ELSEVIER

Journal of Chromatography B, 655 (1994) 97–104

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Analysis of the active lactone form of 9-aminocamptothecin in plasma using solid-phase extraction and high-performance liquid chromatography

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(First received October 25th, 1993; revised manuscript received January 24th, 1994)

Abstract

We have developed a sensitive HPLC assay to quantitate the active lactone form of 9-aminocamptothecin (9AC) in human plasma over the concentration range 10–0.25 n*M* (0.091 ng/ml). Solid-phase extraction separated 9AC lactone from its less active metabolite, 9AC carboxylate, allowing samples to be stored for up to two months prior to reversed-phase HPLC analysis. An acidic (pH 2.55) isocratic HPLC mobile phase was used to enhance 9AC fluorescence resulting in an over 50-fold increase in assay sensitivity compared to previous methods. This assay was able to measure steady-state 9AC lactone concentrations even at the lowest dose level of 9AC used in our Phase I clinical trial.

1. Introduction

9-Aminocamptothecin (9AC, 9-amino-20(*S*)-camptothecin, NSC 629971) (Fig. 1) is a promising new antitumor agent currently undergoing testing in Phase I clinical trials. In preclinical models, 9AC is active against human colon cancer [1], breast cancer [2] and melanoma [3] cell lines. The primary target for all the camptothecin analogues is thought to be the nuclear enzyme, topoisomerase I, which relaxes supercoiled DNA [4,5]. 9AC binds to and stabilizes the covalent cleavable complex formed by topoisomerase I and DNA, ultimately leading to lethal DNA strand breaks.

The activity of 9AC depends upon an intact lactone ring; however, rapid hydrolysis of the lactone at physiologic pH generates the relatively

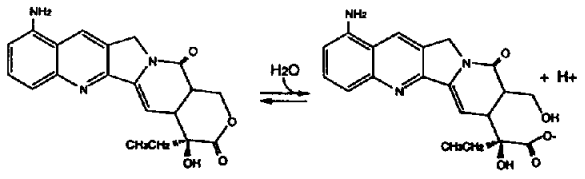

Fig. 1. Hydrolysis of 9-aminocamptothecin (9AC) lactone to 9AC carboxylate.

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0378-4347/94/$07.00
SSDI 0378-4347(94)00051-6

inactive 9AC carboxylate in a reversible, pH-dependent, non-enzymatic reaction as illustrated in Fig. 1 [6]. In human plasma, the equilibrium between these two species greatly favors the more water-soluble, open-ring 9AC carboxylate. This instability of 9AC lactone necessitates the rapid processing and immediate HPLC analysis of clinical samples in order to accurately quantitate drug levels [7,8]. Measurement of 9AC lactone in human plasma is further complicated by the relatively poor fluorescence of the drug compared to the parent compound, camptothecin [7], and by the low plasma concentrations of 9AC lactone (<1 nM) anticipated at the starting dose level used in our Phase I clinical trial. Currently published HPLC assays for 9AC lactone use post-column acidification to enhance drug fluorescence resulting in a lower limit of quantitation of 13 nM [7]. We have developed a simplified, sensitive method for measuring the lactone form of 9AC in plasma which avoids the need for immediate HPLC analysis and post-column acidification. Assay sensitivity has also been improved by over 50-fold with a reproducible lower limit of quantitation of 9AC lactone in plasma of 0.25 nM (0.091 ng/ml).

2. Experimental

2.1. Reagents

9AC lactone and camptothecin [20(S)-camptothecin, NSC 94600] were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). HPLC-grade water and methanol were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). All other chemicals were reagent grade and were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

2.2. Solid-phase extraction

Bond-Elut, 100 mg, C_{18} solid-phase extraction cartridges (Varian, Harbor City, CA, USA) were conditioned by passing 1 ml of methanol, followed by 1 ml of water through the cartridge packing. Blood samples were drawn into heparinized tubes, transported on ice, and immediately centrifuged at 800 g for 10 min at 4°C. A volume of 1 ml of plasma supernatant was combined with 10 μl of 100 nM camptothecin internal standard in a 1.5-ml polypropylene microcentrifuge tube and vortex-mixed for 15 s. The sample was loaded at a rate of 0.25 ml/min onto a solid-phase extraction cartridge and washed twice with 1 ml of water followed by 1 ml of 25% (v/v) methanol to completely remove the 9AC carboxylate. The remaining 9AC lactone and camptothecin internal standard were eluted with 0.75 ml of 75% (v/v) methanol–25 mM KH_2PO_4, pH 2.55. The eluted fraction was combined with 0.25 ml of 25 mM KH_2PO_4 (pH 2.55) to give a final volume of 1 ml before being stored at −70°C for later HPLC analysis.

