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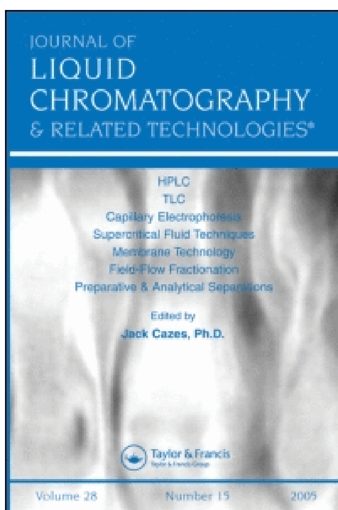

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A Reversed-Phase HPLC Method For Determining Camptothecin In Plasma With Specificity For the Intact Lactone Form of the Drug

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A REVERSED-PHASE HPLC METHOD FOR DETERMINING CAMPTOTHECIN IN PLASMA WITH SPECIFICITY FOR THE INTACT LACTONE FORM OF THE DRUG

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ABSTRACT

Camptothecin is a pentacyclic indole alkaloid with a terminal α-hydroxy-δ-lactone ring, which in aqueous media at physiological pH, exists in equilibrium with the dissociated open-lactone carboxylate. The rate of equilibration between the two components is slow enough to permit their separation by reversed-phase HPLC. Selective determination of the intact lactone form of the drug was achieved by direct analysis of plasma samples immediately upon deproteinization with a solution of the internal standard in methanol chilled to -70°C. Acidification of the sample to pH 2 with perchloric acid prior to protein precipitation effected complete lactonization of the carboxylate and, therefore, provided a measure of total drug levels. Plasma concentrations of the carboxylate may be calculated from the difference between total drug and intact lactone determinations. Chromatography was performed on a 5 μm Ultrasphere ODS column (4.6 mm x 25 cm) preceded by a 1.5 cm RP-18 Brownlee Guard column with an eluent composed of acetonitrile-0.1 M ammonium acetate buffer, pH 5.5 (28:72, v/v) with 1 mM sodium dodecyl sulfate at a flow rate of 1.0 ml/min. The drug was monitored by fluorescence detection with excitation at 347 nm and a 418 nm emission cutoff filter. Approximately 3.0 hr was required to assay an 8 point standard curve and a drug-free plasma sample. Employing 50 μl of plasma, the lowest concentration on the camptothecin lactone and total drug standard curves, 2.82 nM (0.49 ng/ml), was quantified with 3.79 and 5.58% coefficients of variation, respectively. The method has been shown to be specific and reproducible.

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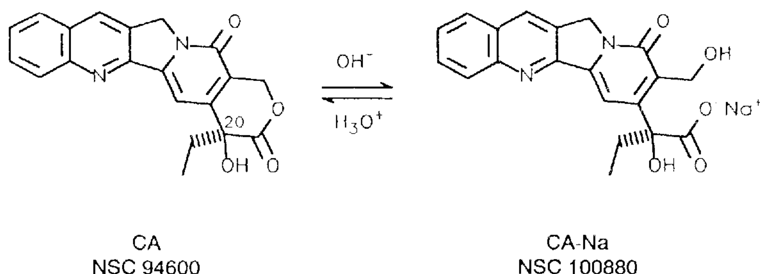

FIGURE 1. Chemical structures of the intact lactone (NSC 94600) and opened ring carboxylate (NSC 100880) forms of camptothecin.

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INTRODUCTION

20(S)-Camptothecin (CA; NSC 94600) is an investigational antitumor agent isolated from *Camptotheca acuminata*, a tree indigenous to southern China (1), with the use of an *in vivo* L1210 screen (2). The terminal α-hydroxy-δ-lactone ring of this pentacyclic indole alkaloid (Figure 1) is a structural requirement for cytotoxic effects (3-7). Configuration of the chiral center at C-20, located adjacent to the lactone carbonyl group, is another determinant of activity. The synthetic 20(R) enantiomer, which does not occur naturally, was found to be essentially devoid of *in vivo* and *in vitro* activity (7-9). An excellent correlation was found between *in vivo* antitumor activity (7-10) and topoisomerase I inhibition (11, 12), the probable mechanism of action of CA and its analogs (13-15).

The poor solubility of CA, conferred by unusually weak basicity of the quinoline nitrogen (1, 2), effectively precluded its use in clinical applications (7). However, as shown in Figure 1, cleavage of the lactone ring in aqueous sodium hydroxide afforded the water soluble carboxylate salt (CA-Na; NSC 100880) (1, 3), which was used in clinical trials despite considerably lower activity against L1210 and P388 murine leukemia than the intact lactone form of the drug (16-19). Following an initial promising report of antitumor activity in colon cancer patients (16), notable responses to CA-Na were not observed in subsequent trials (17-19). It was suggested that dose-limiting hematologic toxicity may be related to variations in renal clearance of the drug (20, 21). The clinical evaluation of CA-Na was discontinued in this country over 15 years ago.

Although the therapeutic and toxic effects of CA appear to be associated with its respective lactone and carboxylate forms, analytical methods for their individual

determination in biological fluids remain unreported. Consequently, all pharmacokinetic information pertaining to CA and its analogs that has appeared in the literature was derived from nonspecific assays. Determinations have been performed microbiologically (22) and by measuring fluorescence of the extracted drug directly (20, 23) or upon HPLC separation (24, 25). However, in each of the fluorometric methods, samples were acidified to effect complete lactonization of the analyte before isolation, providing only a measure of the total drug concentration.

This paper describes an analytical method with specificity to permit quantitation of CA in the presence of its carboxylate form (CA-C) in biological fluids. The assay has been employed during recent preclinical studies to characterize *in vitro* lactone ring opening and total drug, intact lactone and carboxylate plasma pharmacokinetics in mice treated with CA and CA-Na.

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MATERIALS AND METHODS

Reagents and Chemicals

20(S)-Camptothecin (NSC 94600), 20(S)-camptothecin sodium (NSC 100880) and 9-amino-20(RS)-camptothecin (NSC 629971) were supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program, National Cancer Institute. The provided sample of CA ($C_{20}H_{16}N_2O_4$, mol wt 348.36) was used as the analytical reference standard without assigning its purity during the course of this work. Analytical reagent glacial acetic acid from Mallinckrodt (Paris, KY), reagent ACS ammonium acetate and sodium dodecyl sulfate (Eastman Kodak, Rochester, NY) were used. Acetonitrile, methanol and ethyl acetate were OmniSolv grade (EM Science, Gibbstown, NJ). The *p*-toluoyl chloride (Aldrich, Milwaukee, WI), pyridine (Mallinckrodt), chloroform and dimethylformamide (MCB, Cincinnati, OH) were reagent grade. These chemicals were used without further purification. Deionized double distilled water was passed through a 0.2 μm nylon-66 filter (Rainin Instrument Co., Woburn, MA) before use.

