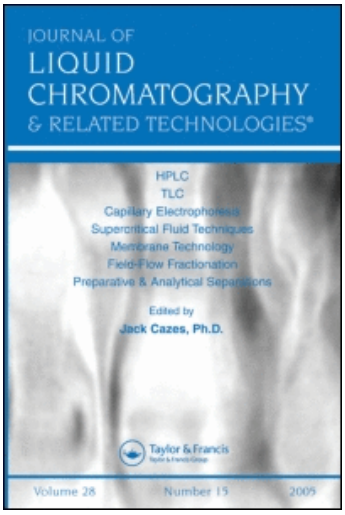

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Supko, Jeffrey G. and Malspeis, Louis(1992) 'Liquid Chromatographic Analysis of 9-Aminocamptothecin in Plasma Monitored by Fluorescence Induced upon Postcolumn Acidification', Journal of Liquid Chromatography & Related Technologies, 15: 18, 3261 — 3283
To link to this Article: DOI: 10.1080/10826079208020883
URL: http://dx.doi.org/10.1080/10826079208020883

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(18), 3261-3283 (1992)

LIQUID CHROMATOGRAPHIC ANALYSIS OF 9-AMINOCAMPTOTHECIN IN PLASMA MONITORED BY FLUORESCENCE INDUCED UPON POSTCOLUMN ACIDIFICATION

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ABSTRACT

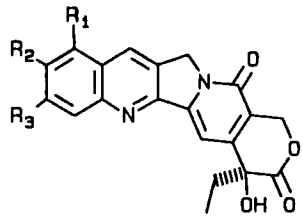
Through preclinical studies by the Developmental Therapeutics Program of the National Cancer Institute with 9-amino-20(S)-camptothecin (AC), this new investigational anticancer agent will soon enter phase I clinical trials in cancer patients. During initial attempts to monitor the drug in biological fluids, it became evident that the presence of an amino group on the camptothecin A-ring suppressed the intense native fluorescence characteristic of the unsubstituted compound. However, subsequent spectrofluorometric studies revealed that the fluorescence of AC was highly pH-dependent in a manner not typically exhibited by aromatic amines. The uncharged species that exists in neutral and weakly acidic solution is nonfluorescent. Protonation of the C-9 amino group proceeds with the development of fluorescence, the intensity of which is optimum in moderately acidic solution of apparent pH 1.7-2.3. Under more strongly acidic conditions, fluorescent intensity again diminishes due to further protonation at the quinoline nitrogen atom. Therefore, postcolumn acidification prior to fluorescence detection provided a convenient and sensitive method to monitor the elution of AC during liquid chromatography. Following protein precipitation induced by mixing with a solution of the internal standard, camptothecin, in methanol chilled to -70°C, plasma samples were separated on a 5 μm Ultrasphere ODS column (4.6 mm x 25 cm) using an isocratic mobile phase composed of acetonitrile-methanol-0.1 M ammonium acetate buffer, pH 5.5 (23:10:67, v/v/v) at a flow rate of 1.0 ml/min. Prior to detection, the column effluent was mixed inline with 0.3 M aqueous trifluoroacetic acid at a flow rate of 0.3

ml/min and ambient temperature. Fluorescence was then monitored using an excitation wavelength of 352 nm and a 418 nm emission cutoff filter. Typical retention times of the drug and internal standard were 7 and 12 min, respectively. Employing sample volumes of 50 μl, the lowest plasma concentration of AC included in the standard curve, 13.3 nM (5.0 ng/ml) was quantified with a 7.63% coefficient of variation (n = 10).

INTRODUCTION

20-(S)-camptothecin (CA; NSC 94600) was discovered in 1966 by the bioassay guided fractionation of stemwood from the Chinese tree *Camptotheca acuminata* Decne using an *in vivo* L1210 leukemia screen (1, 2). The compound is an unusually hydrophobic quinoline alkaloid and its rapid introduction into clinical trials was greatly facilitated by the formation of a water soluble sodium salt (CA-Na, NSC 100880) upon alkaline hydrolysis of the labile terminal α-hydroxy-δ-lactone ring (Figure 1). Evidence available at the time indicated that the two forms of the drug were equipotent (1, 3); however, subsequent studies revealed that the *in vivo* efficacy of CA-Na against L1210 and P388 murine leukemias was considerably lower than the intact lactone structure (4-6). The poor objective response rate among cancer patients treated with the sodium salt, coupled with dose limiting myelosuppression and severe gastrointestinal toxicities, prompted discontinuation of phase II trials in 1972 (7-12).

Seldom has an investigational antitumor agent generated more interest following a highly unfavorable clinical evaluation in the early stages of its development. These endeavors have largely focused upon two primary objectives. Studies undertaken to elucidate the mechanism of antitumor activity have identified CA and its analogs as the first, and currently only, known class of compounds which induce cytotoxic effects through the inhibition of topoisomerase I (13-17). There has also been a continued effort to improve the therapeutic effectiveness of the drug through structural modifications. Molecular modifications have been designed to increase water solubility and thereby facilitate parenteral administration of the intact lactone form of the drug (4, 18-20). Two soluble analogs of CA, topotecan and CPT-11, are currently under clinical evaluation (21, 22). An alternate objective of considerable interest has been to enhance the efficacy of the parent compound (4, 5, 23-25). Among the numerous CA derivatives that have been prepared, the extremely potent 9-amino (AC; NSC 629971) and 10,11-methylenedioxy (MDCA; NSC 606174) analogs synthesized by Wall *et al.* (6) were selected by the Developmental Therapeutics Program of the National Cancer Institute for preclinical evaluation (Figure 1).



CA, $R_1 = R_2 = R_3 = H$
AC, $R_1 = NH_2$, $R_2 = R_3 = H$
MDCA, $R_1 = H$, R_2, $R_3 = OCH_2O$

FIGURE 1. Chemical structures of camptothecin (CA), 9-aminocamptothecin (AC) and 10,11-methylenedioxycamptothecin (MDCA).

We recently reported a reversed-phase HPLC method for determining plasma levels of CA lactone in the presence of its opened ring carboxylate form (26). The intense native fluorescence of CA (27), associated with the highly conjugated quinoline ring system, afforded an extremely sensitive and specific means of detection. The assay was subsequently employed to characterize *in vitro* lactone ring opening and total drug, intact lactone and carboxylate plasma pharmacokinetics in mice treated with CA and CA-Na (28). Although the analytical method developed for CA was suitable for determining MDCA, it was found that AC could not be monitored with fluorescence detection. This paper describes the unusual, marked decrease in native fluorescence imparted by substitution of the camptothecin A-ring with an amino group and the application of postcolumn acidification following liquid chromatography to facilitate detection of AC with acceptable sensitivity.

