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Determination of camptothecin in biological fluids using reversed-phase high-performance liquid chromatography with fluorescence detection

JIANN-PING LOH and AHMED E. AHMED*

Pharmacokinetics Laboratory, Department of Pathology and Cancer Center, University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)

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ABSTRACT

Camptothecin, a plant alkaloid with antitumor activity, is a potent and rapidly acting inhibitor of DNA synthesis. The objective of this study was to develop a sensitive high-performance liquid chromatographic (HPLC) method for the detection and estimation of the camptothecin concentration in biological fluids. Using HPLC coupled with fluorescence detection, at an excitation wavelength of 370 nm and an emission wavelength of 434 nm, we found that the lower limits of detection for camptothecin in aqueous, plasma and urine samples were 0.5, 1 and 10 ng/ml, respectively. The ideal mobile phase used was methanol–10 m*M* potassium phosphate (75:25, v/v, pH 4.0). To determine the utilization of the method in a biological system, we studied the pharmacokinetics of camptothecin in mice. Elimination of camptothecin from mice blood was triphasic and followed first-order kinetics. The half-life of camptothecin in mouse blood was 25.7 min. Our studies indicate that HPLC with fluorescence detection for the determination of camptothecin in different media is a simple, rapid, sensitive and reproducible method.

INTRODUCTION

Camptothecin, 4-(*S*)-4-ethyl-4-hydroxy-1*H*-pyrano[3′,4′,6,7]indolizino[1,2-*b*]quinoline-3,14[4*H*,12*H*]dione, a plant alkaloid, was isolated from *Camptotheca acuminata* Decne (Nyssaceae). Its structure was elucidated by Wall *et al.* [1] and McPhail and Sim [2] as part of a screening program for antitumor agents. Camptothecin 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) [3–8]. Camptothecin has been shown to be a potent inhib-

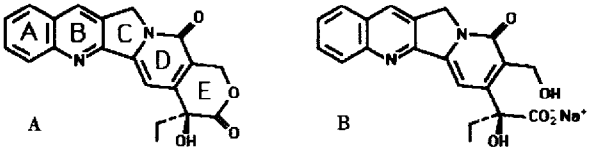

Fig. 1. Structures of (A) camptothecin and (B) camptothecin sodium salt.

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itor of nucleic acid synthesis and a strong inducer of DNA strand breaks in mammalian cells.

Camptothecin (Fig. 1) has two notable chemical properties: (1) Its lack of significant basicity causes it to behave as a neutral molecule. Camptothecin does not form stable salts with mineral acids and it gives negative tests with Dragendorff and Mayer reagents; thus, it is not an alkaloid in the usual sense of the definition. (2) The presence of the C-20 tertiary alcohol imparts an unusual electrophilicity to the lactone carbonyl group, perhaps via a strong intramolecular H-bond [9].

Although much research has been done to study the mechanism of action of camptothecin and its synthetic analogues [10–14], there are few methods of analysis currently available [15–17]. Previous studies on camptothecin analysis used total fluorescence without separation [17], liquid chromatographic separation with UV detection [18] or fluorescence with thin-layer chromatography [17]. These analytical methods have the disadvantage of being (a) incapable of differentiating between camptothecin analogs, (b) not sensitive and (c) elaborate and time-consuming.

The objective of this study was to develop a method to separate, identify, and quantitate camptothecin and its derivatives in biological samples. The assay procedure described herein involves a sensitive, rapid and reproducible method of analysis of camptothecin using high-performance liquid chromatography (HPLC) with fluorescence detection. The significance of this procedure will lie in the chromatographic characterization of the metabolic fate of camptothecin catalyzed by various enzymes *in vivo*.

EXPERIMENTAL

Materials

HPLC-grade methanol, acetonitrile and ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Camptothecin sodium salt (NSC 100880) was obtained from Dr. M. Suffness (Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.). Human plasma was provided by the University of Texas Medical Branch Blood Bank (Galveston, TX, U.S.A.). All water used was doubly distilled and subjected to biological degradation by ultraviolet (UV) light for at least 3 h before use. All other chemicals and reagents were of the highest purity available and were obtained from various commercial sources.