Apparatus

Liquid chromatography was performed using a model 114M pump (Beckman Instruments, Berkeley, CA), a Rheodyne 7125 manual injector (Cotati, CA) with a 200 μL sample loop and a Kratos 980 programmable fluorescence detector (ABI Analytical, Kratos Division, Ramsey, NJ). The detector was fitted with a 5 μL flow cell, deuterium source lamp for excitation at 347 nm (5 nm bandwidth) and a 418 nm emission cutoff

filter. A photomultiplier current of 0.10-0.15 μA and 5 sec rise time were employed. The 1 volt output of the detector was provided as the signal to a model 3393A recording integrator (Hewlett-Packard, Avondale, PA). The integrator was configured to report peak heights using a 0.3 min peak width, a threshold setting of 5 and baseline construction through each detected valley point. A 0.2 cm/min chart speed with an attenuation of 4 were used for plotting the chromatograms. The integrator was interfaced to a 9114B disc drive (Hewlett-Packard) for storage of the bunched signal data.

Milligram quantities of the samples used for preparing stock solutions were weighed on a Cahn 25 electrobalance (Cahn Instruments, Cerritos, CA). Stock solutions were prepared in class A borosilicate glass volumetric flasks. Except were noted, other solutions used in the assay were prepared in screw top borosilicate glass test tubes. The volumetric flasks and test tubes were deactivated with 3% (v/v) Surfasil (Pierce Chemical Co., Rockford, IL) in toluene. An Eppendorf model 5412 microcentrifuge (Brinkmann Instruments, Westbury, NY) was used for centrifuging assay samples contained in 1.5 ml polypropylene tubes (VWR Scientific, Philadelphia, PA) at 12,000 x g.

High resolution electron ionization and fast atom bombardment mass spectra were acquired on Kratos MS-30 and VG 70-250S instruments, respectively, at The Ohio State University Chemical Instrumentation Center.

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Synthesis of 9-(*p*-Toluamido)-camptothecin

A fine suspension of 9-amino-20(RS)-camptothecin (5 mg) in pyridine (0.2 ml) was formed by placing the mixture in an ultrasonic bath for 1 min. After cooling in an ice bath, 3 drops of *p*-toluoyl chloride were added to the suspension, which was stirred and allowed to stand for 16 hr at 5°C. Initial purification was achieved by removing the solvent under reduced pressure and applying the residue, reconstituted in chloroform (2 ml), to a 1.25 x 7.5 cm column of slurry packed silica gel (EM Reagents). Excess reagent and nonfluorescent impurities were eluted with chloroform (75 ml) followed by 95:5 (v/v) chloroform-ethyl acetate (50 ml). The fluorescent components of the reaction mixture were then eluted with ethyl acetate (30 ml).

After removing the solvent on a rotary evaporator, the crude product was dissolved in acetone and applied to six silica gel 60 aluminum backed TLC plates (0.2 mm film thickness) without fluorescent indicator (EM Science). The plates were developed with ethyl acetate three times, with intermittent drying, to effect adequate separation of the major band from more strongly adsorbed impurities. The material comprising the major

fluorescent band was isolated by extraction into ethyl acetate, which upon evaporation, afforded colorless crystals. The product was dried overnight at ambient temperature *in vacuo* (0.1 mm Hg) over P_2O_5: MS (FAB), m/z (ion, relative intensity), 482.23 (M, 27.24), 119.06 (CH_3PhCO, 100); MS (EI-HR), 481.1620941 (M, 0.18), 437.1735840 (M-CO_2, 21.75), 119.0505371 (CH_3PhCO, 100). Calcd for $C_{28}H_{23}N_3O_5$: 481.16389 amu.

Protein Precipitant Solution

The entire amount of purified 9-(*p*-toluamido)-camptothecin afforded from the synthesis described above was initially dissolved in 1 ml of dimethylformamide. A 75 μl aliquot of this solution was diluted to 5 ml with acetonitrile containing 10 mM acetic acid (MeCN-AcOH). Both of these solutions were stored at 5°C. On a daily basis, the solution used in the assay for protein precipitation and drug isolation was prepared by adding 100 μl of the MeCN-AcOH stock solution to 10 ml of methanol, and maintained at approximately -70°C in a dry ice-propanol bath.

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Standard Solutions

Solutions of CA used in the preparation of plasma standards were also made in MeCN-AcOH to prevent the lactone ring of the drug from opening during storage. The initial stock solution, prepared to provide a concentration of approximately 0.1 mg/ml, was quantitatively diluted to 0.01 mg/ml. Varied volumes (10-50 μl) of these two stock solutions were carefully added to 1.0 ml of MeCN-AcOH, providing a series of eight secondary standards which ranged in concentration from 0.1-4.8 μg/ml. The stock and secondary standard solutions of the drug were stored at 5°C.

Each plasma standard was individually prepared immediately prior to assaying in consideration of the lability of the CA lactone ring. Pipetting drug-free human plasma (1.0 ml) into a chilled polypropylene microcentrifuge tube and adding 10 μl of a secondary standard solution, carefully measured with a siliconized 25 μl Hamilton syringe, provided the concentrations of drug which comprised the standard curve: 2.821, 6.948, 13.56, 28.20, 55.85, 82.97, 109.6 and 135.6 nM (0.49-47.22 ng/ml). These solutions were thoroughly mixed by vortexing for 15 sec.

Sample Preparation

Intact lactone determination: To 50 μl of plasma in a microcentrifuge tube was added 150 μl of the cold methanolic internal standard solution. The mixture was vortexed for 10 sec and centrifuged for 2 min. An aliquot of the supernatant (150 μl) was

separated from the precipitated protein, diluted with 125 μl of ice-cold 0.1 M ammonium acetate buffer (pH 5.5), and immediately chromatographed after filling the 200 μl injector loop with the solution.

Total drug determination: The plasma sample (50 μl) contained in a microcentrifuge tube was acidified with aqueous perchloric acid (5 μl, 1.5 M), thoroughly mixed by vortexing for 1 min and permitted to stand for 5 min. The sample was then deproteinized as described above and centrifuged for 5 min. The separated supernatant (150 μl) was transferred to another microcentrifuge tube, treated with 125 μl of aqueous 0.4 M acetic acid-0.1 M potassium acetate, and centrifuged for 1 min before filling the injector loop with the sample.