MATERIALS AND METHODS

Reagents and Chemicals

Camptothecin (CA; NSC 94600) and 9-amino-20(RS)-camptothecin (AC; NSC 629971) were supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program, National Cancer Institute, and used as the analytical reference standards in these studies. Their purity was not assigned during the course of this work.

The following chemicals were used as supplied: analytical reagent glacial acetic acid (Mallinckrodt, Paris, KY) and trifluoroactic acid (Aldrich, Milwaukee, WI); ACS reagent ammonium acetate (Eastman Kodak, Rochester, NY), 60% perchloric acid (G. Frederick Smith, Columbus, OH) and N,N-dimethylformamide (MCB, Cincinnati, OH); OmniSolv grade acetonitrile and methanol (EM Science, Gibbstown, NJ). Deionized double distilled water was passed through a 0.2 μm nylon-66 filter (Rainin Instrument, Woburn, MA) before use.

Apparatus

Liquid chromatography was performed using a model 114M pump (Beckman Instruments, Berkeley, CA) and a manual Rheodyne 7125 injection valve (Cotati, CA) fitted with a 200 μl sample loop. A Waters model 510 HPLC pump (Millipore, Milford, MA), Rheodyne 7040 switching valve and 90° metal-free mixing tee with a 3.1 μl dead volume (Upchurch Scientific, Oak Harbor, WA) were employed for postcolumn treatment of the effluent. Fluorescence was monitored using a Kratos 980 programmable detector (ABI Analytical, Ramsey, NJ) with a deuterium excitation source (5 nm bandwidth) and 5 μl flow cell. The detector inlet was connected directly to the outlet of the mixing tee using 0.010 inch I.D. stainless steel tubing. 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 was used for plotting the chromatograms. The integrator was interfaced to a Hewlett-Packard 9114B disc drive for storage of the bunched signal data. Samples were loaded into the injector using 500 μl Hamilton syringes (Reno, NV) silanized with a 5% solution of Surfasil (Pierce Chemical Co., Rockford, IL) in hexane and dried at ambient temperature. The syringe and injector were thoroughly flushed with methanol and the mobile phase between successive injections. Syringes were cleaned weekly by soaking for 30 min in a hot mixture of sulfuric acid-nitric acid (4:1, v/v) and vigorously rinsed with distilled water followed by methanol.

Milligram quantities of the samples used for preparing stock solutions were weighed on a Cahn 25 electrobalance (Cahn Instruments, Inc., Cerritos, CA). Stock solutions were prepared in class A borosilicate glass volumetric flasks. Except where noted, other solutions used in the assay were prepared in screw top borosilicate glass test tubes. All glassware used to contain solutions of the drug were treated with 3%

Surfasil in toluene. An Eppendorf model 5412 microcentrifuge (Brinkmann Instruments, Westbury, NY) was used for centrifuging assay samples contained in 1.5 ml polypropylene microcentrifuge tubes (VWR Scientific Inc., Philadelphia, PA) at 12,000 x *g*.

Ultraviolet absorption spectra were measured with a model 8452A UV/VIS diode array spectrophotometer (2 nm resolution) controlled through a model 79994A analytical workstation (Hewlett-Packard, Palo Alto, CA). A holmium oxide filter was used to verify wavelength calibration. Uncorrected fluorescence excitation and emission spectra were recorded with a Farrand MK-2 Spectrofluorometer (Farrand Optical Co., Valhalla, NY) using 2.5 nm slits and 0.7 sec time constant at a scan rate of 100 nm/min. Wavelength calibration was ascertained with a 1.0 μg/ml solution of quinine sulfate in 0.1 N sulfuric acid. The spectra were electronically stored by directing the 100 mV output from the instrument to a model 9114B disc drive interfaced through a model 3393A recording integrator (Hewlett-Packard). All spectra were acquired with solutions contained in a 10 mm rectangular quartz cuvette. A Beckman 4500 Digital pH Meter equipped with a 91-03 combination electrode (Orion Research, Cambridge, MA) was used for pH measurements.

UV Absorption and Fluorescence Spectra

Absorbance spectra were determined in acetonitrile-0.1 M ammonium acetate buffer, pH 5.5 (28:72, v/v), the HPLC mobile phase previously used in the CA assay (26), by dissolving AC and CA directly in the solvent system to provide concentrations of 30.2 and 27.3 nM, respectively. Fluorescence excitation and emission spectra of both compounds were measured in solutions prepared by diluting their methanolic stock solutions (5.0 ml) quantitatively to 10 ml with the following aqueous buffers: 0.02 M ammonium phosphate, pH 6.0 and 7.0; 0.02 M ammonium acetate, pH 4.0 and 5.0; 0.02 M ammonium formate, pH 3.0; and 0.02, 0.1, 0.2, 1.0 and 2.0 M HCl. The final concentrations of CA and AC were 2.885 and 29.83 μM, respectively. Scans were initiated immediately after thoroughly mixing the solution and degassing for approximately 1 min in an ultrasonic bath. All spectra were referenced to the appropriate drug-free solvent system. The pH of each solution was measured after acquiring the spectra and reported without activity correction. Consequently, these determinations must be regarded as apparent pH values.

Postcolumn Acidification and Detector Optimization

The concentration and flow rate of aqueous trifluoroacetic acid mixed inline with the column effluent prior to fluorescence detection eliciting maximum response was

initially evaluated. Liquid chromatography was performed according to the conditions specified below. Five replicate injections (50 μl) of a 0.10 ng/μl solution of AC in methanol-water-acetic acid (45:55:0.5, v/v/v) were made under each set of conditions to provide an average chromatographic peak height. The pH of effluent collected at the detector outlet was measured. The optimum excitation wavelength in the 220-370 nm region for detecting AC was then determined from the response of duplicate injections, using a 418 nm emission cutoff filter, 5 sec rise time and 0.10 μA photomultiplier current.

Standard Solutions

Stock solutions of AC were made in dimethylformamide containing 10 mM acetic acid and stored at 5°C to prevent the lactone ring from opening. Serial dilution of the AC stock solution (0.1 mg/mL) with this same solvent system provided a series of nine secondary stock solutions which ranged in concentration from 0.050-37 μg/ml. Plasma standards were individually prepared, immediately prior to assay, by adding 3 μl of the secondary stock solutions to chilled human donor plasma (300 μl) in a microcentrifuge tube and mixing for 15 sec on a vortex action stirrer. The resulting concentrations of AC which defined the standard curve were 0.0, 13.29, 26.58, 53.17, 79.75, 106.3, 265.8, 505.1, 757.6 and 983.6 nM.