Instrumentation

HPLC analyses were run on a Waters Assoc. Model 720 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.). The system consisted of Models 6000A and M45 solvent delivery systems, a Model 720 system controller, a Model 730 data module and a Model 712 WISP sample processor. Detection was achieved

using a Perkin-Elmer Model 650-10M fluorescence detector with a Model 150B xenon lamp power supply. When needed, a Hitachi Model 100-40 spectrophotometer with an Altex Model 155 spectrophotometer flow cell was used as a UV detector. For fluorescence detection, camptothecin was monitored at an excitation wavelength of 370 nm and an emission wavelength of 434 nm, while UV absorbance was measured at 254 nm. Separation was achieved on a reversed-phase Ultrasil ODS column (250 mm × 4.6 mm I.D., 10 μm particle size) (Altex, Berkeley, CA, U.S.A.).

All separations were conducted at ambient temperature at a flow-rate of 0.7 ml/min with an isocratic mobile phase that had been vacuum-filtered (0.22-μm Millipore filters, Millipore, Bedford, MA, U.S.A.) and degassed before use. Aliquots of sample solutions (20 μl) were injected for chromatographic analysis.

Fluorescence spectra were determined on a Perkin-Elmer Model MPG 44A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.). For comparing the sensitivity of both fluorescence and UV detection methods, a mobile phase of methanol–water (75:25, v/v), injections of aqueous camptothecin and/or its sodium salt were analyzed using both UV and fluorescence detectors in series. Simultaneous chromatograms were produced by the data module. The detection limit, the lowest concentration of the sample which is detectable, was then determined and compared for each detection type.

Preparation of biological samples and extraction procedure

Aliquots of camptothecin sodium salt (30–150 μl) were mixed with water, plasma or urine, acidified with 85% phosphoric acid to pH 2, to give a final volume of 3 ml, with drug concentrations in each medium of 0.01, 0.05, 0.1, 0.5 and 1.0 μg/ml; 1 ml of each solution was treated with 2 ml of water-saturated ethyl acetate. The solution was vortex-mixed rigorously for 25 s, centrifuged (IEC HN-SII centrifuge, Damon/IEC Division, Needham Heights, MA, U.S.A.) at 520 *g* for 10 min, and the organic layer was transferred to another tube. The procedure was repeated with another 2-ml portion of water-saturated ethyl acetate, and the two organic extracts were combined. The combined organic extracts were evaporated to dryness using a gentle stream of nitrogen while immersed in a water bath at 60°C. The residue was reconstituted with 1 ml of HPLC-grade methanol and analyzed directly.

For the purpose of removal of biological macromolecules from biological samples (*e.g.*, urine and plasma) the biological samples were diluted with an equal volume (3 ml) of acetonitrile, vortex-mixed and the centrifuged at 520 *g* for 20 min. The resulting supernatant was then subjected to the rest of the extraction procedure described previously.

Effect of pH on camptothecin quantitation

Sample pH study. Aqueous solutions of camptothecin sodium salt (0.1 μg/ml) were prepared at several different pH values (12, 11, 10, 8, 7, 6, 4.5, 3 and 2) by

acidification with 85% phosphoric acid or by increasing the basicity with 3 *M* potassium hydroxide. Using a mobile phase of methanol–water (75:25, v/v) with fluorescence detection, the samples were analyzed as described previously for the composition of both camptothecin (lactone) and camptothecin sodium salt.

Mobile phase pH study. Methanol–water (75:25, v/v) was initially chosen as the mobile phase for the reversed-phase ODS column. Acidification of the mobile phase was carried out using phosphoric acid and potassium phosphate. The mobile phase methanol–aqueous 10 m*M* potassium phosphate (75:25, v/v) was investigated at pH values of 7, 6, 5, 4 and 3. The system and sample pH values were then optimized for use in the remaining analysis. At alkaline pH camptothecin lactone is completely converted to camptothecin sodium salt.

Standard curve of aqueous camptothecin solutions

Using the optimum conditions from the above-mentioned experiments, standard curves for aqueous camptothecin solution were generated using a mobile phase of methanol–10 m*M* potassium phosphate (75:25, v/v, pH 4.0). The camptothecin concentration range investigated was from 0.1 ng/ml to 1.0 μg/ml, and the sample pH was approximately 2.0.

Standard curve of camptothecin in human plasma (in vitro)

Appropriate aliquots of standard camptothecin solutions were added to human plasma to provide concentrations ranging from 0.001 to 2.0 μg/ml. The final pH of the sample was adjusted to 2, using 85% phosphoric acid. Aliquots (1 ml) of camptothecin-spiked plasma were mixed with 1 volume of acetonitrile (1 ml) and vortex-mixed. Macromolecular components precipitated by acetonitrile were separated by centrifugation (520 *g* for 20 min). The clear supernatants were assayed following the extraction process. A mobile phase of methanol–10 m*M* potassium phosphate (75:25, v/v, pH 4.0) was used. Three separate determinations of the standard concentrations *versus* peak areas were conducted. Peak areas were determined for each concentration after deducting the blank values. The concentrations *versus* peak areas were graphed.