Chromatographic Conditions

Reversed-phase chromatography was performed with an isocratic mobile phase composed of acetonitrile-0.1 M ammonium acetate buffer, pH 5.5 (32:68, v/v) with 1 mM sodium dodecyl sulfate on a 4.6 mm x 25 cm stainless steel column packed with 5 μm Ultrasphere ODS (Beckman Instruments). The analytical column was protected by a 0.5 μm postinjector filter and a 1.5 cm Brownlee RP-18 precolumn (Rainin Instrument Co.) Variability in the drug and internal standard retention times between analytical columns was minimized by adjusting the fraction of acetonitrile, typically between 30 to 34% (v/v). The eluent was degassed in an ultrasonic bath for 15 min prior to chromatography. Separations were performed at ambient temperature with a flow rate of 1.0 ml/min.

Samples were loaded into the injector with a 500 μL Hamilton syringe treated with 5% (v/v) Surfasil in hexane at ambient temperature. The syringe and injector were repeatedly rinsed with methanol between successive injections. In addition, syringes were cleaned weekly by soaking for 30 min in hot sulfuric-nitric acid (4:1, v/v) followed by vigorous rinsing with distilled water and methanol. The precolumn was generally replaced on a weekly basis. Analytical columns were periodically flushed after reversing the direction of flow, first with water, then methanol-water (90:10, v/v) containing 0.4% (w/v) sodium dodecyl sulfate for at least 2 hr, followed by 100% methanol for 1 hr.

Quantitation

Standard curves, run on a daily basis, were constructed by plotting the peak height ratios of CA to the internal standard against drug concentration. Linear least squares regression was performed with a weighting factor of reciprocal peak height ratio squared, without inclusion of the origin, to determine the slope, y-intercept and

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correlation coefficient of the best fit line. Drug concentrations in unknown samples were calculated using the results of the regression analysis.

Relative Recovery

The relative recovery of CA and reproducibility of both assay procedures were determined from the calculated drug concentrations in plasma standards assayed during a six week period.

Absolute Recovery

An additional set of secondary standards was made as previously indicated, with methanol-0.1 M potassium hydroxide (1:1, v/v) as the solvent system, to provide CA-C plasma standards. Upon complete hydrolysis of the lactone ring, which occurred within 15 min at ambient temperature, the solutions were stored at 5 °C.

A series of solutions with the same concentrations of drug and internal standard as plasma standards carried through either sample preparation method were made in methanol-water-acetic acid (45:55:1.5, v/v/v) to stabilize the lactone ring toward hydrolysis. Plasma standards containing the intact or hydrolyzed lactone forms of the drug, prepared for analysis according to the appropriate procedure, were chromatographed in pairs with a corresponding nonplasma sample. Five replicate assays were performed for both sets of solutions at concentrations spanning the range of the standard curve. Accordingly, ratios of the CA peak height for the precipitated plasma standard to that of the paired nonplasma sample provided a measure of absolute recovery. Absolute recovery of the internal standard was similarly determined.

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Optimization of the Fluorescence Detector

The fluorescence excitation wavelength in the region from 225-375 nm was optimized by determining the average peak height from duplicate 25 µl injections of a 50 ng/ml drug solution in methanol-water-acetic acid (45:55:1.5, v/v/v) with varied excitation wavelengths, while maintaining a constant photomultiplier current. The emission filter which provided maximum response at the optimum excitation wavelength was similarly determined. Rise time and photomultiplier current were then varied to further maximize detector response while maintaining an acceptable signal to noise ratio.

Stability of Camptothecin Lactone

The loss of CA in human donor plasma was studied at -22, 0, 25, and 37 °C. Three kinetic runs at a single initial drug concentration of 135.6 nM were performed. The

experiment at -22°C was carried out by initially spiking chilled plasma (1.0 ml) with the drug, from which 50 μl aliquots were rapidly transferred to individual microcentrifuge tubes, flash frozen in dry ice-propanol and placed in a freezer until removed for assaying. Stability studies at 0, 25 and 37°C were conducted in a borosilicate glass weighing bottle, containing a magnetic stir bar, submerged in a water bath maintained at the desired temperature. The plasma (5.0 ml) was permitted to equilibrate in the stoppered bottle for 1 hr, with gentle stirring, before adding 50 μl of the secondary CA standard. Aliquots of the solution were periodically removed for assaying. In addition, CA stability in the supernatant of deproteinized plasma samples stored at -22 and -70°C was determined during a seven day period. Individually frozen aliquots (150 μl) of the protein-free supernatant were removed from the freezers at 24 hr intervals, thawed and chromatographed immediately upon dilution.

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Dosing and Sample Collection

Unfasted male $CD2F_1$ mice were treated with 10 mg/kg doses of either CA or CA-Na by 1 min tail vein (iv) injections, performed without anesthesia using a 27 gauge, 0.5 inch Yale hypodermic needle (Becton-Dickinson). The injectable solutions were formulated immediately before use to deliver the desired dose in a volume of 100 μl as follows. A dimethylsulfoxide solution of CA was diluted 1:4 (v/v) with 50% (v/v) polyethylene glycol 400 in sterile water acidified to pH 3.0-3.5 with phosphoric acid (10 mM) to prevent lactone ring opening. CA-Na was dissolved in sterile water for injection.

The mice were bled 60 min after treatment through the retro-orbital plexus under ether anesthesia using heparinized microhematocrit capillary tubes (American Scientific Products). The whole blood was rapidly cooled in dry ice-propanol for several seconds, without freezing, prior to centrifugation at 12,000 x g for 1 min. Three 50 μL aliquots of the separated plasma were transferred to individual microcentrifuge tubes and deproteinized according to the intact lactone assay procedure. These samples were either chromatographed immediately upon addition of buffer or stored directly at -20°C. The remaining plasma was then separated from the erythrocytes and stored at -20°C until assayed for total drug.