Sample Preparation

A stock solution of the internal standard, CA (2 μg/ml), was made in acetonitrile containing 10 mM acetic acid and stored at 5°C. The deproteinizing reagent was prepared daily by diluting this solution (10 μl) with methanol (10 ml) and maintained at -70°C in a dry ice-propanol bath. The subsequent procedures to prepare plasma specimens for analysis were reported previously (26). Briefly, to specifically determine the intact lactone form of the drug, plasma samples (50 μl) were treated with 150 μl of the cold deproteinizing reagent in a microcentrifuge tube. The mixture was thoroughly stirred by vortexing for 10 sec and centrifuged for 2 min. An aliquot of the supernatant (150 μl) was separated from the pellet, diluted with 125 μl of ice-cold ammonium acetate buffer (0.1 M, pH 5.5), and immediately chromatographed after filling the injector loop with the solution. Total drug levels were separately quantified by initially acidifiying the plasma specimen (50 μl) with aqueous perchloric acid (5 μl, 1.5 M). The mixture was vortexed for 1 min and permitted to stand for an additional 5 min at ambient temperature before deproteinization. The pH of the separated supernatant was adjusted to approximately 3 with 25 mM potassium acetate (125 μl) and centrifuged for 5 min just prior to chromatography.

Chromatographic Conditions

Chromatography was performed with an isocratic mobile phase of acetonitrile-methanol-0.1 M ammonium acetate buffer, pH 5.5 (23:10:67, v/v/v) 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., Emeryville, CA). Variability in the retention times of the drug and internal standard between analytical columns was minimized by adjusting the fraction of methanol, typically from 8-12% (v/v), to effect elution of AC within 7.0-7.5 min. The eluent was degassed in an ultrasonic bath for 15 min before use. Separations were performed at ambient temperature with a flow rate of 1.0 ml/min.

Quantitation

Standard curves constructed by plotting the drug to internal standard peak height ratio *versus* AC plasma concentration were run on a daily basis. Linear least squares regression was performed with a weighting factor of $1/y^2_{obs}$, without inclusion of the origin, to determine the slope, *y*-intercept and correlation coefficient of the best fit line. Drug concentrations in unknown samples were calculated using the results of the regression analyses.

Recovery and Assay Reproducibility

AC Carboxylate Plasma Standards: Solutions comprising a series of concentrations analogous to the secondary stock solutions were made by serially diluting the 0.1 mg/ml stock solution of AC with 0.1 M aqueous potassium hydroxide and incubating at 37°C for 15 min to effect complete hydrolysis of the lactone ring. Plasma standards of AC carboxylate were obtained upon 100-fold dilution of these solutions with human plasma.

Nonplasma Standard Solutions: A set of solutions was prepared in a methanol-water-acetic acid (45:55:0.5, v/v/v) solvent system such that AC lactone and the internal standard were present at concentrations corresponding to the plasma standards processed for analysis assuming quantitative recovery.

Plasma standards containing the intact or hydrolyzed lactone forms of the drug were prepared for analysis according to the appropriate procedure and 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. Absolute recovery was calculated from the drug to internal

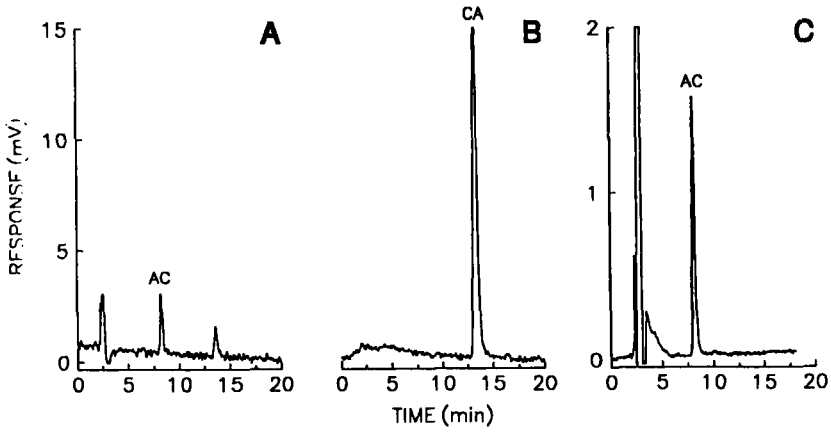


FIGURE 2. Liquid chromatograms of reference solutions determined using a mobile phase of acetonitrile-methanol-0.1 M ammonium acetate buffer, pH 5.5 (23:10:67, v/v/v) at 1.0 ml/min. (A) 10 ng of AC with UV detection at 332 nm; (B) 10 ng of AC detected by fluorescence with excitation at 347 nm; (C) 2.5 ng of CA detected by fluorescence with excitation at 347 nm.

standard peak height ratio for the plasma standards relative to the nonplasma samples. Backcalculated concentrations determined from results of linear regression analyses for calibration curves of AC in plasma run during a 3 month period provided a measure of relative recovery and assay reproducibility.

RESULTS

Liquid Chromatography

The capacity factor of AC under isocratic reversed-phase conditions was much lower than that of CA and particularly sensitive to the fraction of acetonitrile in the eluent (Figure 2). Incorporating methanol in the mobile phase significantly improved the chromatographic behavior of AC, providing narrower bands than realized with buffered eluents modified with acetonitrile alone. Employing a mobile phase composed of acetonitrile-methanol-0.1 M ammonium acetate buffer, pH 5.5 (23:10:67, v/v/v) at a flow rate of 1.0 ml/min, AC and CA eluted from a 4.6 mm x 25 cm column of 5 μm Ultrasphere ODS with R_T of 8.2 and 13.4 min, respectively. Thus, based upon the separation

achieved, CA was selected as the internal standard (IS) for quantifying its 9-amino analog.

The liquid chromatograms shown in Figures 2A and 2B demonstrate the extremely poor fluorescence of AC using the optimum conditions for detecting CA (excitation at 347 nm with a 418 nm emission cutoff filter). The relative response of the AC chromatographic peak was only 3.5% of that realized for CA. This was similar to the sensitivity attained when the elution of AC was monitored by UV absorption at 332 nm (Figure 2C).

UV Absorption Spectra

UV spectra of CA and AC measured in the HPLC mobile phase showed that absorptivity at 347 nm was similar for both compounds (Figure 3). However, the presence of the exocyclic amino group resulted in several prominent alterations in the parent chromophore, indicative of a strong influence upon the electron density distribution within the ring system. Amino substitution produced an apparent bathochromic shift of the 254 nm band in the spectrum of CA to 262 nm with considerable broadening and diminished intensity. Absorptivity of the CA band evident as a shoulder near 335 nm appeared greatly enhanced in the spectrum of AC. The minor peak at 288 nm was completely obscured with absorption tailing well into the visible region.