Standard curve of camptothecin in human urine (in vitro)

Aliquots of standard camptothecin solutions were added to human urine to provide concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 μg/ml. The final pH was adjusted to 2 using 85% phosphoric acid. Aliquots of camptothecin-spiked urine and blank urine samples were assayed directly, using a mobile phase of methanol–10 m*M* potassium phosphate (75:25, v/v, pH 4.0).

Two separate trials were conducted, and peak areas were obtained for each concentration. After subtraction of blank urine values (Fig 2A and B) the concentrations *versus* peak areas were plotted.

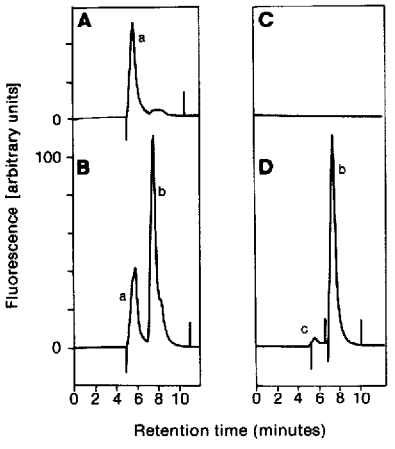

Fig. 2. HPLC profiles of camptothecin lactone in biological fluids. (A) Human urine (blank); (B) camptothecin-spiked human urine; (C) mouse blood (blank); (D) camptothecin-spiked mouse blood. Samples in A, B, C and D were acidified and extracted as described in Experimental. Peak b is camptothecin lactone.

Time-course study of camptothecin levels in mice

Four male mice (~24 g, Harlan Sprague–Dawley, Houston, TX, U.S.A.), were treated orally with 40 mg/kg camptothecin sodium salt. Blood samples (~50 μl) were taken from the ocular vein using capillary tubing and were collected at 5, 15, 30 90, 120, 180, 240 and 360 min, and 24 and 48 h after treatment. Each sample was mixed with 100 μl of 1 *M* hydrochloric acid, extracted and analyzed as described previously (Fig. 2C and D).

The triphasic curve obtained when the blood concentrations of camptothecin were plotted *versus* time is described by the following equation

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-k_{01}t}$$

where: C_t = blood concentration at time t, α = apparent rate of distribution to the peripheral compartment, β = apparent rate of elimination from the central compartment, k_{01} = absorption rate of central compartment, A = intercept of distribution slope α with ordinate, B = intercept of elimination slope β with ordinate and C = intercept of absorption slope K_{01} with ordinate.

A, B, C, α, β and k_{01} were obtained by using a curve-fitting computer program, JANA (Statistical Consultants, Lexington, KY, U.S.A.). These estimates were used to calculate various pharmacokinetic parameters using non-linear regression computer analysis (NONLIN, Statistical Consultants). Values of pharmacokinetic parameters were calculated using the equation for a two-compartment model with excretion occurring mainly from the central compartment.

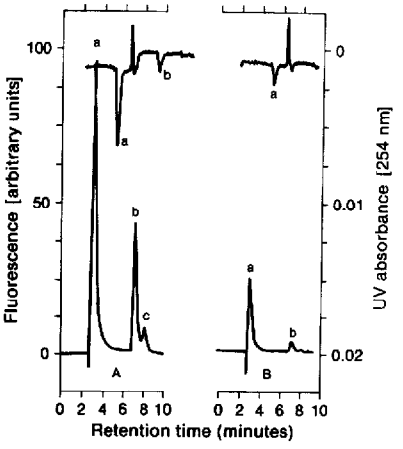

Fig. 3. Comparison of HPLC profiles of camptothecin sodium salt using UV and fluorescence detection. Upper trace, UV (0.02 a.u.f.s.; 254 nm); lower trace, fluorescence (course × 3; excitation, 370 nm; emission, 434 nm). Peaks: a = camptothecin sodium salt; b = camptothecin; c = sample impurity. Injection volume, 10 μl. Mobile phase, methanol–water (75:25). (A) 2.5 μg/ml; (B) 0.5 μg/ml.