RESULTS

Fluorescence Detector Optimization

As the position of excitation maxima are often affected by matrix and instrumental factors, the excitation wavelength of the analytical detectors was optimized about the

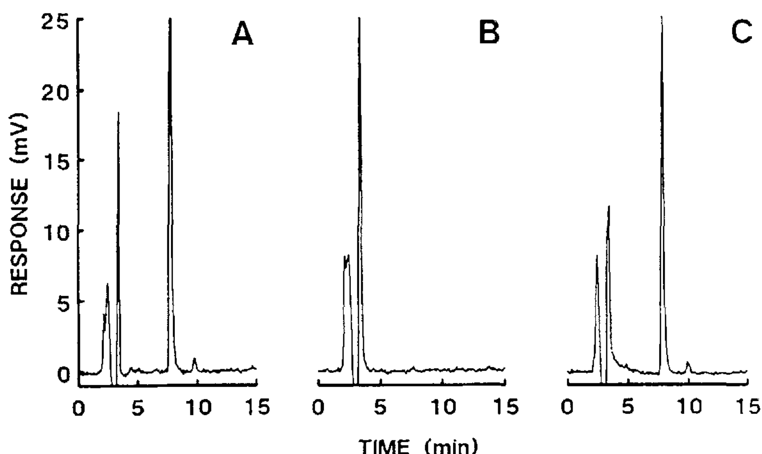

FIGURE 2. Liquid chromatograms of plasma samples containing camptothecin lactone (A) and carboxylate (B) prepared for analysis by deproteinization with methanol at -70°C. (C) The plasma sample with camptothecin carboxylate processed for chromatography with acidification prior to protein precipitation. Chromatographic peaks: CA carboxylate, 3.25 min; CA lactone, 6.8 min.

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253 and 368 nm UV absorption bands of CA. The chromatographic peak height for a constant amount of the drug was determined at systematically varied excitation wavelengths while maintaining all other instrumental parameters. For each of the three Kratos 980 fluorescence detectors in our laboratories, the excitation maxima of CA were located at 254 and 347 nm. Fluorescence intensity emitted upon excitation at 254 nm was approximately 2-fold greater than at 347 nm. The other instrumental parameters affecting sensitivity are the emission cutoff filter, photomultiplier current (PMT), voltage and rise time. Detector response was greatest with a 418 nm emission cutoff filter in comparison to filters rated at 389, 440 and 470 nm. A further enhancement in sensitivity was achieved by using the highest PMT that provided an acceptable baseline noise level with a 5 sec rise time, which ranged from 0.10-0.15 μA for the different detectors.

Sample Preparation and Chromatography

The liquid chromatograms shown in Figure 2 demonstrate that interconversion between the lactone and carboxylate forms of CA during sample preparation was effectively inhibited by rapidly deproteinizing plasma specimens containing the drug with

methanol at -70°C. Samples were chromatographed immediately after dilution of the supernatant with 0.1 M ammonium acetate buffer (pH 5.5) to adjust the solvent strength. CA eluted as a sharp peak (Figure 2A) well resolved from poorly retained CA-C (Figure 2B). The chromatogram of CA-C in plasma showed no peaks with a capacity factor similar to CA. However, acidifying plasma samples to approximately pH 2 with perchloric acid before protein precipitation resulted in the rapid and complete lactonization of the carboxylate (Figure 2C).

Typical liquid chromatograms of plasma standards encompassing the range of concentrations which comprised the standard curve are shown in Figure 3. The chromatogram of drug-free human plasma was free of endogenous components which could interfere with detection of the analyte or internal standard. The inclusion of sodium dodecyl sulfate in the mobile phase served to sharpen the peak associated with polar endogenous plasma constituents and prolong column lifetime. It did not alter the chromatographic properties of either form of the drug at the concentration employed. Although not significantly affected by the presence of buffer components or ion pairing agents, the retention of CA exhibited extreme sensitivity to the fraction of acetonitrile in the mobile phase. Retention times (mean ± SD, n = 14) of CA and the internal standard obtained from plasma samples chromatographed on separate days using a single analytical column were 7.52 ± 0.31 and 13.25 ± 0.93 min, respectively. Chromatograms acquired from the higher concentration plasma standards show a small peak following the drug attributed to a minor unknown impurity. With an 18 min run time for a single sample, a calibration curve consisting of 8 plasma standards and a drug-free sample was analyzed in less than 3 hr.

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Calibration Curves

Figures 4A and 4B represent typical calibration curves for the intact lactone and total drug assays, respectively, in which the CA to internal standard peak height ratio is proportionate to analyte concentration. The standard curves included 8 points ranging from 2.8 to 135.6 nM. Linear least squares regression was performed with a weighting factor of $1/y^2_{obs}$, without inclusion of the origin, to determine the best fit line. Correlation coefficients over this concentration range were generally greater than 0.998 for either assay procedure.

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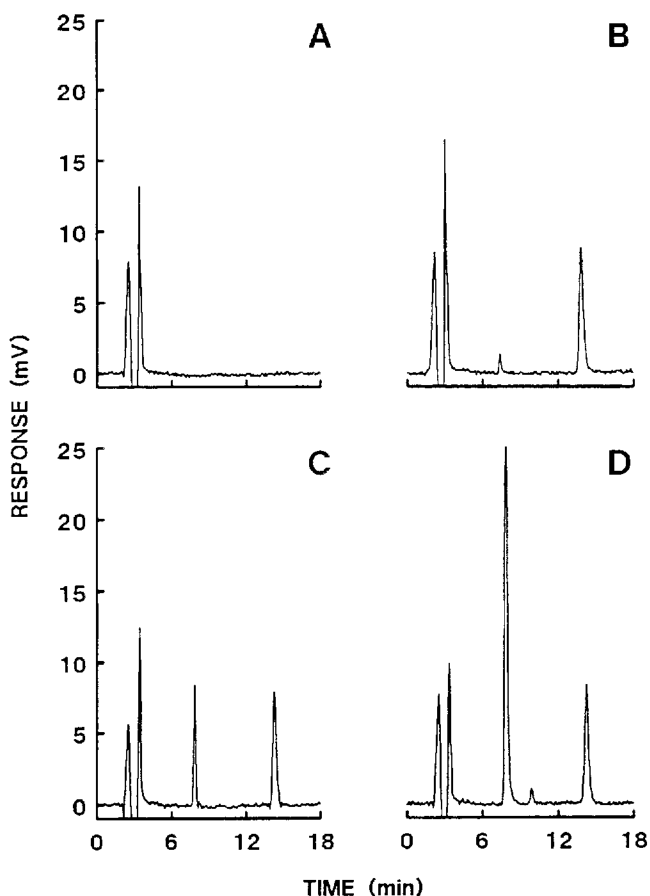

FIGURE 3. Representative liquid chromatograms of standard curve samples prepared by spiking drug-free human plasma with camptothecin lactone and assayed according to the direct deproteinization method. (A) Drug-free plasma; (B) 2.82 nM CA; (C) 28.19 nM CA; (D) 135.6 nM CA. Chromatographic peaks: CA, 7.29-7.36 min; IS, 14.57-14.71 min.