Spectrofluorometric Studies

The uncorrected fluorescence excitation spectrum of CA in 50% (v/v) aqueous methanol, pH 5.0, was characterized by a major band with λ_{max} 376 nm, a shoulder near 360 nm and a low intensity band at 255 nm (Figure 4A). The corresponding emission spectrum consisted of a single major band with λ_{max} 430 nm (Figure 4B). Fluorescence intensity was invariant above pH 4 but diminished with increasing acidity to insignificance. A plot of fluorescence intensity *versus* apparent pH exhibited a single inflection point near an apparent pH of 1.8 (Figure 4C). Therefore, fluorescence of the molecule is lost upon protonation of the weakly basic quinoline nitrogen atom.

A similar experiment with AC confirmed the observation that the compound was nonfluorescent during liquid chromatography at pH 5.5 (Figure 5). However, fluorescence developed with increasing acidity, becoming relatively invariant from pH 2.3 to 1.7, although it again vanished as the solutions were made strongly acidic. The inflection points of this bell-shaped relative fluorescence-pH profile occurred near pH 1.2

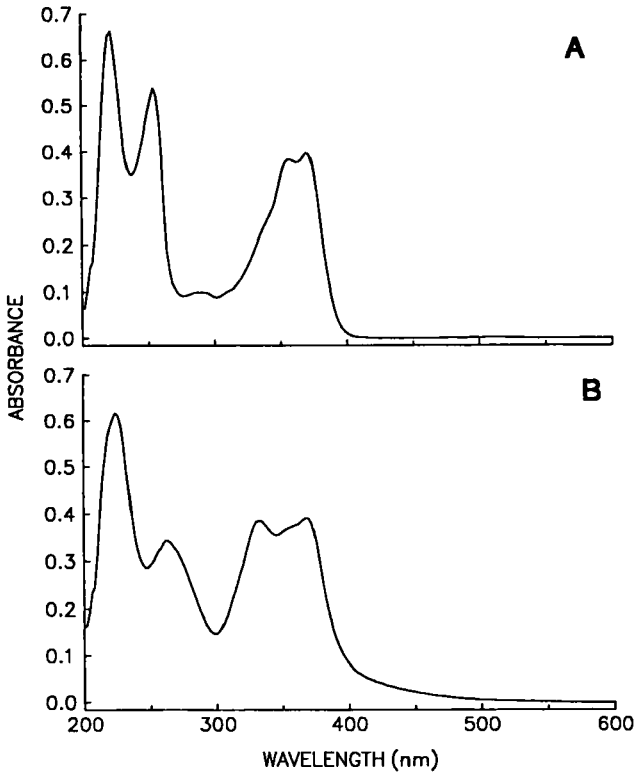


FIGURE 3. UV absorption spectra determined in acetonitrile-0.1 M ammonium acetate buffer, pH 5.5 (28:72, v/v): (A) 27.3 μM CA, λ_{max} 222, 254, 288, 358 and 370 nm; (B) 30.2 μM AC, λ_{max} 224, 262, 332 and 368 nm.

and 3.1 (Figure 5C). Uncorrected excitation and emission spectra in moderately acidic solution exhibited λ_{max} at 372 and 455 nm, respectively.

These observations are consistent with the reaction scheme shown in Figure 6. The development of AC fluorescence is associated with initial protonation at the C-9 amino group. Further protonation of the molecule occurs at the quinoline nitrogen atom with a concurrent diminution of fluorescent intensity. Comparison of the maximum fluorescence intensities observed for both compounds suggested that the fluorescent yield of AC under optimum conditions was approximately 6.3-fold lower than CA.

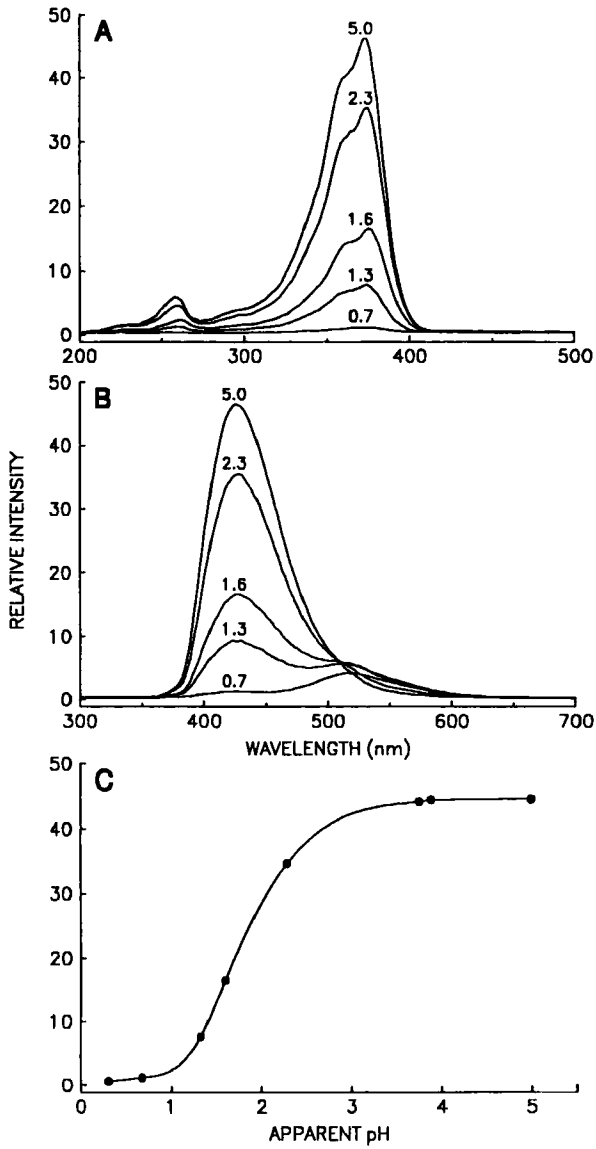


FIGURE 4. Influence of pH on CA fluorescence in 50% (v/v) aqueous methanol solutions (2.87 μM) with apparent pH 0.68-5.00: (A) excitation spectra determined with λ_{em} 430 nm; (B) emission spectra measured with λ_{ex} 376 nm; (C) relative fluorescence-pH profile.

