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Journal of Chromatography B, 764 (2001) 141–159

JOURNAL OF CHROMATOGRAPHY B

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Review

Separation methods for camptothecin and related compounds

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Abstract

This paper reviews working procedures for the analytical determination of camptothecin and analogues. We give an overview of aspects such as the chemistry, structure–activity relationships, stability and mechanism of action of these antitumor compounds. The main body of the review describes separation techniques. Sample treatment and factors influencing high-performance liquid chromatography development are delineated. Published high-performance liquid chromatographic methods are summarized to demonstrate the variability and versatility of separation techniques and a critical evaluation of separation efficiency, detection sensitivity and specificity of these methods is reported.

Keywords: Reviews; Camptothecin; Irinotecan; SN-38; Topotecan; Lurtotecan; DX 8951

Contents

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00319-X

1. Introduction

Camptothecin is a natural, water-insoluble alkaloid produced by two Asian trees, *Camptotheca acuminata* [1] and *Mappia foetida* [2]. In the 1970s preliminary clinical trials with camptothecin demonstrated promising antitumor activity, for example, in gastric cancer. Unfortunately, there were also severe side effects, including poorly predictable hemorrhagic cystitis, gastrointestinal toxicities and myelosuppression [3–5]. It was not until the early 1980s that the mechanism of antitumor activity of camptothecin was identified as the inhibition of topoisomerase I [6,7]. Researchers then began to modify camptothecin and created a host of analogues with the aim of overcoming the two key factors that hampered clinical application of the parent drug: poor water-solubility and severe toxicity. A number of camptothecin analogues are currently at different stages of clinical development, including topotecan (TPT), irinotecan (CPT-11), lurtotecan (LRT), 9-aminocamptothecin (9-AC), 10-hydroxycamptothecin (HCPT) and 9-nitrocamptothecin (9-NC) (Fig. 1). Thus far, most clinical experience relates to the water-soluble derivates topotecan and irinotecan. In this review we describe the analytical quantification of those camptothecin analogues currently in clinical trials. We provide an overview of the relevant chemistry, mechanisms of action, and stability of these compounds. Detailed reviews of the status of clinical applications and clinical phamacokinetics of camptothecins have been described elsewhere [8–10].

2. Chemistry, structure–activity relationships and stability of camptothecin analogues

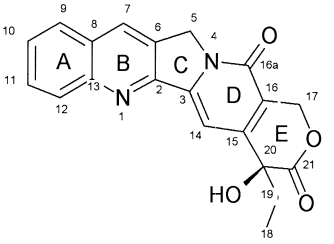
2.1. Chemistry and structure–activity relationships

Camptothecin (CPT) is a natural alkaloid which is relatively insoluble in aqueous solutions. A variety of analogues have been synthesized in an attempt to: (1) define the features of the molecule that are essential for cytotoxicity and (2) produce derivatives with increased solubility under physiological conditions.

Hydrolysis of the lactone ring of CPT produces a hydroxy acid and these two species are in equilibrium in aqueous buffers. Subsequent studies have suggested that the open form, the hydroxy acid, is a less potent inhibitor of topoisomerase I [11,12] and a much less potent antitumor agent [11,13–15]. Thus, the closed lactone ring appears to be important for activity. All camptothecin analogues share similar chemistry with different substitutions on C-7, C-9, C-10 and C-11 (Fig. 1). Typical chemical features are the planar aromatic five-ring system with a lactone moiety and *S*-configuration at C-20. Structure–activity relationships indicate that substitutions at positions 7, 9 and 10 generally enhance the inhibitory activity of topoisomerase I [11,12,16], whereas substitutions at C-12 and, to a lesser degree, at C-11 neutralize such activity [16]. Water solubility was improved by introduction of a basic dimethylamine group at C-9 for topotecan and a bipyridine carbonyloxy group at position 10 for irinotecan, which allow these compounds to be formulated as hydrochloric salts. The chemically unstable E-ring lactone of camptothecin is conserved for all active derivatives. Camptothecin derivatives with more then one stable E-rings (21-*S*-lactam and 20-deoxy-camptothecin) are inactive [17].

2.2. Stability

Because an intact lactone group is essential for interaction with the DNA–enzyme complex, all camptothecin derivatives presently in clinical trials possess an E-ring lactone. This lactone moiety is chemically unstable and undergoes reversible hydrolysis to a hydroxyl carboxylate form, which is devoid of topoisomerase I inhibitory activity (Fig. 2) [11,12,16,17]. The hydrolysis rate is dependent on several factors including pH [18], ionic strength [19] and protein concentration [20,21]. Under acidic conditions ($pH<4$) the lactone structure predominates, whereas at pH values >10 the open-ring form is exclusively present [19,22]. At physiological pH, equilibrium processes favor conversion to the carboxylate form for all camptothecins except for SN-38, the main metabolite of CPT-11. Both the lactone/carboxylate ratio at equilibrium and the rate of conversion between the two forms is affected by the pH [22]. Increased temperature increases the rate of interconversion, without affecting the equilibrium



Compound	Substituent Group C11	C10	C9	C7
Topotecan	H	OH	CH_2-N(CH_3)(CH_3)	H
CPT-11	H	4-piperidinyl-1-carbonyloxy	H	CH_2CH_3
SN-38	H	OH	H	CH_2CH_3
CPT	H	H	H	H
9-aminocamptothecin (9-AC)	H	H	NH_2	H
9-nitrocamptothecin (9-NC)	H	H	NO_2	H
10-hydroxycamptothecin (HCPT)	H	OH	H	H
Lurtotecan (LRT)	1,4-dioxane-2,3-diyl		H	4-methylpiperazin-1-ylmethyl
DX-8951	F	CH_3	CH_2-CH-NH_2	

Fig. 1. Structures of camptothecin analogues: topotecan, irinotecan (CPT-11) and its main metabolite SN-38, camptothecin (CPT), 9-aminocamptothecin (9-AC), 9-nitrocamptothecin (9-NC), 10-hydroxycamptothecin (HCPT), lurtotecan (LRT) and DX-8951.

itself. In vivo the equilibrium also depends on binding to albumin [21]. Human serum albumin (HSA) binds the carboxylate form of camptothecin with a 200-fold greater affinity than the lactone form [23]. These interactions result in camptothecin opening more rapidly and completely in the presence of HSA than in the absence of the protein [20,24,25]. In human plasma, at 37°C and pH 7.4, camptothecin lactone opens rapidly and completely to the carboxylate form and at equilibrium only 0.2% is lactone. In whole blood as opposed to plasma, camptothecin is more stable and at equilibrium, 5.3% is lactone. The enhanced stability of camptothecin lactone in human blood was found to be due to associations with the lipid bilayers of red blood cells [24]. Camptothecin binds to erythrocyte membranes, localizes within the acyl chain region, and thus remains protected from hydrolysis [24,26,27]. In the

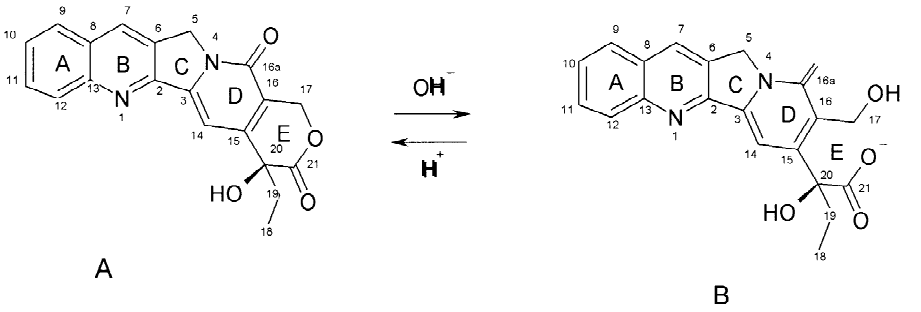


Fig. 2. Schematic representation of the reversible hydrolysis of the lactone ring (A) into the open-ringed carboxylate form (B) of camptothecin analogues.

cases of topotecan, CPT-11, and SN-38, there is no observed preferential binding of the carboxylate form by HSA. In contrast to the situation with camptothecin and its 9-amino analogue, HSA preferentially binds the lactone form of SN-38 which promotes high circulatory levels of this biologically active species [21].