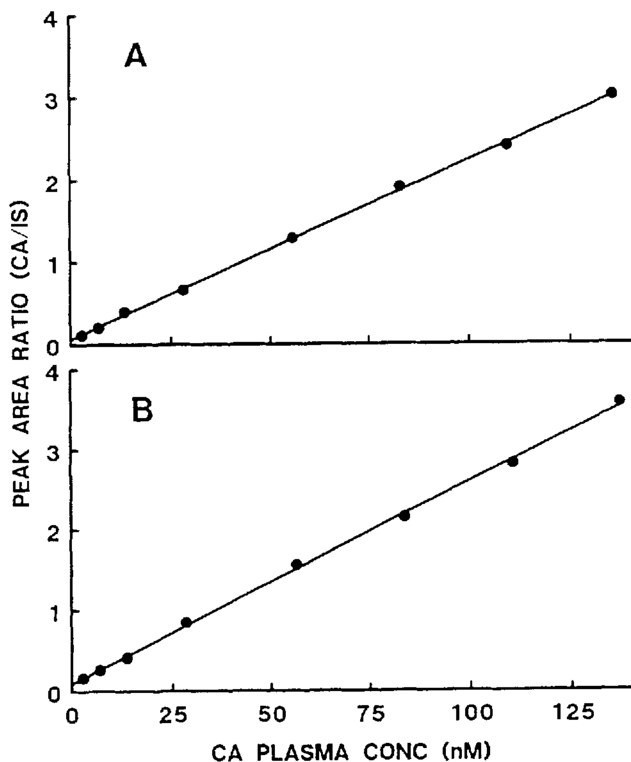

FIGURE 4. Typical standard curves of camptothecin in human plasma determined according to the analytical methods for the intact lactone (A) and total drug (B). Parameters describing the best fit lines are: (A) slope, 0.02194; y-intercept, 0.05519; corr, 0.9986; (B) slope, 0.02503; y-intercept, 0.09053; corr, 0.9990.

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Assay Reproducibility

Plasma standards prepared from a single stock solution of CA were assayed according to the intact lactone and total drug procedures on 10 days during a 6 week period. The relative recoveries of the drug from plasma and assay reproducibility for the range of concentrations included in the standard curves during this period are summarized in Table 1. Coefficients of variation for the replicate determinations ranged from 3.44-5.89% for the direct analysis method and 3.48-7.32% for total drug assays. The respective values for the lowest plasma concentration of CA quantitated, 2.819 nM

TABLE 1

Relative Recovery and Reproducibility of the Analytical Methods for Intact Camptothecin Lactone and Total Drug in Human Plasma

Amount Added nM	N	Mean Amount Found nM	Recovery %	CV[a] %
Intact Lactone				
2.819	10	2.750	97.55	3.79
6.943	10	7.212	103.87	5.89
13.56	9	14.32	105.61	3.59
28.19	9	27.74	98.39	4.19
55.82	10	53.89	96.55	5.31
82.91	10	82.36	99.34	3.44
109.5	10	107.9	98.52	4.92
135.6	10	141.3	104.20	3.95
Total Drug				
2.819	10	2.786	98.82	5.58
6.943	10	7.151	103.00	6.62
13.56	10	13.31	98.16	7.32
28.19	10	28.43	100.86	3.71
55.82	10	57.50	103.01	4.69
82.91	10	83.13	100.26	5.06
109.5	10	110.6	101.04	3.48
135.6	10	136.4	100.61	4.55

[a] Coefficient of variation.

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(0.49 ng/ml), were 3.79 and 5.58%. The minimum detectable plasma concentration of 1.4 nM was not quantified with acceptable reproducibility. Relative recoveries were 96.55-105.61% and 98.16-103.01% for the intact lactone and total drug methods, respectively. Inasmuch as these calibration curves were assayed using several different analytical columns over an extended period of time, the coefficients of variation for the slopes of the 10 linear regression curves, 8.21% for intact lactone and 6.99% for total drug, demonstrate that the method proved to be consistent during this period.

Absolute Recovery

Absolute recoveries were determined by comparing the peak heights from plasma standards containing either the pure lactone or carboxylate form of the drug carried

TABLE 2

Absolute Recovery of Camptothecin Lactone and Carboxylate from Human Plasma

Amount Added nM	Intact Lactone Recovery[a] %	Intact Lactone CV[b] %	Carboxylate Recovery[a] %	Carboxylate CV[b] %
2.821	97.64	12.27	100.08	13.43
6.948	99.67	5.22	102.39	6.69
28.20	100.87	3.37	98.44	3.25
82.97	99.44	2.95	106.11	8.39
137.5	98.16	1.47	99.21	5.67

[a] Mean absolute recovery, n = 5.
[b] Coefficient of variation.

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through the assay procedure to directly injected solutions of CA and the internal standard (Table 2). The absolute recovery (mean ± SD) of CA lactone from plasma was 99.16 ± 1.28%, calculated from the individual mean recoveries (n = 5) for 5 different concentrations spanning the range of the standard curve. Similarly evaluated, 101.25 ± 3.10% of the CA-C was recovered from plasma by the acidification procedure. The mean absolute recovery of the internal standard was 98.96 ± 5.51% (n = 25).

Stability of the Camptothecin Lactone Ring

Information on the temperature dependence of CA lactone hydrolysis in human donor plasma was desired for the development of a sample acquisition and storage protocol for use during pharmacokinetic studies. At temperatures ranging from -22 to 37°C, the concentration of CA in plasma decayed in an apparent first-order manner during time periods which encompassed up to 95% loss of the added drug (Figure 5A). Thereafter, the rate of lactone hydrolysis diminished until the establishment of equilibrium, which was approximately 99.4% in favor of CA-C at 37°C. Total drug recovery at the end of each kinetic run was essentially quantitative.