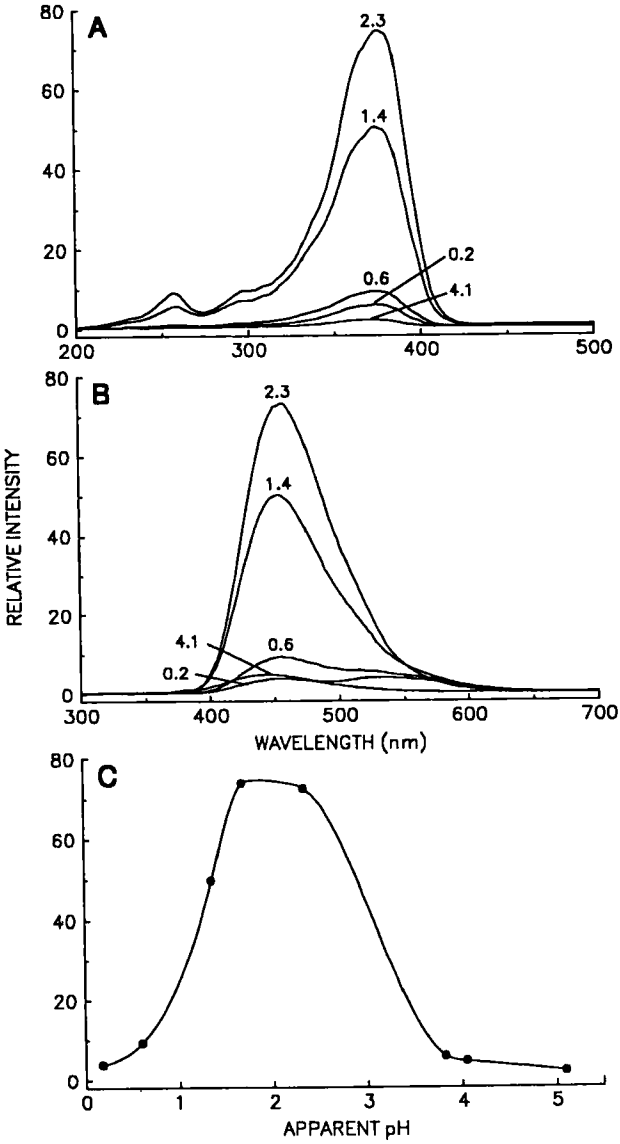


FIGURE 5. Influence of pH on AC fluorescence in 50% (v/v) aqueous methanol solutions (29.8 μM) with apparent pH 0.60-4.05: (A) excitation spectra determined with λ_{em} 455 nm; (B) emission spectra measured with λ_{ex} 372 nm: (C) relative fluorescence-pH profile.

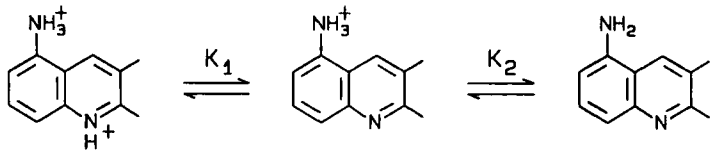


FIGURE 6. Scheme for the protonation of 9-aminocamptothecin.

HPLC with Fluorescence Detection Following Postcolumn Acidification

As suggested by the hydronium ion concentration dependent behavior of AC fluorescence, postcolumn acidification provided a convenient means to monitor the drug upon reversed-phase separation. The column effluent and aqueous acid were directed into the orthogonal ports of a low dead volume 90° mixing tee interfaced directly to the detector with 0.010 inch I.D. stainless steel tubing. Although the magnitude of baseline disturbances introduced by several dedicated commercial postcolumn reagent pumps was unacceptable, excellent results were obtained when employing an HPLC pump for this purpose.

Figure 7 depicts the decrease in apparent pH imparted to the eluent and corresponding height of the AC chromatographic peak using aqueous solutions of trifluoroacetic acid (TFA) as the postcolumn reagent. Maximum peak height was realized with 0.3 M TFA at a flow rate of 0.3 ml/min. The diminished response observed at higher reagent flow rates was associated with band broadening in addition to formation of the diprotonated species in the presence of excess acid (Figure 7B). Sensitivity was not improved when the 0.010 inch I.D. tubing between the mixing chamber and detector was replaced with a 3 m x 0.5 mm I.D. knitted TFE Teflon delay tube (Supelco, Bellfonte, PA).

Optimum Conditions for Fluorescence Detection

Measurement of the AC chromatographic peak as the wavelength of excitation (λ_{ex}) was incremented from 220 to 370 nm afforded an apparent hydrodynamic excitation spectrum. The two strongest bands occurred at λ_{ex} 250 and 352 nm with relative intensities of 100 and 55.5, respectively (Figure 8). Detector response was greater with a 418 nm emission cutoff filter than filters rated at 389, 440 and 470 nm.

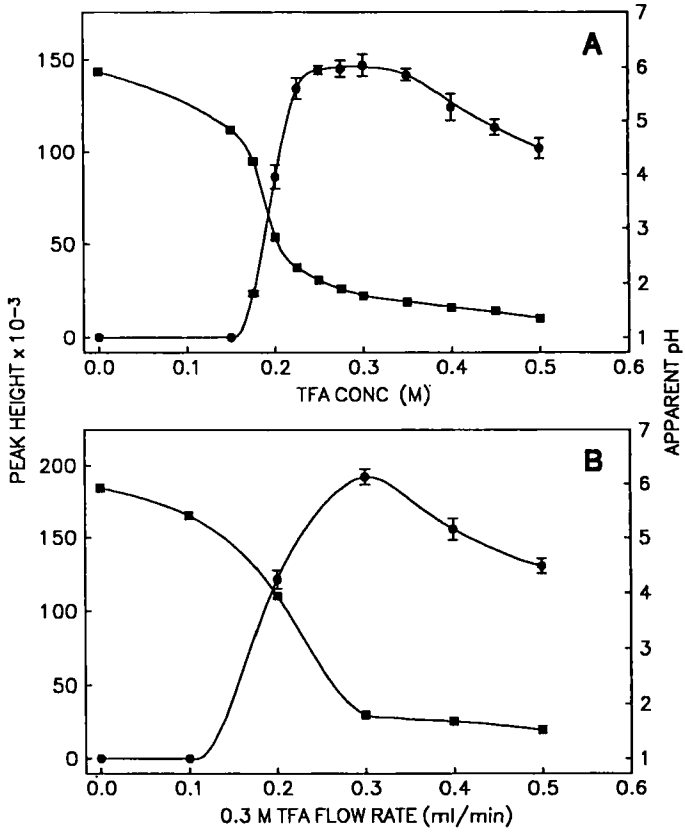


FIGURE 7. Optimization of the concentration and flow rate of the postcolumn reagent, trifluoroacetic acid. HPLC conditions were as described in "Materials and Methods". Initially, the TFA concentration was varied at a constant flow rate of 0.25 ml/min (A) followed by variation of the flow rate of 0.3 M acid (B). Curves: (•) height of the chromatographic peak for 5 ng of AC; (■) apparent pH of effluent emerging from the detector. *Points*, mean of $n = 5$; *bars*, SD.