3. Mechanism of action

3.1. Normal function and regulation of activity of DNA topoisomerase I

The target of camptothecins in a mammalian cell is topoisomerase I (topo I), a nuclear enzyme, comprised of a monomeric 100 000 M_r polypeptide [34–36] encoded by a single-copy gene [37,38]. Like all topoisomerases, topo I relaxes torsionally strained (supercoiled) duplex DNA [28–33]. To accomplish this relaxation, topo I introduces a single-strand nick in the phosphodiester back-bone of the DNA [39], allows the intact strand to pass through the nick, and then rejoins the nicked strand of DNA (Fig. 3) and dissociates from the DNA so that the replication process can proceed. Topo I is involved in the transcription process. Transcription results in positive supercoiling ahead of the moving transcription apparatus and negative supercoiling behind [40,41]. It has been proposed that topo I senses and relieves this transcription-associated torsional strain [42–44].

The promotor for the topo I gene has the features of a constitutively expressed gene [45]. Published data suggest that topo I levels in tumor samples might differ substantially from levels in corresponding normal tissues. Relative to normal tissues, significantly more topo I was detected in surgical specimens of colon adenocarcinoma [46], several types of non-Hodgkin lymphoma, specimens of leukemia [47], and 25 xenograft lines of human colon adenocarcinoma, carcinoma of the stomach, breast, lung, and malignant melanoma.

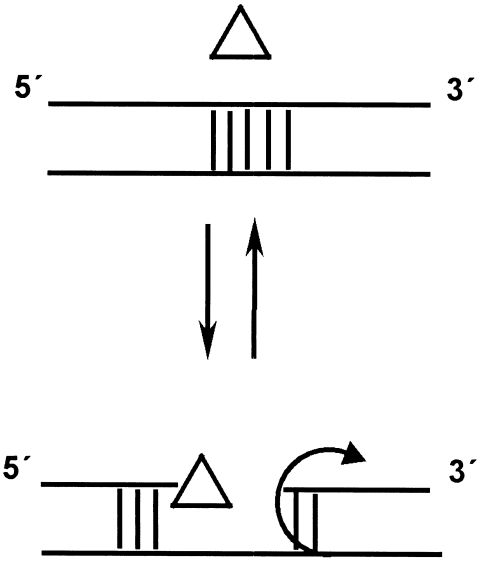


Fig. 3. Catalytic cycle of topo I. During the course of its catalytic cycle, topo I becomes covalently bound to the 3′ end of the phosphodiester backbone, concomitantly creating a DNA–protein crosslink and a nick in the phosphodiester backbone.

3.2. Interaction of camptothecin analogues with DNA topoisomerase I

Camptothecin analogues interact specifically with topoisomerase I [16]. Camptothecins bind covalently to and stabilize the cleavable DNA–enzyme complex and thereby delay re-sealing of DNA. CPT appears to stabilize adducts containing topo I covalently bound to the 3′-phosphate group of the DNA backbone. Such adducts are similar to those formed during the normal catalytic cycle of topo I [6]. Treatment of intact mammalian cells with CPT or analogues enhances the stability of topo I–DNA adducts [48–51,7]. A one-to-one relationship between the CPT-stabilized topo I–DNA adducts and CPT-induced DNA single-strand breaks has been observed [50]. CPT is capable of stabilizing these adducts even if it is added after the cleavage of the DNA has occurred [52]. In view of these results, it has been suggested that CPT binds to a site that is created when topo I is covalently bound to specific sequences of DNA [53]. CPT preferentially binds to a topo I–DNA complex rather than to topo I alone or DNA alone [6,54], although some binding to DNA has also been observed [55].

Structure–activity studies have revealed a direct relationship between the ability of CPT analogues to inhibit topo I catalytic activity and their potency as cytotoxic agents [11,12,16,56]. Cytotoxicity of camptothecins can be attributed to the arrest of the replication fork by the stabilized drug–DNA–enzyme complex. Such arrest results in double strand breaks and cessation of RNA synthesis [57]. Thus, cytotoxicity requires fork movement and this implies that camptothecins are specific to the S-phase of the cell-cycle. The mechanism of action and cell-cycle specificity would suggest that the activity of camptothecins should be highly schedule-dependent. Indeed, in in vitro and preclinical in vivo studies the greatest cytotoxicity was observed with continuous and intermittent dosing schedules [58,59]. The cytotoxic potency of the camptothecins against HT-29 human colon carcinoma cells and isolated nuclei varied greatly with the following order of potency (from greater to lesser): SN-38 (the active metabolite of irinotecan), CPT, 9-AC, TPT, CPT-11 [60]. DNA–enzyme complexes stabilized with camptothecin, topotecan, and 9-AC broke up within minutes but complexes stabilized with SN-38 took longer to dissassociate. The effect of irinotecan is minimal and the clinical activity of this drug may therefore strongly depend on its hydrolysis to SN-38. Although in vitro analyses do not necessarily reflect chemotherapeutic efficacy, the differences in cellular and molecular pharmacologic properties may in part explain differences in antitumour efficacy among the various camptothecin derivatives currently in clinical trials [60].

CPT analogues do not kill cells solely by diminishing the total catalytic activity of topo I. The concentration of CPT analogues that kill mammalian cells are 50- to 100-fold lower than the concentrations required to inhibit topo I catalytic activity by 50% [11,56]. It has been proposed that CPT analogues induce an accumulation of topo I–DNA adducts with the concomitant formation of DNA single strand breaks. This, rather than the total inhibition of cellular topo I catalytic activity, may be the critical event leading to cytotoxicity. CPT is particularly toxic to S phase cells [61–63]. Current models [64] suggest that CPT kills cells by stabilizing topo I–DNA adducts, some of which are converted to irreversible double-strand breaks through an interaction with the replication machinery.

In addition to their cytotoxic effects, CPT analogues reportedly cause several other changes in tissue culture cells. CPT is a potent inducer of sister chromatid exchanges [65]. Further studies are required to determine whether CPT analogues enhance the rate of topo I-mediated non-homologous recombination and whether this process contributes to CPT induced cytotoxicity. CPT also inhibits DNA and RNA synthesis within minutes of being added to tissue culture cells [51].

CPT has been reported to induce the morphological changes and endonucleolytic DNA damage that are characteristic of apoptosis [66–68].

4. Making therapeutic drug monitoring work to optimize cancer chemotherapy

Several factors can limit the use of therapeutic drug monitoring (TDM) for cancer chemotherapeutic agents, including poorly defined concentration–effect relationships for many antineoplastic agents such as

the camptothecins. TDM clearly has the potential to improve the clinical use of antineoplastic agents, most of which have very narrow therapeutic indices and highly variable pharmacokinetics.