Observed first-order rate constants for lactone decay (k_{obs}) were determined from unweighted linear regression analysis of the semilogarithmic geometric mean plasma concentration-time profiles. Kinetic parameters corresponding to these fits are

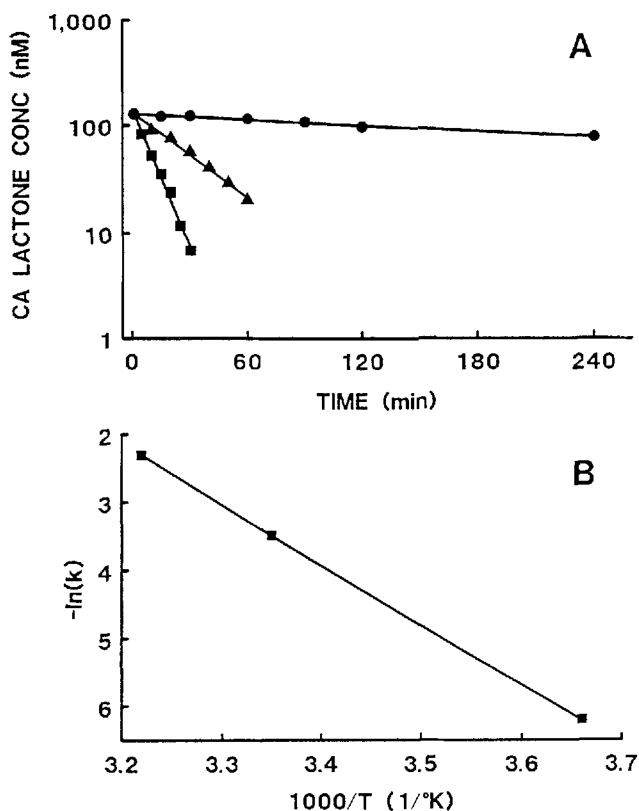

FIGURE 5. (A) Time courses of camptothecin lactone in human donor plasma at 0 (•), 25 (▲) and 37°C (■). (B) Arrhenius plot showing the temperature dependence of apparent first-order rate constants characterizing the loss camptothecin in human donor plasma from 0-37°C: slope, -8.823°K; y-intercept, 26.09; corr, 0.9999.

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summarized in Table 3. The best fit lines drawn through the experimental points of each profile were generated from the linear regression analyses (Figure 5A). As shown in Figure 5B, the temperature dependence of lactone decay in donor plasma afforded a linear Arrhenius plot of $\ln(k_{obs})$ *versus* reciprocal absolute temperature. The activation energy calculated from the slope, 17.5 kcal/mol, was similar in magnitude to typical values of nonenzymatic ester hydrolysis (26).

Directly freezing plasma samples at -22°C did not adequately stabilize CA toward lactone ring opening, as 5% of the initial concentration was lost within 16.8 hr. There

TABLE 3

Temperature Dependence of Camptothecin Lactone Stability in Human Plasma

t, °C	T, °K	k_{obs},[a] min^{-1}	$t_{1/2}$, min
-22	251.16	5.080 ± 0.093 x 10^{-5}	1.36 x 10^{4} [b]
0	273.16	2.035 ± 0.258 x 10^{-3}	340.9
25	298.16	3.157 ± 0.992 x 10^{-3}	22.61
37	310.16	9.925 ± 1.405 x 10^{-2}	6.98

[a] Apparent first-order rate constant ± 95% confidence interval.

[b] Time for 5% degradation ($t_{0.95}$), 16.8 hr.

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was no apparent loss of CA when the supernatant of plasma samples deproteinized with methanol was stored at either -22 or -70°C during a 7 day period. This indicated that plasma samples acquired during a kinetic study could be stored in a conventional freezer upon deproteinization and subsequently assayed for the intact lactone. Total drug levels may be determined upon storing plasma samples directly at -20°C.

In Vivo Drug Monitoring

Figures 6 and 7 show liquid chromatograms of plasma specimens obtained from mice 60 min after iv treatment with 10 mg/kg of CA-Na or CA, respectively, assayed according to the intact lactone and total drug procedures. Fluorescent components which could interfere with detection of either the drug or internal standard were not apparent when pretreatment plasma was assayed by either technique (Figures 6A, 7A). Peaks corresponding to potential metabolites of the drug were not evident in the chromatograms of mouse plasma obtained after dosing with either formulation.

Figure 6B demonstrates that systemic levels of the active lactone form of the drug were generated following iv administration of CA-Na to mice. However, the total drug plasma concentration, 261 nM, was more than 2-fold greater than CA itself (127 nM), suggesting that the drug remained predominantly in the carboxylate form 60 min after dosing (Figure 6C). At the same time subsequent to treatment with a similar dose of CA, the respective intact lactone and total drug plasma levels in mice were 1311 and 1521 nM (Figures 7B and 7C). Thus, compared to CA-Na, not only were significantly greater total drug levels provided, but a larger fraction of the drug in plasma was present in the

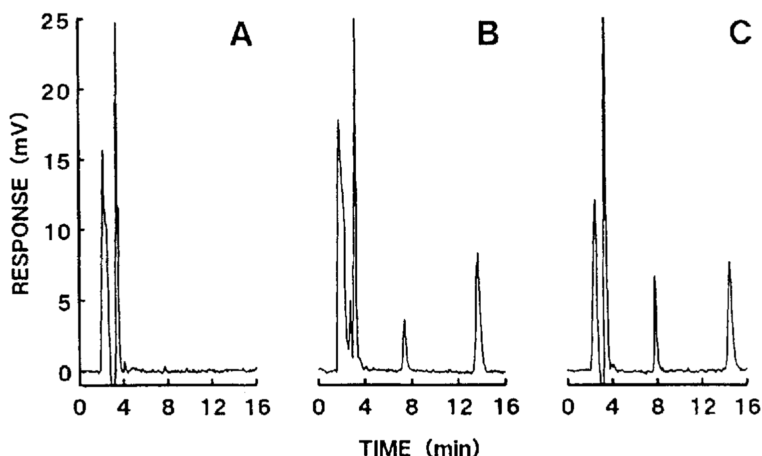

FIGURE 6. Liquid chromatograms of plasma specimens acquired from a mouse treated with 10 mg/kg of camptothecin sodium by iv injection. (A) Pretreatment plasma prepared for analysis according to the intact lactone method. Plasma samples obtained 60 min after dosing were assayed upon 10-fold dilution with drug-free plasma by the intact lactone (B) and total drug procedures (C). The concentrations of CA lactone and total CA were 127 and 261 nM, respectively.