RESULTS AND DISCUSSION

Camptothecin has significant antitumor activity in rodents, and because of its potential value as a chemotherapeutic agent in man, knowledge of the physiologic disposition of this agent is essential before rational therapeutic studies can be instituted. Different substituted analogues with different activities and toxicities have been developed. Nevertheless, it is necessary to develop a suitable specific assay for analyzing camptothecin and its derivatives and metabolites *in vitro*. Separation, quantitation and identification of camptothecin and its derivatives is essential for the understanding of the mechanism of action of camptothecin *in vivo*.

Simultaneous detection of camptothecin sodium salt, using UV and fluorescence detectors (Fig. 3), illustrates the increased sensitivity of analysis of the fluorescence technique. The fluorescence detector was set at an excitation wavelength of 370 nm and an emission wavelength of 434 nm because fluorescence spectra for camptothecin sodium salt and camptothecin lactone reveal excitation and emission peaks at these wavelengths. There is an excitation peak at 250 nm, but in biological media, other compounds also absorb at this wavelength, thus making measurements inaccurate.

The chromatograms shown in Fig. 4 illustrate the effect of sample pH on analysis of aqueous solutions of camptothecin sodium salt. When the sodium salt is dissolved in water, the solution produced has a pH of ~6.5 (Fig. 4B) and contains both camptothecin lactone (peak b) and the ionized sodium salt (peak

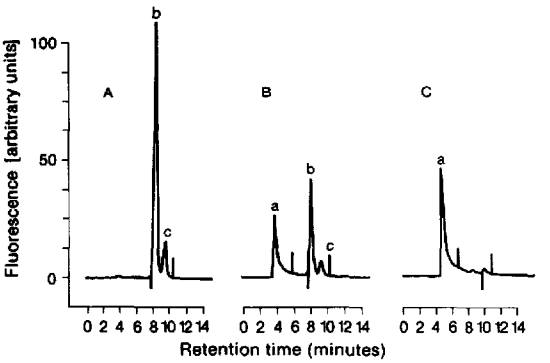

Fig 4. Effect of sample pH on rate of equilibrium between camptothecin (lactone) and camptothecin sodium salt in neutral mobile phase. Mobile phase, methanol–water (75:25, v/v), flow-rate, 0.7 ml/min. Peaks: a = camptothecin sodium salt; b = camptothecin; c = sample impurity. Camptothecin concentration, 0.1 μg/ml: (A) pH 2; (B) pH 6.5; (C) pH 12.

a); peak c is sample impurity. In an acidic environment with pH 2, a complete conversion of camptothecin sodium salt to lactone occurred (Fig. 4A). In a basic solution with pH 12, conversion of camptothecin lactone to the sodium salt is observed (Fig. 4C). Fig. 4 also shows the marked increase in sensitivity of fluorescence detection of the lactone (Fig. 4A) over the sodium salt (Fig. 4C). The two- to three-fold increase [peak area $148 \cdot 10^6$ *versus* $60 \cdot 10^6$ (Fig. 4C)] is in agreement with the findings of Hart *et al.* [17].

Given these findings, we focused our study on the effect of mobile phase pH on the quantitation of camptothecin. Acidification of the mobile phase to pH 4 optimized the conditions for analysis of acidified camptothecin samples. Under these conditions using a mobile phase of pH 4, the limit of detection was 0.5 ng/ml and the sensitivity of the detection was two- to three-fold higher than the

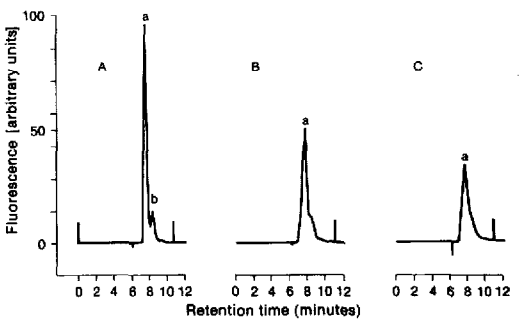

Fig. 5. Effect of sample pH on HPLC profiles of camptothecin (lactone) in acidic mobile phase. Mobile phase, methanol–10 m*M* KH_2PO_4 (75:25, v/v), pH 4.0. Peaks: a = camptothecin; b = sample impurity. Camptothecin concentration, 1.0 μg/ml. (A) pH 2.0; (B) pH 6.5; (C) pH 11.

TABLE I

RECOVERIES OF CAMPTOTHECIN AFTER TWO CONSECUTIVE ETHYL ACETATE EXTRACTIONS FROM DIFFERENT MEDIA

Concentration (μg/ml)	Recovery (%)		
	Water	Plasma	Urine
0.01	88	62	55
0.05	89	86	75
0.1	90	90	72
0.5	90	91	88
1	96	94	95

sensitivity when using a mobile phase of pH 7.0. Furthermore, the effect of sample pH at the optimal mobile phase condition (pH 4) was also studied and the results are shown in Fig. 5. As indicated, increasing the acidity of the sample enhanced the sensitivity as well as the peak symmetry (Fig. 5A).