Until the 1980s, the routine use of therapeutic drug monitoring in antineoplastic drug therapy was limited to methotrexate. During the last few years however, routine drug monitoring in oncology has been extended to other drugs. There are limitations however [69–71]. First, there is an incomplete understanding of the pharmacology and pharmacokinetics of most anticancer agents; the plasma drug concentration is an indirect measure of the amount of drug in the target tissue and there is typically a long time lag between the measurement of drug in plasma and assessment of the final pharmacodynamic effect. Secondly, defining concentration–effect relationships is further complicated by the fact that cancer is almost always treated with multiple drugs given in combination [72]. Combination chemotherapy not only complicates assessment of therapeutic effects, it also often complicates the pharmacodynamics of drug toxicity. Hence, it is difficult to establish ranges which are the essential elements for TDM. Furthermore, cellular characteristics contribute to variability in drug response among patients and limit the precision of ''therapeutic ranges'' for anticancer agents.

Cancer chemotherapy with camptothecins could be improved by the application of TDM because, like other anticancer drugs, the camptothecins have highly variable pharmacokinetics and narrow therapeutic indices. Maximal efficacy of cancer chemotherapy is of prime importance because of the enormous consequences to cancer patients. Knowledge about the therapeutic range of camptothecins is of great clinical utility in order to maximize efficacy and minimize undesirable adverse effects. Although the final objective of TDM is to individualize chemotherapy, other potential benefits include enhancement of compliance, minimization of pharmacokinetic variability among patients, dose adjustment in patients with hepatic and(or) renal disfunction [73] and detection of drug interactions [74].

The successful implementation of TDM requires a well-coordinated multidisciplinary effort. The proper execution of TDM includes the correct administration of drug, correct collection and processing of blood samples, precise and accurate measurement of drug and metabolites and appropriate interpretation of results. An accurate and precise quantification of drugs and metabolites is critical in TDM. Thanks to advanced technology, quantification of many commonly monitored drugs or metabolites is automated with commercially available reagents. However, this is not the case for anticancer drugs, most of which must be measured by high-performance liquid chromatography (HPLC) or gas chromatography based methods. For the analysis of human plasma levels of camptothecins, most assays developed to date are HPLC based. Whether TDM is automated or not, it is essential to utilize well characterized and fully validated analytical methods to quantify the drugs and metabolites monitored. Ideally, the selected assay should be accurate, precise, simple, rapid and sensitive, and with minimal matrix effects. The method should be clinically and financially feasible if it is to be performed on a routine basis and be cost-effective.

5. Separation methods

Several methods have been described for the measurement of CPT analogues using HPLC and fluorescence detection. There are methods developed for the determination of camptothecin derivatives in buffered solutions [75,76], and in biological fluids [75,77,78].

(a) Sample pretreatment: sample pretreatment procedures vary greatly and most of them involve several phases such as addition of an internal standard (I.S.) and deproteinization of plasma by means of a precipitating agent. Deproteinization of chilled blood samples with cold methanol, the most typical precipitating agent, is usually fast and effective [78–85]. Some published methods include long and tedious liquid–liquid extraction procedures [85–87] or solid-phase extraction on disposable extraction C_{18} columns [88–93]. All methods also incorporate at least one acidification or alkalinization step.

(b) Quantification methods: several methods have been described for the camptothecins; involving quantification of the lactone form alone [91,92,94–97], for the simultaneous determination of the lactone and carboxylate forms [19,78,80,98–101] and

methods quantifying total concentrations of camptothecins [77,82,85,92,87,88]. In general, carboxylate forms elute with the solvent front; if the sample is acidified to convert all the drug into its lactone form then it is only possible to estimate the total concentration of CPT. When samples are collected in the appropriate fashion (rapid cooling of the blood, centrifugation and protein precipitation) the hydrolysis of lactone to its corresponding carboxylate is stopped [96,98]. The concentration of the lactone form present in the plasma can then be estimated selectively. The total concentration of CPT from acidified samples can be estimated and then, the carboxylate form can be determined by subtraction. Usually, such techniques require the use of different HPLC conditions and two injections of a single sample. Since lactone is the active form of these anticancer agents, discriminating between lactone and carboxylate forms would seem to be an important objective of quantitative methods. However, clinical applicability of this kind of assay is hampered by stability problems associated with lactone–carboxylate interconversion [85]. Since hydrolysis of lactones may occur with improper storage or handling of the samples, the resulting data will be unreliable. However, for practical purposes, monitoring of total camptothecins has essentially the same clinical significance as monitoring of lactone [102].

(c) Development of the HPLC methodology: developing a HPLC method for the analysis of camptothecins can be very difficult, due to the numerous variables that must be considered. In the simplest case of lactone separation, two or three components are normally present in the mobile phase. A buffer is used to control the pH of the mobile phase, while acetonitrile is used to control retention of the lactone form of the analogue. A third component is also frequently added to control peak tailing due to interactions between the analyte and underivatized silanols on the column wall. This interaction results in a dual retention process, which produces peak tailing that makes quantification more difficult. The addition of a positively charged substance, such as sodium dodecyl sulfate (SDS), to the mobile phase counteracts peak tailing by interacting with the silanols.

For the simultaneous determination of the lactone and carboxylate forms of a camptothecin, an additional component, namely an ion-pairing agent, must also be employed. Without this agent, the chromatographic behavior of the charged carboxylate species is such that it will not be retained on the C_{18} column to an appreciable extent, precluding separation of the lactone and carboxylate form of the drug. The ion-pairing agent tends to mask the charge of the camptothecin carboxylate species, increasing its affinity for the column [103]. Tetrabutylammonium phosphate (TBAP) is a common ion-pairing reagent normally added to the buffered mobile phase causing an increased retention of the carboxylate forms of CPT analogues such that they can be kept back from the solvent front.

In the development of a HPLC based method for the analysis of camptothecins the following parameters must be optimized: (i) type and concentration of ion-pairing reagent; (ii) type, concentration and pH of buffer; (iii) amount of organic modifier; (iv) amount of SDS or other agent capable of masking silanols.

The resulting HPLC method can therefore be very time-consuming and complicated.

5.1. HPLC analysis of camptothecin

CPT, [4-(*S*)-4-ethyl-4-hydroxy-1H-pyrano(3′,4′,6,7)indolizino(1,2-b)quinolone-3,14(4H,12H)dione], a plant alkaloid, was isolated from *Camptotheca acuminata*, Decne (Nyssaceae). Its structure was elucidated by Wall et al. [1] and McPhail and Sim [123] as part of a screening program for antitumor agents. CPT is a yellow opaque crystalline powder that inhibits the growth of a wide range of experimental tumors, including murine leukemia L1210, Walker 256 rat carcinosarcoma, L5178Y, K1964 and P388 leukemia, Novikoff hepatoma in vitro, mastcell P815 sarcoma and reticulum cell sarcoma (A-RCS) [124–128].