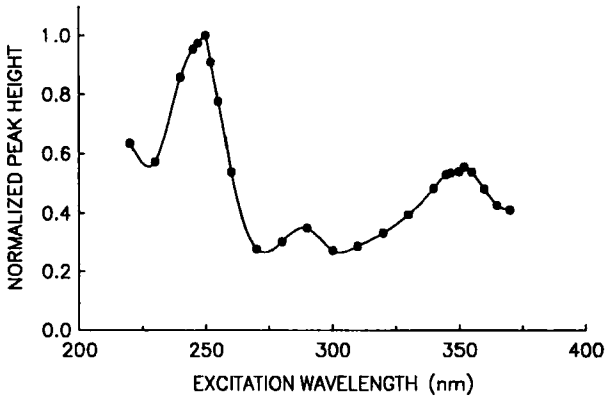


FIGURE 8. Hydrodynamic fluorescence excitation spectrum of AC determined by liquid chromatography with postcolumn acidification prior to detection using 0.3 M TFA at a flow rate of 0.3 ml/min. Excitation maxima: 250, 290 and 352 nm. *Points*, mean of two determinations.

Calibration Curves

The relatively facile hydrolysis of the AC lactone ring at physiological pH required that plasma standards be individually prepared immediately prior to assay. Representative liquid chromatograms of plasma standards at a range of concentrations encompassing the calibration curve are shown in Figures 9B-D. There were no endogenous components in human plasma that interfered with detection of AC or the internal standard (Figure 9A). The negatively charged AC carboxylate was poorly retained and eluted with partial obscurement by the solvent front.

Figure 10 shows a typical calibration curve constructed by plotting the drug to internal standard peak height ratio against the concentration of AC in plasma. The lowest concentration of AC in 50 μl of plasma amenable to quantitation was 13.3 nM (5.0 ng/ml). Standard curves having concentrations ranging from 13.3 to 980 nM exhibited good linearity with a correlation coefficient (mean ± SD) of 0.997 ± 0.001 (n = 10) and slope of 0.00207 ± 0.00019.

Relative Recovery

Relative recovery of the drug from plasma and assay reproducibility for the range of concentrations included in the standard curves during a 3 month period are

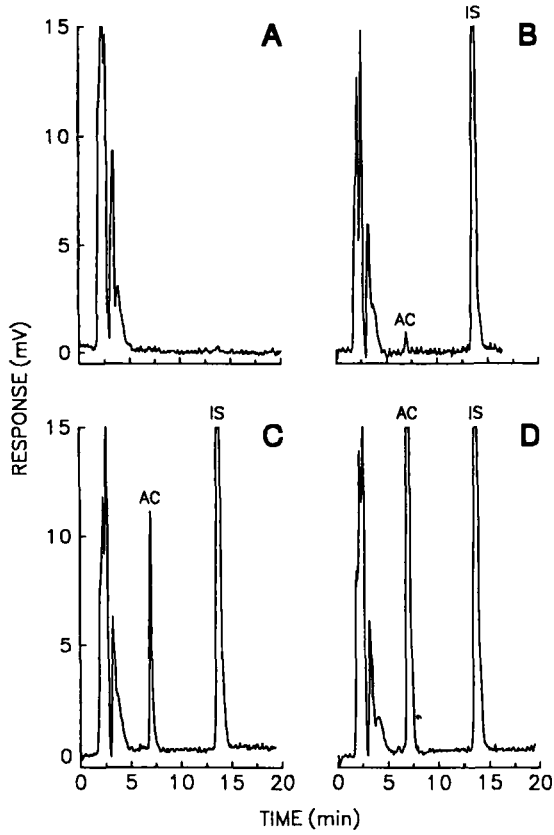


FIGURE 9. Representative liquid chromatograms of standard curve samples prepared by adding AC to drug-free human plasma to afford concentrations of (A) 0.0 nM; (B) 13.29 nM; (C) 505.1 nM and (D) 983.6 nM. Chromatographic peaks: AC, 6.02-6.26 min; IS, 11.14-11.42 min.

summarized in Table 1. Relative recoveries ranged from 98.01 to 103.9%. Coefficients of variation for the replicate determinations (n = 10) ranged from 3.72 to 8.61%, with a value of 7.63% for the lowest plasma concentration of AC quantitated. The analytical method is considered reproducible inasmuch as these calibration curves were assayed using several different analytical columns over an extended period of time.

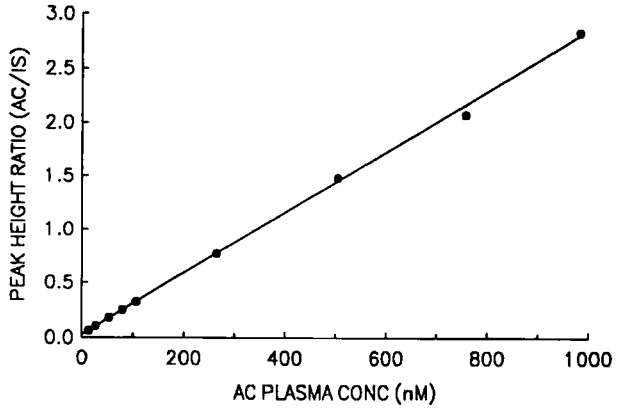


FIGURE 10. Typical standard curve of AC in human plasma: slope, 0.002820; *y*-intercept, 0.02603; corr, 0.9994.

TABLE 1

Relative Recovery and Reproducibility of the Analytical Method for 9-Aminocamptothecin in Human Plasma

Amount Added nM	N	Mean Amount Found nM	Recovery %	CV[a] %
13.29	10	13.42	101.98	7.63
26.58	10	26.05	98.01	6.48
53.17	10	53.60	100.81	8.61
79.75	10	79.52	99.71	6.66
106.3	10	110.4	103.86	5.14
265.8	10	264.3	99.44	3.92
505.1	10	504.6	99.90	4.34
757.6	10	746.6	98.55	4.31
983.6	10	983.7	100.01	3.72

[a] Coefficient of variation.

