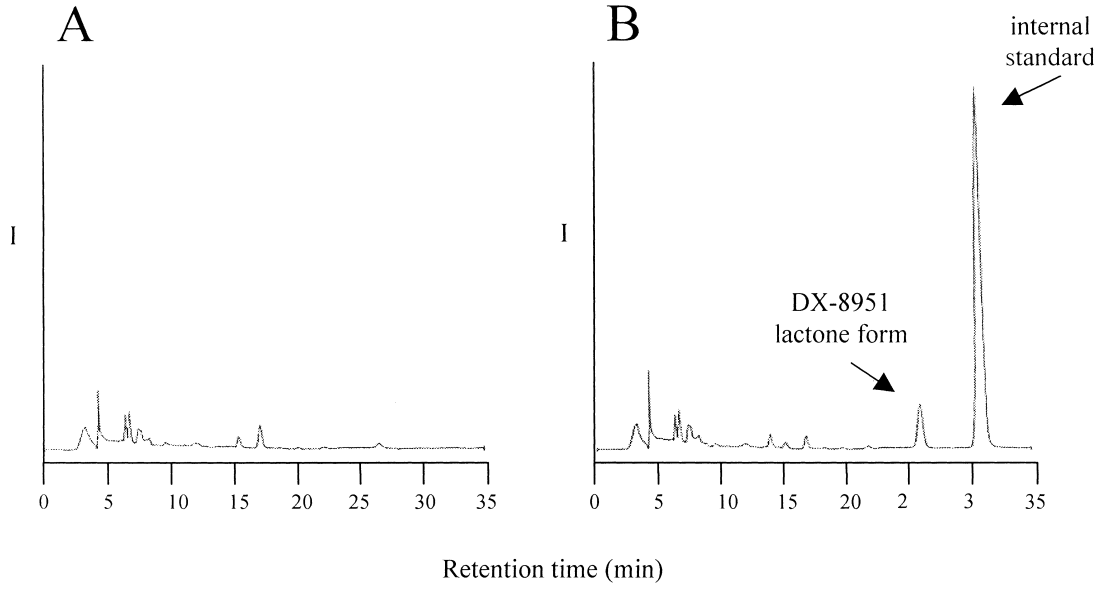


Fig. 10. HPLC separation of the lactone form of DX-8951. (A) Human plasma. (B) Human plasma containing 0.186 ng/ml of DX-8951. The four desmethyl derivatives are used as an internal standard. Column: a reversed-phase TSKgel ODS-80Ts (250×4.6 mm I.D.) column and thermostatted at 30°C. Mobile phase: 18:82 (v/v) acetonitrile–0.05 *M* potassium dihydrogen phosphate, pH 3.0 with phosphoric acid. Flow-rate 1.0 ml/min. Detection: fluorescence. Excitation 365 nm, emission 445 nm.

7. Conclusions

The development of adequate methods for separation and quantitative determination of camptothecin-related anticancer drugs represents a basic requirement to perform useful uptake and clinical pharmacodynamic investigations to optimize dosage, schedule and route of administration. In addition, valuable information can be recovered concerning the drugs' metabolism and excretion in biological fluids.

The drug instability renders it necessary to validate appropriate sample storage and pre-treatment procedures to avoid chemical reactions to occur between withdrawal and processing of the sample. These procedures generally include solid-phase or liquid-phase extraction of the biological matrix. In addition, care should be taken during the extraction process, which should be as mild as possible if the lactone form is to be quantified. In the case of determination of total drug, acidification procedures produce the stable open form quantitatively. Care should additionally be taken to avoid the photochemical modifications reported upon sample irradiation.

HPLC reversed-phase methods have been appropriately developed to separate the lactone from the carboxylate form in the original compound and in its metabolites. This allows in some instances simultaneous determination of several species, with obvious advantages.

In the vast majority of the reports, detection is performed fluorometrically. This takes advantage of the high fluorescence quantum yield of the drugs and of the possibility of setting excitation and emission wavelengths in spectral regions (visible or near UV), characterized by a low probability of interference with other cell components.

For all CPT compounds extracted from biological matrices the limits of detection are a few (0.5–5) ng/ml and the reproducibility quite satisfactory. Hence, the techniques thus far developed are sensitive and precise enough to be compatible with accurate determination of the pharmacologically relevant concentrations of the drugs during anticancer treatment.

Novel camptothecin derivatives are being continuously synthesized with the aim of improving the pharmacokinetics and of overcoming the limits (in particular resistance and severe systemic toxicity) of the presently administered compounds. Indeed, many of them are now beginning clinical trials. This will surely prompt further investigations concerning the methods of detection, separation and quantitative determination of the new species and their metabolites.

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