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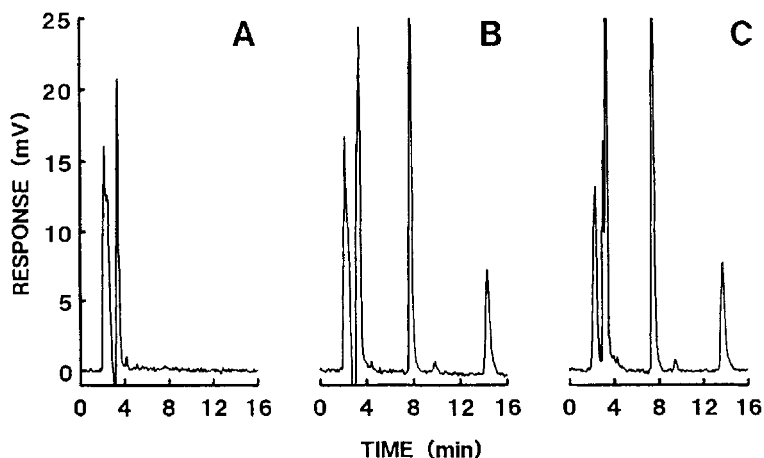

FIGURE 7. Liquid chromatograms of plasma specimens acquired from a mouse treated with 10 mg/kg of camptothecin by iv injection. (A) Pretreatment plasma prepared for analysis according to the total drug procedure. Plasma samples obtained 60 min after dosing were assayed upon 10-fold dilution with drug-free plasma by the intact lactone (B) and total drug methods (C). The concentrations of CA lactone and total CA were 1311 and 1521 nM, respectively.

therapeutically active form. The higher drug levels observed after dosing with the intact lactone formulation indicates that CA-C is eliminated from plasma more rapidly than CA.

DISCUSSION

During the past 20 years since the CA-Na clinical trials were terminated, there has been a continued effort to identify structural analogs of the drug which may prove to be more suitable from therapeutic considerations than the parent compound. In addition to improving efficacy (5, 7, 8, 10, 27), molecular modifications were designed to increase water solubility and thereby facilitate parenteral administration of the intact lactone form of the drug without diminishing its activity (7, 28, 29). Information derived from comparative disposition studies may be of particular advantage to the preclinical evaluation of promising CA analogs, since the dose-limiting toxic effects of CA-Na were apparently associated with its urinary excretion (21) and accumulation in the gastrointestinal tract (30). Unfortunately, analytical methods utilized to determine drug plasma levels during each of the previously reported pharmacokinetic investigations of CA and related compounds did not differentiate their intact lactone and carboxylate forms (20-25, 31, 32).

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Hydrolysis of the CA lactone ring is a reversible reaction influenced by solution pH and composition (1, 3, 7). In general, the rate and extent of lactone hydrolysis are augmented by increasing hydroxide ion concentration while ring closure becomes favored under acidic conditions. However, although cyclization of CA-Na at physiological pH was considered to be improbable (7), it has been recently demonstrated in our laboratories and elsewhere (33) that, in neutral aqueous solution and biological media, CA and its analogs exist as a mixture of their intact and opened lactone ring forms, the composition of which changes at a relatively slow rate until equilibrium is established.

The intense fluorescence and lipophilicity of CA is diminished upon cleavage of the lactone moiety (1, 23). Accordingly, each of the fluorometric methods developed to quantify CA and related compounds in biological media utilized an acidification procedure to effect lactonization of the analyte prior to isolation and detection (20, 23-25). This provides a measure of the total drug level, a composite value corresponding to the sum of the intact and opened lactone concentrations. Consequently, all information pertaining to the individual concentration of either component is lost when samples are

assayed in this manner. However, consideration of the relatively slow rate of lactone-carboxylate equilibration and the dissimilar solubility properties of these reversibly associated species indicates that their pharmacokinetic behavior will differ appreciably. The appropriate relationship between the lactone and carboxylate forms of CA following administration of either pure component is probably more analogous to a parent drug and derived metabolite than the conjugate acid-base pair of an ionizable xenobiotic. It is, therefore, inappropriate to establish the disposition of CA exclusively from total drug determinations.

An analytical method with specificity to permit quantitation of both forms of CA in plasma necessarily depended upon the successful development of a procedure to suppress interconversion between the intact lactone and CA-C during sample preparation for chromatography. We explored the use of a water miscible organic solvent for isolating CA with coincident deproteinization in consideration of favorable analyte solubility properties and general rapidity of the procedure. Lactone-carboxylate interconversion was evident when plasma samples spiked with either pure form of the drug were deproteinized with methanol at ambient temperature. Reducing the solvent temperature to approximately -70°C in dry ice-propanol effectively arrested both directions of the reaction.

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Optimum reversed-phase liquid chromatographic separations were achieved with an Ultrasphere ODS analytical column and mobile phase composed of acetonitrile-0.1 M ammonium acetate, pH 5.5 (28:72, v/v). The pH of the eluent buffer was selected to coincide with the minimum in the pH-rate profile of the CA lactone-carboxylate equilibrium reaction (33), which was slow enough to permit chromatographic separation of the two components. There were only two peaks observed upon HPLC analysis of reference solutions containing the drug, eluting shortly after the solvent front and at 7.5 min, respectively assigned to CA-C and CA. Consideration of the known pK_a values of other α-hydroxy carboxylic acids, such as lactic acid (pK_a 3.79) and gluconic acid (pK_a 3.60) (34), suggests that CA-C will be entirely dissociated in a mixed aqueous solution of this composition. Furthermore, identification of the retained component as CA lactone is also consistent with the appreciably weaker basicity of the CA quinoline nitrogen, pK_a 1.18 (33), than quinoline itself (pK_a 4.5) (34).

Incorporating tetrabutylammonium hydrogen sulfate (2-5 mM) in the mobile phase resolved CA-C from the solvent front without affecting the chromatographic behavior of CA. Even though this permitted concurrent detection of both components without interference, direct quantitation of CA-C could not be achieved with the sensitivity and

precision that was desired. When visualized at sensitivities approaching the limit of detection, the ion-paired carboxylate peak exhibited unacceptable tailing on a variety of stationary phases (octadecylsilane from several sources, cyano and phenyl) and eluents composed of acetate buffer modified with methanol, acetonitrile or their mixtures. This behavior was evident even when the peak was eluted with a capacity factor less than half that of CA. Furthermore, as previously indicated, fluorescence intensity of CA-C is two to three-fold lower than CA (23), although UV absorption spectra of the two components are not appreciably different (1, 2).

Consequently, an indirect approach for quantifying CA-C was pursued, based upon the difference between the total drug and intact lactone plasma concentrations. Total drug plasma levels were determined essentially in the same manner as the intact lactone following acidification of the sample with perchloric acid to effect the rapid and complete lactonization of CA-C. The use of formic, hydrochloric or phosphoric acid in this application compromised the effectiveness of methanol as a deproteinizing agent. Acidification of the supernatant from deproteinized samples resulted in less than quantitative drug recovery, which indicated that CA-C was not completely extracted into the methanolic liquid phase.