Studies on camptothecin analysis have used total fluorescence without separation [129], liquid chromatographic separation with ultraviolet detection [130] or fluorescence with thin-layer chromatography [129]. These analytical methods have the disadvantage of being incapable of differentiating between camptothecin analogues, of not being sensitive and of being elaborate and time-consuming. In initial HPLC methodologies utilizing fluorescence detection

for camptothecin analysis only the lactone species was retained on the C_{18} column, with the carboxylate species being eluted in the void volume [95,96]. For the detection and estimation of the camptothecin concentration in biological fluids, Loh and Ahmed [95] developed a sensitive HPLC method in which the best mobile phase was methanol–10 m*M* phosphate (75:25, v/v, pH 4). Under these conditions, i.e., using a mobile phase at pH 4, the sensitivity of detection was two- to threefold higher than that when using a mobile phase at pH 7. Furthermore, the effect of sample pH at the optimal mobile phase condition (pH 4) was also studied and the results indicated that increasing the acidity of the sample enhanced the sensitivity as well as the peak symmetry (Fig. 4). Supko and Malspeis [96] employed a system with ammonium acetate buffer–acetonitrile–SDS as mobile phase. Two injections were required for complete determination. The first injection was to determine the amount of lactone species present in the sample. Then, the sample was acidified in order to drive the lactone–carboxylate equilibrium towards lactone, and injected again to determine the amount of total drug in the sample. The difference determined indirectly the amount of carboxylate species present. More recent HPLC methodologies use ion-pairing to obtain a simultaneous separation of the lactone and carboxylate species [19,100,101]. Beijnen et al. [100] used TBAP as ion-pairing agent in the mobile phase to allow separation of the carboxylate species from the void volume. Warner and Burke [75] used a mobile phase composed only of triethylamine acetate (TEAA) buffer and acetonitrile to simultaneously separate the carboxylate and lactone forms of camptothecin in less than 10 min (Fig. 4). Ahmed et al. [131] developed a sensitive HPLC assay to quantify total drug and lactone. They used sodium heptanesulfonate as ion-pairing agent in an isocratic mobile phase of acetonitrile–25 m*M* KH_2PO_4 (35:65) at pH 4.5 and achieved enhanced sensitivity as well as peak symmetry. Lactone and total CPT were extracted using solid-phase extraction and liquid–liquid extraction, respectively. The extracted lactone samples could be stored without immediate HPLC analysis. This is an important advantage respect to previous assays for the two forms of CPT, such as the assays of Loh and Ahmed [95] (Fig. 4) and Supko and Malspeis [96], where, due to the instability of the lactone, immediate extraction and HPLC analysis were required. Fig. 5 shows a representative total and lactone profile in a patient after a single dose (A) and multiple doses (B). The average lactone to total ratio in the samples was about 9.6/100. The two forms had similar disposition profiles.

There are several analytical methods for camp-

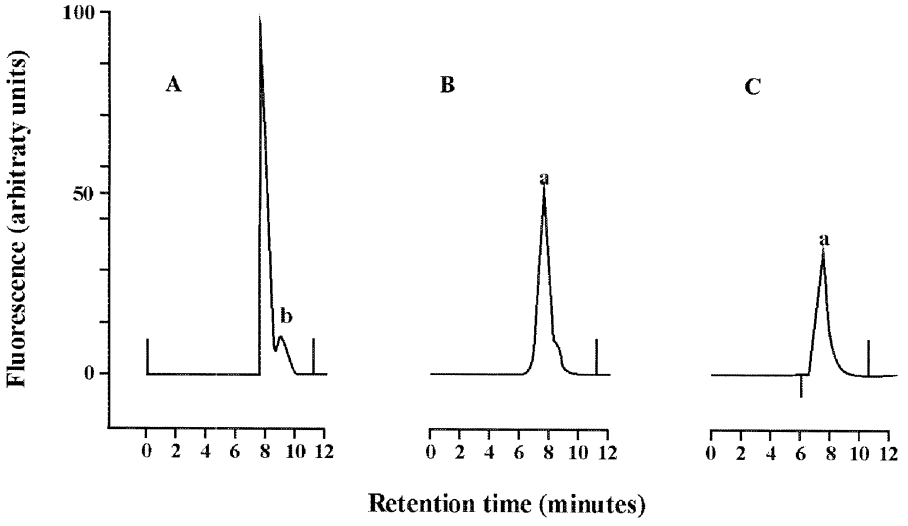


Fig. 4. Effect of sample pH on HPLC profiles of camptothecin (lactone form) in an acidic mobile phase. Mobile phase: methanol–10 m*M* KH_2PO_4 (75:25, v/v), pH 4.0. Peaks: a=camptothecin; b=sample impurity. Camptothecin concentration: 1.0 μg/ml. (A) pH 2.0; (B) pH 6.5; (C) pH 11 (from Loh and Ahmed [95] with permisison).

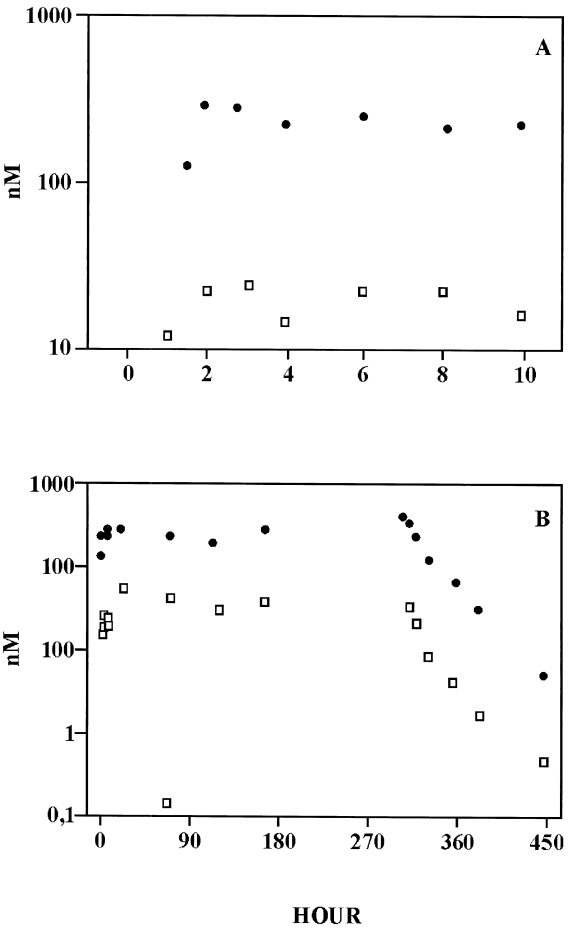


Fig. 5. CPT total (●) and lactone (□) concentrations in a patient after a single dose (A) and during multiple doses (B) (from Ahmed et al. [131] with permission).

tothecin but Tsai et al. [132] designed the first method using microdialysis and microbore HPLC. The authors developed a method to evaluate the biliary excretion of unbound camptothecin by on-line microdialysis coupled to microbore liquid chromatography with fluorescence detection. The microdialysis sampling technique was originally developed for in vivo neurotransmitter release in the rat brain [133,134]. More recently, however, application of microdialysis sampling has extended into pharmacology and pharmacokinetics [135,136]. Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted into the tissue space being dialyzed. In the Tsai et al. [132] study a flow-through microdialysis probe was constructed for bile sampling. For automatic analysis of microdialysate, an on-line injector was connected to a microbore HPLC column with fluorescence detection. The microdialysis technique provides protein-free samples (because protein molecules are too large to pass through the dialysis membrane with a molecular mass cut-off 13 000) which can be directly injected onto a liquid chromatographic system for continuous in vivo monitoring of unbound drugs in bile. Further, this sampling method facilitates pharmacokinetic studies, which reduce the effects of disturbances in biological fluid volume. Another study by Tsai et al. [137] used the microdialysis technique to characterize the pharmacokinetics of protein-free camptothecin in blood and brain. In this study, they constructed blood and brain microdialysis probes, then inserted them into rat jugular veins and brain striata for sampling of camptothecin from biological fluids after camptothecin was given intravenously. The quantitative analysis was carried out using validated microbore HPLC methods with fluorescence detection. Several other in vivo techniques have been described for brain pharmacokinetic studies, including autoradiography, imaging methods (positron emission tomography, PET) and nuclear magnetic resonance (NMR), cerebral fluid sampling, in vivo voltammetry. However, these techniques are too expensive for general laboratory use. Although intracerebral microdialysis has the disadvantage of being invasive, it is much cheaper than PET scanning or NMR and can be used in the general laboratory [138].