TABLE 2

Absolute Recovery of 9-Aminocamptothecin Lactone and Carboxylate from Human Plasma

Amount Added nM	Intact Lactone		Carboxylate	
	Recovery[a] %	CV[b] %	Recovery[a] %	CV[b] %
13.29	99.56	1.59	97.61	2.57
53.17	97.13	3.83	94.09	3.87
106.3	98.23	3.48	98.10	7.11
505.1	95.75	2.91	98.03	0.89
983.6	96.61	2.73	102.00	7.52

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

Absolute Recovery

The absolute recovery (mean ± SE) of AC lactone from plasma was 97.46 ± 1.48%, calculated from the individual mean recoveries ($n = 5$) for five different concentrations spanning the entire range of the standard curve (Table 2). Similarly evaluated, 97.97 ± 2.80% of AC carboxylate was recovered from plasma by the total drug method of sample preparation. Absolute recovery of the internal standard was 101.9 ± 10.6% (mean ± SD, $n = 25$).

DISCUSSION

Introducion of an aromatic amino substituent at the C-9 position of CA abolished the intense native fluorescence of the parent compound in neutral aqueous and organic solutions. This behavior is unusual, since the presence of an amino group typically enhances the fluorescent yield of an aromatic compound. Studies to characterize the influence of pH on the fluorescence spectra of these compounds demonstrated that protonation of the CA quinoline nitrogen atom quenched fluorescence of the molecule. However, initial protonation of AC occured at the more strongly basic exocyclic amino group, preventing the resonance interaction between the lone pair electrons and the

aromatic ring system which apparently makes fluorescent energy transitions unfavorable. The absence of AC fluorescence in strongly acidic media occurs upon subsequent protonation of the ring nitrogen atom.

Alternative approaches to visualize the compound following HPLC were therefore considered. Liquid chromatography performed with a mobile phase sufficiently acidic to effect the first protonation of AC had the primary disadvantage of being restricted to total drug determinations. The rapid lactonization of the carboxylate form of CA and its analogs in aqueous solution more acidic than pH 3 precluded specific quantitation of the intact lactone species (26, 29). Employing the sample preparation methods developed for the analysis of CA (26), using UV detection at 336 nm, the minimum detectable concentration of AC in plasma was only 80 nM (30 ng/ml). This sensitivity was inadequate for drug level monitoring during preclinical pharmacokinetic studies at the doses of interest. Although the aromatic amino group was subject to oxidation with a glassy carbon electrode at +0.90 V, interferences from endogenous constituents at this potential precluded detection of the drug in a 126.5 nM (47.1 ng/ml) plasma sample.

Postcolumn derivatization techniques prior to fluorescence detection proved to be a feasible means for detecting AC with the sensitivity desired. In addition to protonation, localization of the lone pair electrons of the C-9 amino group upon acylation also resulted in intense fluorescence, as exemplified by the 9-*p*-toluamido derivative employed as the internal standard in the analytical method for CA (26). This suggested the potential use of postcolumn acylation with a nonfluorescent reagent such as acetic anhydride. However, postcolumn derivatization with a fluorigenic reagent offered the potential for a substantial improvement in detectability. Fluorescence from the chromophore introduced with an appropriate reagent could complement the inherent fluorescence of the ring system upon derivatization of the amino group. Unfortunately, the reaction rates between AC and all reagents examined, which included aliphatic and aromatic acid anhydrides, dansyl chloride and OPA, were insufficiently rapid at conditions adaptable to an HPLC postcolumn derivatization system.

Consequently, reversed-phase liquid chromatography followed by inline postcolumn acidification to pH 1.8-2.2 prior to fluorescence detection offered a convenient method for monitoring AC in the HPLC effluent. Implementation of the chromatographic procedure required an investigation of the effect of postcolumn reagent concentration and flow rate on detector response. Trifluoroacetic acid was selected for use as the acidifying reagent in consideration of its acid strength (pK_a 0.3) and compatability with stainless steel HPLC hardware (30). Conditions resulting in

maximum height of the AC chromatographic peak were postcolumn acidification with 0.3 M aqueous TFA at a flow rate of 0.3 ml/min followed by fluorescence detection with excitation at 352 nm and a 418 nm cutoff filter. Although the sensitivity achieved with excitation at 352 nm was approximately 50% less than at 250 nm, interfering endogenous plasma components were observed at the lower wavelength. Excitation at the longer wavelength provided adequate selectivity at the expense of somewhat diminished sensitivity.

Postcolumn acidification preceding fluorescence detection therefore proved to be a convenient technique to permit the determination of AC in plasma with far superior sensitivity and selectivity than could be acheived with UV or electrochemical detectors. With typical R_T for AC lactone and the internal standard of 7 and 12 min, respectively, approximately 3.0 hr was required to assay 9 plasma standards. The sample preparation procedures developed to assay intact lactone and total drug levels of CA in plasma were employed for AC without significant alteration (24). Selective determination of AC lactone was achieved by direct analysis of plasma samples immediately upon deproteinization. Acidification of the sample to approximately pH 2 with perchloric acid prior to protein precipitation, effected complete and rapid lactonization of AC carboxylate, which provided a measure of total drug levels. The AC carboxylate levels were calculated as the difference between the total drug and AC lactone plasma concentrations. Employing sample volumes of 50 μl, the lowest plasma concentration of AC included in the standard curve, 13.3 nM (5.0 ng/ml) was quantified with a 7.63% coefficient of variation (n = 10).

ACKNOWLEDGMENTS

The authors wish to thank Ms. Margaret E. Lyon, Ms. Patricia Calligan-Snuffer and Mr Sean Mullroy for their skillful technical assistance. The laboratory work of this study was conducted at The Ohio State University, College of Pharmacy with support from contract N01-CM-97619 from the National Cancer Institute, NIH, DHHS.

REFERENCES

1. Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhail, A. T., and Sim, G. A., Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from *Camptotheca acuminata*, J. Am. Chem. Soc., 88, 3888, 1966.

2. Wall, M. E., Wani, M. C. and Taylor, H. L., Isolation and Chemical Characterization of Antitumor Agents from Plants, Cancer Treat. Rep., 60, 1011, 1976.

3. Hartwell, J. L. and Abbott, B. J., Antineoplastic Principles in Plants: Recent Developments in the Field, Adv. Pharmacol. Chem., 7, 117, 1969.

4. Wani, M. C., Ronman, P. E., Lindley, J. T. and Wall, M. E., Plant Antitumor Agents. 18. Synthesis and Biological Activity of Camptothecin Analogues, J. Med. Chem., 23, 554, 1980.

5. Wall, M. E., Wani, M. C., Natschke, S. M. and Nicholas, A. W., Plant Antitumor Agents. 22. Isolation of 11-Hydroxycamptothecin from *Camptotheca acuminata* Decne: Total Synthesis and Biological Activity, J. Med. Chem., 29, 1553, 1986.