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The total drug method of sample preparation has been carefully validated by demonstrating that a known amount of CA-C added to drug-free plasma was quantitatively recovered as the lactone upon acidification of the sample followed by deproteinization with methanol. However, the question of specificity upon applying this technique to samples of physiological origin remains to be addressed. The hydroxyl group at C-20 invests the carbonyl carbon of the lactone moiety with strong electrophilic character, as evidenced by the facile synthesis of amides at C-21 (2, 28, 35). The amides readily return to the lactone by intramolecular attack of the resultant primary alcohol in acidic aqueous solution.

The preceding discussion suggests the possibility that CA and its analogs may react with endogenous nucleophiles (27). However, all previous efforts to detect fluorescent metabolites of the drug in biological fluids obtained from mice (23, 31), rats (21, 31), dogs (23) and man (16, 20, 21) treated with CA-Na have proven negative. Furthermore, studies conducted in our laboratories have shown that the polar drug derived component isolated without acidification from plasma specimens obtained from mice treated with CA or CA-Na exhibited the same retention time as authentic CA-C when liquid chromatography was performed with an ion pairing agent in the mobile phase. When these same samples were assayed according to the total drug procedure,

the polar peak vanished together with an enhancement of the later eluting lactone peak. It is extremely unlikely that this behavior could be associated with another drug derived component. However, the potential formation of nonfluorescent and highly polar metabolites of CA which, consequently, elude detection or isolation by the techniques employed for monitoring the parent drug cannot be discounted.

It has been a general practice in our laboratories to initially assay samples acquired during a pharmacokinetic study in duplicate. A third assay is performed in cases where the individual determinations deviate from their average by more than 10%. In the present method, the volume of plasma assayed was 50 μl, selected as the largest sample size accommodating triplicate determinations of the intact lactone and total drug concentration in each specimen acquired from a mouse. With a fixed sample size, the primary determinants of sensitivity are analyte recovery, efficiency of the chromatographic separation and detector optimization.

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The sample preparation procedure was devised to maximize the fraction of the initial sample ultimately available for analysis after processing for liquid chromatography. Quantitative isolation of CA from plasma was effected with adequate deproteinization using only 150 μl of methanol. Prior to chromatography, the solvent strength of the protein-free supernatant was adjusted to minimize band broadening by dilution with 125 μl of aqueous buffer. Thus, with an injection volume of 200 μl, more than 80% of the analyte present in the sample was introduced into the chromatograph. Although acetonitrile is a more effective macromolecule precipitant than methanol, distortion of the CA peak upon HPLC necessitated greater dilution of the sample. However, the proportionately larger injection volume required for loading a similar fraction of the sample into the system resulted in a significantly broader peak than was achieved with methanol.

The uncorrected excitation and emission spectra of CA measured in 95% ethanol were reported to consist of single bands with maxima at 370 and 434 nm, respectively (23). However, when determined during liquid chromatography using a deuterium light source, the CA fluorescence excitation spectrum appeared very similar to its UV absorption spectrum, characterized by three principal bands near 220 nm (ϵ 37,300 $M^{-1}cm^{-1}$), 254 (29,200) and 370 (19,900) (1). Although excitation at the more intense bands would afford the greatest sensitivity, interfering endogenous plasma constituents were evident when wavelengths below 300 nm were used. Thus, with optimized detection parameters, which included excitation at 347 nm and a 418 nm emission cutoff filter, the lower limit of quantitation for CA in a 50 μl plasma sample was 2.82 nM (0.49 ng/ml).

The compound which served as the internal standard, 9-(*p*-toluamido)-camptothecin, was synthesized on a micropreparative scale by the reaction between 9-amino-20(RS)-camptothecin and *p*-toluoyl chloride in pyridine. Initially, CA analogs were not considered suitable for use as internal standards in the intact lactone assay because of the lability associated with the lactone ring. However, it was subsequently discovered that the lactone moiety was sufficiently stable in methanol when maintained at -70°C to permit incorporation of the 9-*p*-toluamido derivative directly in the protein precipitant.

Hydrolysis of the CA lactone ring was detected in an acetonitrile stock solution of the drug within one week after preparation. There was no apparent loss of CA or the internal standard, associated with lactone cleavage or degradation otherwise, during a four week period when stock solutions were made with acetonitrile containing 10 mM acetic acid. However, as a consequence of lactone instability in plasma, standard solutions were individually prepared on a daily basis immediately prior to analysis.

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Lactone hydrolysis was not evident during 12 hr after plasma samples containing the drug were prepared for analysis according to the acidification procedure. This suggested that an automatic injector may be utilized for overnight analysis to facilitate an increased rate of data acquisition. Unfortunately, both types of automatic samplers at our disposal, the Waters Associates WISP model 712 and Spectra-Physics model 8780, proved to be unsatisfactory. Residual traces of the drug, which exhibited a propensity to adsorb onto solid surfaces, were not adequately removed between injections because of limitations regarding the volume and composition of solvent systems that may be used for flushing these devices. Similarly, all glassware used to contain solutions of the drug or internal standard should be deactivated by siliconization.

In summary, an isocratic reversed-phase HPLC method was developed for the determination of CA in biological fluids. Isolation of the drug from plasma was achieved by macromolecule precipitation with a solution of the internal standard in methanol chilled to -70°C. The selective determination of CA was achieved by direct analysis of plasma samples immediately upon deproteinization. Acidification of the sample with perchloric acid prior to protein precipitation, effecting complete lactonization of CA-C, provided a measure of total drug levels. Plasma concentrations of CA-C may, if desired, be calculated from the difference between total drug and CA determinations.

Chromatography was performed on a 5 μm Ultrasphere ODS column (4.6 mm x 25 cm) preceded by a 1.5 cm RP-18 Brownlee Guard column with an eluent composed of acetonitrile-0.1 M ammonium acetate buffer, pH 5.5 (28:72, v/v) at a flow rate of 1.0

ml/min. The drug was monitored by fluorescence detection with excitation at 347 nm and a 418 nm emission cutoff filter. With an 18 min chromatographic run time, approximately 3.0 hr was required to assay an 8 point standard curve and a drug-free plasma sample. Employing 50 μl of plasma, the lowest drug concentration on the CA standard curve, 2.82 nM (0.49 ng/ml), was quantitated with a 3.79% coefficient of variation (n = 10). The method has been shown to be specific and reproducible.

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