5.2. HPLC analysis of CPT-11 and its metabolite SN-38

CPT-11, {7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin}, is a water soluble analogue of camptothecin first synthesized by Yokokura and co-workers at the Yakult Central Institute (Tokyo, Japan) in an attempt to identify derivatives with greater aqueous solubility and antitumor activity than CPT. Also known as Irinotecan (CPT-11), it is supplied as a yellow crystalline powder that is soluble in water and slightly soluble in aromatic solvents. The molecular mass of the hydrochloride trihydrate salt is 677 and, the free base is 587. Unlike CPT and TPT, CPT-11 has little inherent antitumor

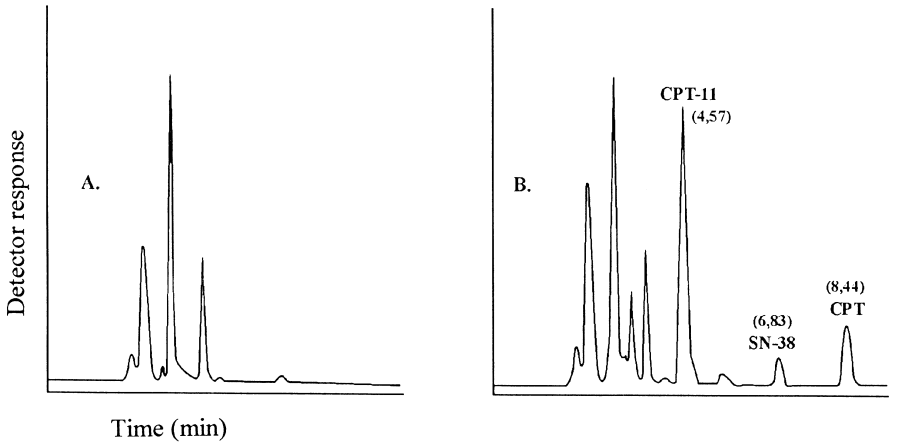


Fig. 6. HPLC of a human plasma extract after administration of CPT-11. Peaks: CPT-11 (4.57 min), SN-38 (6.83 min) and internal standard CPT (8.44 min) (from Escoriaza et al. [114] with permission).

activity in vitro. Instead, CPT-11 is a pro-drug that undergoes deesterification in vivo (Fig. 1) to yield SN-38, a metabolite that is 1000-fold more potent than the parent compound in vitro [107,108]. SN-38 has been shown to undergo glucuronic acid conjugation to form the corresponding glucuronide (SN-38G) which is the first step in the principle elimination pathway of SN-38 [109–112]. CPT-11 demonstrated substantial activity in vivo against a broad spectrum of tumor xenografts when administered by intraperitoneal, intravenous, or oral routes [113].

Although initial HPLC methods resolved only the intact lactone forms of CPT-11 and SN-38 [92], to be useful, HPLC methods should be able to separate lactone and carboxylate species of both drugs (four components in total) within a reasonable quickly amount of time. The availability of such analytical methods is particularly important as only the lactone forms of CPT-11 and SN-38 are active as antitumor agents [54]. The method employed by Rivory and Robert [78], which used TBAP as ion-pairing reagent with an ammonium acetate buffer and acetonitrile as mobile phase and with a traditional C_{18} column, allowed the simultaneous determination of both forms of CPT-11 and SN-38 in plasma samples after the precipitation of plasma proteins with an ice-cold mixture of acetonitrile and methanol. Although all four components were separated, significant peak tailing precluded baseline separation. Warner and Bourke [75] used a mobile phase composed of only TEAA buffer and acetonitrile to perform the separation of all four components in less than 13 min with an optimum peak shape.

Although lactone is the active form of these antineoplastic agents and discriminating between lactone and carboxylate forms would appear to be an important aim, for practical purposes, monitoring of total CPT-11 and SN-38 has essentially the same clinical significance as monitoring of lactone CPT-11 and SN-38. This is because the pharmacokinetics of total CPT-11 and total SN-38 are significantly correlated with those of lactone CPT-11 and lactone SN-38, respectively [78,102,115]. Chabot [116] reported that the area under the curve (AUC) ratio of lactone to total forms appeared to be constant between patients, and that monitoring the total form was probably a simpler and less biased alternative as it was more convenient to assay in a clinical setting. The HPLC method developed by Escoriaza et al. [114] determines total concentrations of CPT-11 and SN-38 in plasma (Fig. 7). By means of the incorporation of internal standard (I.S.) (CPT) to plasma samples in acidified acetonitrile solution, Escoriaza et al. [114] condensed in just one phase three common operations other methods develop separately: addition of I.S., acidification of plasma and protein precipitation. In this way, the assay gains in simplicity and rapidity reducing sample preparation time to a minimum, shortening analysis time and reducing solvent waste. After deproteinization, sam-

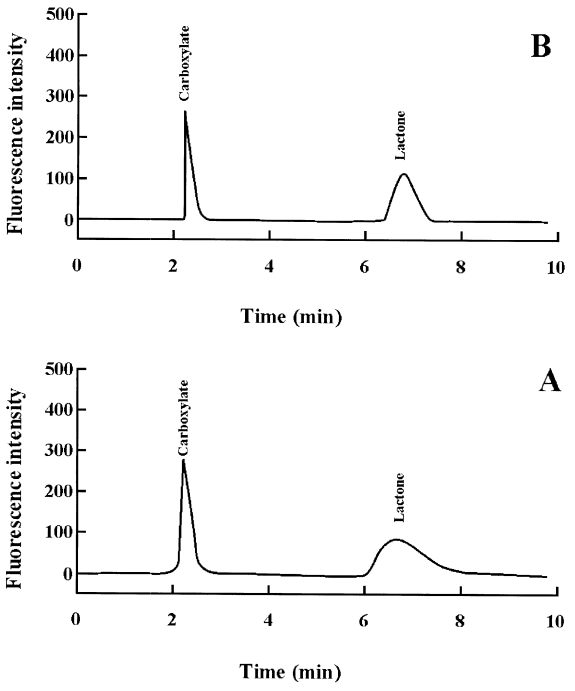


Fig. 7. HPLC separation of topotecan carboxylate and lactone species. Fluorescence detection wavelengths are 350–470 nm (excitation) and 510–650 nm (emission). (A) HPLC separation of topotecan using 1% TEAA. Mobile phase was acetonitrile–TEAA (1%, v/v), pH 5.5 (14:86, v/v). (B) HPLC separation of topotecan using 2% TEAA. Mobile phase conditions were acetonitrile–TEAA buffer (2%, v/v), pH 5.5 (12:88, v/v) (from Warner and Burke [75] with permission).

ples were treated with potassium dihydrogenphosphate (0.1 *M*) and injected into a Nucleosil C_{18} 5 μm column. The mobile phase consisted of potassium dihydrogenphosphate (0.1 *M*)–acetonitrile (67:33), at a flow-rate of 1 ml/min. CPT-11, SN-38 and CPT are detected by fluorescence with the excitation wavelength set at 228 nm and emission wavelengths of CPT-11, SN-38 and CPT fixed, respectively, at 450, 543 and 433 nm. Fig. 7 is a chromatogram, obtained under such chromatographic conditions, of a human plasma extract after administration of CPT-11. This assay provides high recoveries for CPT-11 and SN-38 and is precise and accurate and at least as sensitive as other assays for the simultaneous determination of these drugs [75,77,78,82,85,92,117].