6. Wani, M. C., Nicholas, A. W. and Wall, M. E., Plant Antitumor Agents. 23. Synthesis and Antileukemic Activity of Camptothecin Analogues, J. Med. Chem., 29, 2358, 1986.

7. Gottlieb, J. A., Guarino, A. M., Call, J. B., Oliverio, V. T. and Block, J. B., Preliminary Pharmacologic and Clinical Evaluation of Camptothecin Sodium (NSC-100880), Cancer Chemother. Rep., 54, 461, 1970.

8. Moertel, C. G., Schutt, A. J., Reitmeier, R. J. and Hahn, R. G., Phase II Study of Camptothecin (NSC-100880) in the Treatment of Advanced Gastrointestinal Cancer, Cancer Chemother. Rep., 56, 95, 1972.

9. Gottlieb, J. A. and Luce, J. K., Treatment of Malignant Melanoma with Camptothecin (NSC-100880), Cancer Chemother. Rep., 56, 103, 1972.

10. Muggia, F. M., Creaven, P. J., Hansen, H. H., Cohen, M. H. and Selawry, O. S., Phase I Clinical Trial of Weekly and Daily Treatment with Camptothecin (NSC-100880): Correlation with Preclinical Studies, Cancer Chemother. Rep., 56, 515, 1972.

11. Creaven, P. J., Allen, L. M. and Muggia, F. M., Plasma Camptothecin (NSC 100880) Levels During a 5-day Course of Treatment: Relation to Dose and Toxicity, Cancer Chemother. Rep., 56, 573, 1972.

12. Creaven, P. J. and Allen, L. M., Renal Clearance of Camptothecin (NSC 100880): Effect of Urine Volume, Cancer Chemother. Rep., 57, 175, 1973.

13. Jaxel, C., Kohn, K. W., Wani, M. C., Wall, M. E. and Pommier, Y., Structure-Activity Study of the Actions of Camptothecin Derivatives on Mammalian Topoisomerase I: Evidence for a Specific Receptor Site and a Relation to Antitumor Activity, Cancer Res., 49, 1465, 1989.

14. Hsiang, Y., Liu, L. F., Wall, M. E., Wani, M. C., Nicholas, A. W., Manikumar, G. Kirscenbaum, S., Silber, R. and Potmesil, M., DNA Topoisomerase I-mediated DNA Cleavage and Cytotoxicity of Camptothecin Analogues, Cancer Res., 49, 4385, 1989.

15. Hsiang, Y., Hertzberg, R., Hecht, S. and Liu, L. F., Camptothecin Induces Protein-linked DNA Breaks via Mammalian DNA Topoisomerase I, J. Biol. Chem., 260, 14873, 1985.

16. Mattern, M. R., Mong, S. M., Bartus, H. F., Mirabelli, C. K., Crooke, S. T. and Johnson, R. K., Relationship Between the Intracellular Effects of Camptothecin and the Inhibition of DNA Topoisomerase I in Cultured L1210 Cells, Cancer Res., 47, 1793, 1987.

17. Eng, W., Faucette, L. Johnson, R. K. and Sternglanz, R., Evidence that DNA Topoisomerase I is Necessary for the Cytotoxic Effects of Camptothecin, Mol. Pharmacol., 34, 755, 1989.

18. Adamovics, J. A. and Hutchinson, C. R., Prodrug Analogues of the Antitumor Alkaloid Camptothecin, J. Med. Chem., 22, 310, 1979.

19. Kunimoto, T., Nitta, K., Tanaka, T., Uehara, N., Baba, H., Takeuchi, M., Yokokura, T., Sawada, S., Miyasaka, T. and Mutai, M., Antitumor Activity of 7-Ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, a Novel Water-Soluble Derivative of Camptothecin, Against Murine Tumors, Cancer Res., 47, 5944, 1987.

20. Vishnuvajjala, B. R. and Garzon-Aburbeh, A., Water Soluble Prodrugs of Camptothecin, U.S. Patent 4,943,579, Jul. 24, 1990.

21. Chabot, G. G., Barilero, I., Armand, J. P. Gandia, D., Mathieu-Boue, A. and Gouyette, A., Pharmacokinetics of the Camptothecin Analog CPT-11 and Its Active Metabolite SN-38 in Cancer Patients, Proc. Am. Asssoc. Cancer Res., 32, 175, 1991.

22 Recondo, G., Abbruzzese, J., Newman, B., Newman, R., Kuhn, J., von Hoff, D., Garteiz, D. and Raber, M., A Phase I Trial of Topotecan Administered by a 24-Hour Infusion, Proc. Am. Asssoc. Cancer Res., 32, 206, 1991.

23 Sugasawa, T., Toyoda, T., Uchida, N. and Yamaguchi, K., Experiments on the Synthesis of *dl*-Camptothecin. 4. Synthesis and Antileukemic Activity of *dl*-Camptothecin Analogues, J. Med. Chem., 19, 675, 1976.

24. Wani, M. C., Nicholas, A. W., Manikumar, G. and Wall, M. E., Plant Antitumor Agents. 25. Total Synthesis and Antileukemic Activity of Ring-A Substituted Camptothecin Analogues. Structure-Activity Correlations, J. Med. Chem., 30, 1774, 1987.

25. Wani, M. C., Nicholas, A. W. and Wall, M. E., Plant Antitumor Agents. 23. Synthesis and Antileukemic Activity of Camptothecin Analogues, J. Med. Chem., 29, 2358, 1986.

26. Supko, J. and Malspeis, L., A Reversed-Phase HPLC Method for Determining Camptothecin in Plasma with Specificity for the Intact Lactone Form of the Drug, J. Liq. Chromatogr., 14, 1779, 1991.

27. Hart, L. G., Call, J. B. and Oliverio, T., A Fluorometric Method for Determination of Camptothecin in Plasma and Urine, Cancer Chemother. Rep., 53, 211, 1969.

28. Supko, J. and Malspeis, L., Pharmacokinetics of Camptothecin, 9-Aminocamptothecin and 10,11-Methylenedioxycamptothecin in the Mouse, Proc. Am. Asssoc. Cancer Res., 32, 345, 1991.

29. Fassberg, J. and Stella, V. J., A Kinetic and Mechanistic Study of the Hydrolysis of Camptothecin and Some Analogs, J. Pharm Sci., *In Press*, 1992.

30. 'The Merck Index,' Budavari, S. ed., 11th ed., Merck & Co., Rahway, NJ, 1989, p. 9599.

Received: April 1, 1992
Accepted: May 27, 1992