Using these ideal conditions a standard curve was generated for the camptothecin concentration ranges 0.0005–0.01 and 0.01–1 μg/ml. The equations determined were $y = 1.18 \cdot 10^8 x$ ($r = 0.99$) and $y = 1.09 \cdot 10^8 x$ ($r = 0.99$), respectively, where x is the concentration of camptothecin and y is the relative peak area. The coefficients of variation for multiple samples were calculated for 1.0 μg/ml (6.9%) and for 5 ng/ml (6.7%).

The standard curve for camptothecin in human plasma provided a linear equation of $y = 1.18 \cdot 10^8 x$ ($r = 0.99$) for the concentration range 0.01–1 μg/ml. For concentrations below 0.01 μg/ml the equation was $y = 1.28 \cdot 10^8 x$ ($r = 0.99$). In human urine, the equation derived was $y = 1.11 \cdot 10^8 x$ ($r = 1.00$). Comparison of peak areas of camptothecin-spiked plasma samples to those of aqueous camptothecin samples indicated that the recovery of camptothecin from plasma was 90 $\pm$ 4%. The recovery of camptothecin from urine was 93 $\pm$ 10%. Under these conditions no interference was observed from normal biological molecules in plasma.

The recoveries of camptothecin from the biological samples following the extraction procedure described are presented in Table I. There was a steady increase in recovery as the concentration of camptothecin in each media increased. The extraction recoveries would increase slightly with a third extraction, but was determined to be inefficient.

The results of the analysis of time-dependent camptothecin concentration in blood of mice receiving a single oral dose of 40 mg/kg are shown in Fig. 6. The peak camptothecin concentration in blood was observed at 15 min following administration. Camptothecin blood levels started to decline with a half-life of about 26 min (α-phase elimination). A very slow β-phase with a half-life of 252

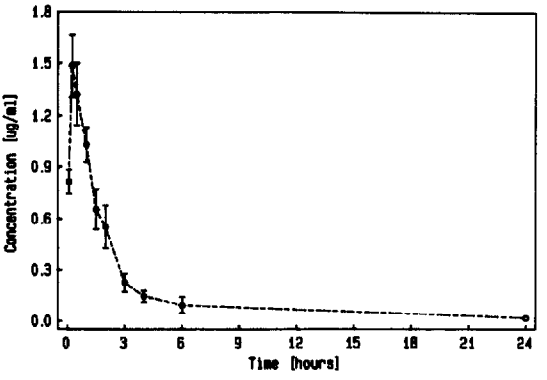

Fig. 6. Time-course of camptothecin in mouse blood following a single oral dose of camptothecin (40 mg/kg). Each point represents mean ± S.E.M. of four animals.

min was observed (Table II). The absorption of camptothecin from the gastrointestinal tract was high with an absorption half-life of 9.0 min (Table II). No camptothecin was detected 48 h after oral administration.

In conclusion, the methanol–water–potassium phosphate mobile system with an ODS reversed-phase column and fluorescence detection was used for the analysis of camptothecin in urine, plasma and water. The fluorescence detection used in this study provided a simple, reproducible method with excellent sensitivity and selectivity and little interference from other constituents found in biological or aqueous media. The application of this method in biological systems was indicated by the study of camptothecin pharmacokinetics in mice. Using this method, we found that camptothecin is absorbed fast from the gastrointestinal tract, undergoes a fast α-phase elimination half-life and slow β-elimination half-life. Camptothecin elimination from blood followed a two-compartment model with first-order input and first-order output. Using this method, further investigation on the metabolic degradation of camptothecin and its derivatives in a biological system *in vivo* will be carried out.

TABLE II

PHARMACOKINETIC PARAMETERS OF CAMPTOTHECIN IN MICE

Parameter	Value
Area under blood level curve (μg/ml per min)	181.64 ± 19.44
Elimination half-life (min)	45.81 ± 10.13
Fast α-elimination half-life (min)	25.69 ± 12.65
Slow β-elimination half-life (min)	251.81 ± 36.08
Volume of distribution (ml)	0.38 ± 0.12
Elimination rate constant (min^{-1})	0.016 ± 0.0035
Absorption half-life (min)	9.03 ± 4.41

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