Kurita and Kaneda [118] established an HPLC method with a fully automated on-line solid-phase extraction system, PROSPEKT, for the simultaneous determination of CPT-11 and its metabolites SN-38 and SN-38 glucuronide (SN-38G) in rat plasma. Samples were pretreated with 0.146 *M* H_3PO_4 to inactivate carboxylesterase and β-glucuronidase activity and to transform the carboxylate forms of CPT-11, SN-38 and SN-38 glucuronide (SN-38G) to the lactone forms. With other methods, the determination of the conjugate of SN-38, SN-38G, is both time-consuming and problematic with regard to precision because it is determined as SN-38 deconjugated by β-glucuronidase. The method of Kurita and Kaneda [118] simultaneously determines CPT-11, SN-38 and SN-38G (which meets the criteria of Shah et al. [119] for the concentration ranges of 5–25 000, 5–2500 and 2.5–500 ng/ml, respectively), directly and with high precision even when the concentration of SN-38G is much lower than that of SN-38. At such concentrations, SN-38G was often calculated as a negative value by previous conventional methods. Compared with conventional methods, Kurita and Kaneda [118] method has several advantages, such as the rapid sample preparation with a fully automated on-line solid-phase extraction system (PROSPEKT), time-saving by the simultaneous determination of three compounds, and the direct determination of SN-38G. The use of PROSPEKT enables the processing of many samples within a relatively short time and with higher repeatability, in terms of precision and accuracy, compared to manual sample preparation. The use of an automatic wavelength change fluorescence detector enabled the exact determination of CPT-11 and SN-38 at low concentrations. For the determination of SN-38G the detector was set at 373 nm, 428 nm, and ×100 (excitation and emission wavelengths, and gain, respectively), for SN-38 the settings were 380 nm, 540 nm, and x100 and for CPT-11 and the internal standard CPT the detector was set at 373 nm, 428 nm, and ×10.

To date, HPLC methods for the determination of camptothecin derivatives mainly use fluorimetric detection after a liquid–liquid or solid-phase extraction. Recently, HPLC–atmospheric pressure chemical ionization tandem mass spectrometry (APCI-MS–MS) has been used, after a solid-phase extraction, to assess the presence and structure of several metabolites of CPT-11 in biological fluids [120]. Ragot et al. [121] developed a specific method using liquid chromatographic–electrospray mass spectrometry (LC–ESI-MS) for the determination of

the total concentration of CPT-11 and SN-38 in human serum. Owing to MS, this method is one of the most specific. The quantification limit was similar to those obtained with conventional HPLC techniques. The SN-38 limit of quantification (LOQ) was considerably lower than those generally reported for HPLC with UV and fluorescence detection (between about 2 and 5 ng/ml) [77,92,122]. Only Rivory et al. [88] reported a fluorescence technique which was more sensitive that other published methods [78,82,92], with an LOQ at the low pg/ml level, suitable for the measurement of trace concentrations (as low as 10 p*M* of total SN-38) in human plasma after acidification and transformation to the lactone form [88]. De Bruijn et al. [87] developed an HPLC method for SN-38 quantification which was similar in assay sensitivity to that of Rivory et al. [88] but without the laborious solid-phase extraction. The most important advantage of the De Bruijn et al. [87] method is that sample pretreatment involves only a protein precipitation step of 1 ml samples with 2 ml of acetonitrile, followed by a one-step solvent extraction with 5 ml of chloroform. However, with UV and fluorescence detection, other compounds in samples from patients administered with multiple drugs may sometimes interfere with detection.

5.3. HPLC analysis of topotecan

TPT is supplied as a lyophilized, buffered, light yellow powder that is soluble in water. The molecular mass of the hydrochloride salt is 458. TPT, [(*S*)-9-dimethylaminomethyl - 10hydroxy-camptothecin] (SKF 104864-A), was identified by Kingsbury et al. at Smithkline Beecham Pharmaceutical Company as a potent, water-soluble CPT analogue which acted directly and not as a pro-drug [56]. Subsequently, large proportions of human colorectal, breast, non-small-cell lung, ovarian, and renal cell carcinomas were shown to be sensitive to TPT in an in vitro clonogenic study [58]. The results of pre-clinical and clinical studies indicate enhanced antineoplastic activity of TPT when administered daily for prolonged periods of time [46,58,104–106].

Previous HPLC methodologies using fluorescence detection for topotecan have been performed for the simultaneous separation of the carboxylate and lactone species [80,98,99]. Beijnen et al. [98] had developed, for the analysis of the closed-ring lactone form of topotecan and the open-ring form in human plasma, an HPLC assay with a limit of quantification for both compounds of 1 ng/ml. Loos et al. [99] developed a new methodology because the plasma concentrations in their clinical study, where TPT was administered orally for prolonged periods of time, were anticipated to be much lower than the lower limit of quantification (LLQ) of the previously developed methodology. Plasma sample treatment was carried out immediately after sample collection by deproteinizing the sample with cold methanol, as previously described by Beijnen et al. [100]. The separation was done on a Shandon Hypersil BDS C_{18} column, with 3 μm particle size and the mobile phase consisted of 10 m*M* potassium dihydrogenphosphate containing 25% methanol and 0.2% triethylamine with pH adjusted to pH 6 by the addition of orthophosphoric acid. Because of the complexity of the mobile phases employed by these authors, (containing three to four components), preparation is tedious which makes these mobile phases unattractive for routine use, and development of these assays can be a laborious process. Warner and Bourke [75] developed a simple HPLC methodology where the mobile phase employed included only TEAA buffer and acetonitrile. TEAA serves multiple roles; as the ion-pairing reagent, as a masking agent for underivatized silanols and as the major buffer component. This simplified mobile phase has been used to simultaneously separate the carboxylate and lactone species of TPT in less than 8 min (Fig. 4). Topotecan contains a dimethylaminomethyl group which can potentially interact with residual silanols, so the percentage of TEAA in the buffer was increased from 1 to 2% to significantly improve the peak shapes of the lactone and carboxylate analytes. Fluorescence detection wavelengths were 350–470 nm (excitation) and 510–650 nm (emission). The specific mobile phase for the optimum separation was acetonitrile–TEAA buffer (2%, v/v), pH 5.5 (12:88, v/v). Using this mobile phase composition, the carboxylate form elutes in 2.2 min while the lactone form elutes in 6.8 min.

5.4. HPLC analysis of 9-aminocamptothecin

9AC [9-amino-20(*S*)-camptothecin, NSC629971] was the first synthetic camptothecin derivative selected for advanced in vivo testing and possible clinical

development but due to its limited water solubility, unlike other newly designed camptothecin analogues, has only recently been subjected to clinical testing. 9AC is active against human colon cancer [46], breast cancer [13], and melanoma [139] cell lines. The activity of 9AC depends upon an intact lactone ring, but in human plasma, equilibrium between the lactone and carboxylate forms greatly favors the more water-soluble, open-ring 9AC carboxylate form. This instability necessitates the rapid processing and immediate HPLC analysis of clinical samples in order to accurately quantify drug levels [140]. 9AC measurement in human plasma is further complicated by the relatively poor fluorescence of the drug compared to the parent compound, camptothecin [141]. HPLC assays for 9AC lactone use post-column acidification to enhance drug fluorescence and achieve a lower limit of quantification of 13 n*M* [141]. Takimoto et al. [90] have developed a sensitive HPLC assay to quantify the active lactone form of 9AC in human plasma. The assay uses solid-phase extraction in order to separate 9AC lactone from its less active metabolite, 9AC carboxylate, and so samples can be stored for up to 2 months prior to reverse HPLC analysis. Assay sensitivity was over 50-fold better than that of Supko and Malspeis [141]; by using an acidic (pH 2.55) isocratic HPLC mobile phase to enhance 9AC fluorescence the assay has a reproducible LLQ of 9AC lactone in plasma of 0.25 n*M* and avoids the need for post-column acidification. Solid-phase extraction also enhanced assay sensitivity by removing interfering endogenous plasma compounds and by deproteinating the extract without diluting the sample. Acidification of plasma samples prior to solid-phase extraction allowed for the determination of total plasma levels. The 9AC carboxylate level could be determined by subtracting 9AC lactone concentration from total plasma 9AC level. Because the pharmacodynamic characteristics of camptothecin analogues are still incompletely defined, both the active lactone and the total plasma levels of these drugs should be measured in order to provide a complete pharmacokinetic profile of these agents.

Loos et al. [86] described two sensitive reversed-phase HPLC fluorescence methods which avoid one of the major drawbacks of the previous procedure; the laborious solid-phase extraction technique. For the determination of 9AC lactone, the sample preparation was a liquid–liquid extraction with acetonitrile–*n*-butyl chloride (1:4, v/v), whereas the sample preparation for total 9AC (lactone plus carboxylate) was a simple deproteinization with 5% perchloric acid–methanol (1:1, v/v), which converts the carboxylate into the lactone form. The lower limits of quantification were 50 pg/ml and 100 pg/ml for 9AC lactone and total 9AC, respectively. The plasma concentration–time curves of the 9AC lactone and the total 9AC from a patient treated orally with 2.7 mg of 9AC, are given in Fig. 8. The methodology described permits the analysis of patient samples, and will be implemented in future investigations on the clinical pharmacology of 9AC.

5.4.1. Observations on 9-nitrocamptothecin

9-NC is supplied as a yellow opaque crystalline powder that is water insoluble. It is generated from CPT. In vivo 9-NC is metabolized to 9-AC [142]. The drug is interesting as a clinical alternative of 9-AC because it is easier to produce than 9AC and possesses greater stability. The percentage of 9-NC present in plasma in the lactone form is about 15% [143]. No direct correlation could be established between drug concentrations and toxicity or antitumor activity [144].

5.5. HPLC analysis of 10-hydroxycamptothecin

Recently, water-insoluble CPT and its natural analogues have been shown to have a wide spectrum

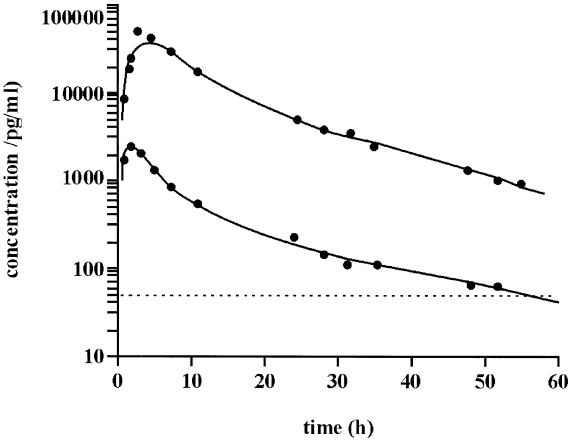


Fig. 8. Plasma concentration–time curves of 9AC lactone and total 9AC in a patient after oral administration of 2.7 mg of 9AC (from Loos et al. [86] with permission).

of anticancer activity and greater potency than water soluble CPT analogues [145]. One of the natural CPT analogues, HCPT, has been shown to have a strong antitumor activity against gastric carcinoma, hepatoma, leukemia, and tumor of head and neck, and furthermore, HCPT is more potent and less toxic in experimental animals and in human trials compared to CPT [146]. Although the mechanisms responsible for the anticancer activity are not fully understood, this drug has been demonstrated to be a DNA topoisomerase I inhibitor with a specific S-phase cell killing effect [147]. Li and Zhang [148] developed a reversed-phase HPLC method with fluorescence detection for the simultaneous analysis of the lactone and carboxylate forms of HCPT in plasma, urine, feces and tissue. Biological samples were prepared by a liquid–liquid extraction method using ice-cold methanol–acetonitrile (1:1, v/v). The mobile phase was 0.075 *M* ammonium acetate buffer (pH 6.4)–acetonitrile (70:30, v/v) to which TBAP was added to a final concentration of 5 m*M*. Eluent was monitored spectrofluorometrically with the excitation wavelength set at 363 nm and the emission wavelength set at 550. Because HCPT, with its substitute hydroxy group at the 10-position, has different water solubility from CPT, the cationic ion-pairing reagent TBAP made it possible to completely separate HCPT from CPT under the stated chromatographic conditions (Fig. 9). Like all known active derivatives of CPT, the lactone form of HCPT, the most important form for anticancer activity, is reversibly hydrolyzed to the less active carboxylate form. Therefore, as with other CPT derivatives, it is essential to quantify both species in biological samples for proper interpretation of pharmacokinetic–pharmacodynamic data [92].

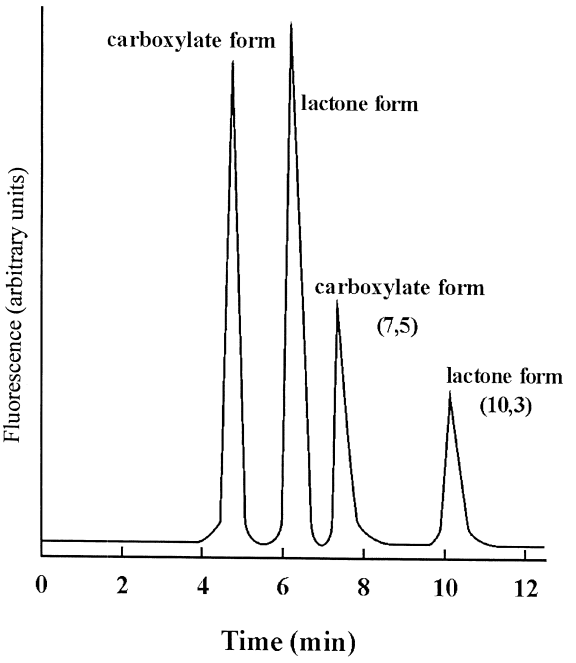


Fig. 9. Chromatogram of HCPT and CPT. Chromatographic conditions: mobile phase, 0.075 *M* NH_4AC (pH 6.4)–acetonitrile (70:30) to which TBAP was added to a final concentration of 0.005 *M*; flow-rate, 0.8 ml/min; column, 250×4.6 mm I.D., RP-18 coupled with inline guard column. Fluorescence detection wavelengths were set at 363 nm (excitation) and 550 nm (emission). Retention times: carboxylate HCPT, 7.5 min; lactone CPT, 10.3 min. Concentrations used: lactone and carboxylate HCPT, 0.5 μg/ml and carboxylate, 5 μg/ml (from Li and Zhang [148] with permission).

5.6. HPLC analysis of lurtotecan

LRT [7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(*S*)-camptothecin; also known as GI147211] is a novel semisynthetic analogue of camptothecin. LRT has previously been shown to have significant activity in both in vitro cytotoxicity assays and in vivo tumor model systems [149–151], and has been recently introduced in clinical trials [152–154].

HPLC methodologies with fluorescence detection have been developed for the determination of the lactone species of lurtotecan [91,97]. These methods employ a mobile phase with ammonium acetate buffer and acetonitrile. For the simultaneous separation of the carboxylate and lactone forms of lurtotecan, Warner and Burke [75] published a method with a specific mobile phase content of acetonitrile–TEAA buffer (1%, v/v), pH 5.5 (19:81, v/v). The method takes less than 10 min.

In recent years, considerable effort has been put into the development of alternative formulations that would allow prolonged systemic exposure to the pharmacologically active drug form. One of these approaches is the incorporation of the lactone forms of camptothecins in liposomal particles. Recent preclinical studies indicated that liposomal encapsu-

lation of the topoisomerase I inhibitors; topotecan [155,156], camptothecin [157,158] and irinotecan [159,160] effectively reduced opening of the lactone ring, increased antitumor activity in experimental tumor models and dramatically enhanced tissue distribution and the systemic availability. Loos et al. [161] developed a sensitive RP-HPLC method with fluorescence detection (λ_{ex}=378 nm; λ_{em}=420 nm) using a sample clean-up procedure which disrupts the liposomes, to determine total drug levels in plasma and urine samples following NX211 administration (NX211 is a new liposomal formulation of LRT; NeXstar Pharmaceuticals). Disruption of liposomes while maintaining the physiologic lactone to carboxylate ratio may not be feasible, so only total concentrations could be measured. In greater detail: to determine whether the therapeutic efficacy of LRT in patients could be improved, the drug was encapsulated in liposomes (NX211; Gilead Sciences). Sample pretreatment involved deproteinization with 10% (w/v) aqueous perchloric acid–acetonitrile (2:1, v/v), and chromatographic separations were achieved on an Inertsil-ODS 80A analytical column. The mobile phase was composed of 1.0 *M* aqueous ammonium acetate (pH 5.5)–water–acetonitrile (10:72.5:17.5, v/v). The choice of the I.S. (6,7-dimethoxy-4-methylcoumarin), and the optimal fluorescence wavelength couple of LRT (378/420 nm) was based on earlier work described for determination of LRT in human blood and in dog plasma by Selinger et al. [97] and Stafford and St. Claire [91], respectively. The assay sensitivity could be significantly improved in order to achieve sub-nanogram per milliliter determinations by increasing the fluorescence intensity of LRT through a modification of the detection procedure using a photochemical reaction to increase the native fluorescence of LRT. LRT is known to be slightly light sensitive [97] and photochemical reactor units in combination with HPLC have been described for a wide variety of other compounds [162–166], where from 2- to 80-fold increases in detector signal outputs have been described. Post-column exposure of LRT to UV light (254 nm) results in a loss of the piperazinomethylene moiety on C7 of the LRT molecule. As shown in Fig. 10, the fluorescence intensity of LRT increased 9- to 15-fold depending on the flow-rate used (0.5–2.00 ml/min). The use of a flow-rate set at 0.75 ml/min resulted in a 14-fold increase in the fluorescence signal of LRT, and was associated with an acceptable total run time (35 min) with retention times of 19 and 24 min for LRT and the I.S., respectively.

5.7. HPLC analysis of DX-8951

DX-8951 is a synthetic water-soluble derivative with a unique hexacyclic structure. DX-8951 is superior to SN-38 and other currently available camptothecins in terms of stabilization of the DNA–enzyme complex, inhibition of topoisomerase I and resulting cytotoxic activity in vitro [167]. The drug exhibited high topoisomerase I inhibition and also high antitumor activity in vitro and was found to affect the transport of P-glycoprotein-mediated multidrug resistant cell lines [168–170]. As with the other camptothecin derivatives, the lactone form, with greater antitumor activity, is in equilibrium with the

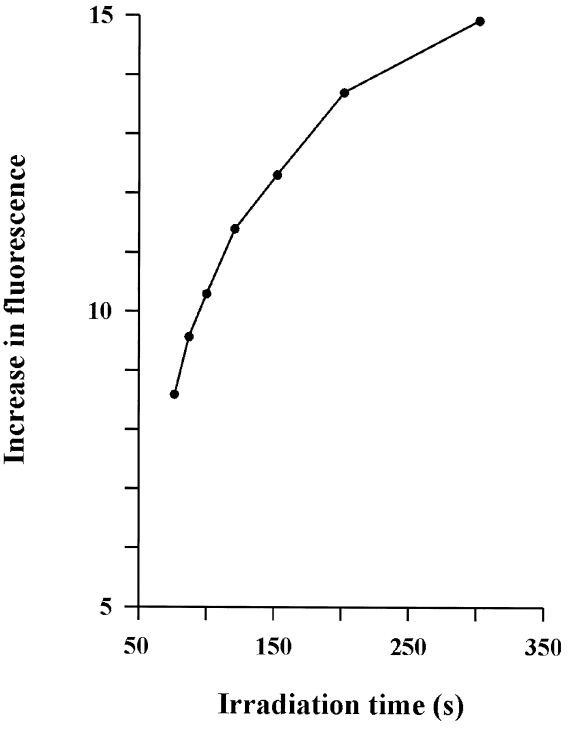


Fig. 10. Influence of the photochemical reaction unit on the fluorescence intensity of LRT (from Loos et al. [161] with permission).

hydroxy-acid form and separate quantification of these two forms is important to evaluate the pharmacokinetics and pharmacodynamics of DX-8951 in clinical trials. Oguma et al. [171] described a new HPLC method for the quantification of the lactone form and the lactone plus hydroxy-acid forms of DX-8951. This assay method consisted of two analytical procedures, one in which the lactone form was collected by the stepwise separation on a C_{18} cartridge and the other in which the lactone plus hydroxy acid forms were collected, from another batch of the plasma sample, by co-elution of the two forms from a C_{18} cartridge under acidic conditions. The hydroxy-acid form of DX-8951 was quantified as the difference between the lactone plus hydroxy-acid forms and the lactone form. The plasma samples in the validated range (0.2–50 ng/ml) were assayed by reversed-phase HPLC with a spectrofluorometer as detector.

6. Conclusion

In the last few years there have been important advances in the development of analytical methods designed to quantify CPT and its analogues. These drugs are strong cytotoxic agents and promising in the treatment of different kinds of tumors, fundamentally gastrointestinal and lung carcinomas.

The big increase in clinical applications of CPT and its analogues and the development of clinical pharmacokinetics as an important tool in controlling the dosage of these agents, intensify the interest in rapid, sensitive and specific analytical methods to quantify them.

7. Nomenclature

9-AC	9-Aminocamptothecin
AUC	Area under the plasma concentration–time curve
CPT-11	Irinotecan
DNA	Desoxyribonucleic acid
HCPT	10-Hydroxycamptothecin
HPLC	High-performance liquid chromatography
HPLC–APCI-MS–MS	High-performance liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry
HSA	Human serum albumin
I.S.	Internal standard
LC–ESI-MS	Liquid chromatography–electrospray mass spectrometry
LLQ	Lower limit of quantification
LOQ	Limit of quantification
LRT	Lurtotecan
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
9-NC	9-Nitrocamptothecin
PET	Positron emission tomography
SDS	Sodium dodecyl sulfate
SN-38	7-Ethyl-10-hydroxy-camptothecin
SN-38G	SN-38 glucuronide
TBAP	Tetrabutylammonium phosphate
TDM	Therapeutic drug monitoring
TEAA	Triethylamine acetate
TPT	Topotecan
Topo I	Topoisomerase I
UV	Ultraviolet

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