2.3. High-performance liquid chromatography

HPLC separation was achieved using a C_{18} reversed-phase, 250 × 4.6 mm I.D., 5 μm, Ultrasphere ODS column (Beckman Instruments, San Ramon, CA, USA). A Waters 510 HPLC solvent delivery system (Millipore Corp., Milford, MA, USA) was used to deliver an isocratic mobile phase of 45% (v/v) methanol–25 mM KH_2PO_4, pH 2.55, at a flow-rate of 1 ml/min. The detection of the 9AC lactone peak was maximized using a Waters 470 scanning fluorescence detector at an excitation wavelength of 365 nm and an emission wavelength of 440 nm with an 18-nm bandwidth. Stored plasma extracts were thawed and a volume of 125 μl was injected onto the HPLC system using a 250-μl syringe (Hamilton, Reno, NV, USA) which had been treated with 3% Surfasil (Pierce Chemicals, Rockford, IL, USA). The syringe was washed with six volumes of methanol between injections. The number of theoretical plates for the HPLC column was calculated from the formula: theoretical plates = $5.54 \cdot (t_r/W_{1/2})^2$, where t_r is the retention time from the point of injection and $W_{1/2}$ is the peak width measured in units of time at one-half the maximal peak height.

2.4. Standard solutions

Stock solutions of 9AC lactone in DMSO were stored at 4°C in class A volumetric flasks which had been treated with 3% Surfasil. These solutions were stable under these conditions for at least 2 months. Plasma calibration standards were prepared by adding 10 μl of different stock solutions of 9AC lactone in DMSO to 1 ml of donor plasma to give final drug concentrations of 0.25, 0.5, 1.0, 2.5. 5.0, and 10.0 n*M*. Stock solutions of 100 n*M* camptothecin, the internal standard, were prepared and stored under similar conditions. Stock solutions of 9AC carboxylate were prepared immediately prior to use by diluting stock 9AC lactone into 0.1 *M* NaOH followed by a 15-min incubation at room temperature to facilitate the complete hydrolysis of the lactone ring.

2.5. Absolute recovery

The absolute recovery of drug from plasma samples was determined by analyzing 1-ml samples of donor plasma which were spiked to give final 9AC lactone concentrations of 0.25, 2.5, and 10 n*M*. Relative peak heights of these extracts were compared to unextracted 9AC lactone standards at the same concentrations made by directly diluting drug into 1 ml of elution buffer. The percent absolute recovery was defined as: (peak height of the plasma standard/peak height of the elution buffer standard) · 100. The amount of drug which was removed from the solid-phase extraction cartridge by the wash steps was measured by acidification of the wash fractions followed by HPLC analysis. Similar measurements were made for the camptothecin internal standard.

2.6. Calculations

Standard curves were generated by plotting the 9AC lactone/camptothecin peak-height ratio as the ordinate and the 9AC lactone plasma concentration as the abscissa. The data were fitted using least squares linear regression with a weighting factor of $1/(y_{obs})^2$, not including the origin. Standard curves were analyzed daily and unknown plasma samples were prepared in triplicate. If two replicates of an unknown sample differed from each other by more than 15%, which occurred *ca.* 25–30% of the time, then the third sample was analyzed and the average of all three measurements was used.

2.7. Accuracy, reproducibility, and stability of stored samples

Ten different standard curves of 9AC lactone in plasma were analyzed over a 3-month period (concentration range 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 n*M*). The measured amount of 9AC lactone was determined for each individual plasma standard by fitting its 9AC lactone/camptothecin peak-height ratio to that day's standard curve. Coefficients of variation were also determined for each concentration measured in the 10 standard curves. The stability of samples stored at −70°C over time was determined in separate experiments by repeated analysis of the same set of plasma standards and 3 previously extracted patient plasma samples on 4 separate days over a 2 month period.

2.8. Hydrolysis of 9AC lactone in plasma

Uncorrected pH measurements were made in fresh human plasma using a KCl glass electrode (Beckman Instruments). Plasma (10 ml) containing 100 n*M* 9AC lactone was incubated at 0°C, 25°C, and 37°C and aliquots were removed at various time points and the amount of 9AC lactone was determined. The concentration *vs.* time data were fitted to a first-order exponential decay curve, $C = C_0\exp(-kt)$, where C_0 is the initial concentration, k is the first-order rate constant, and t is time. The half-life was calculated by dividing 0.693 by the rate constant, k.

2.9. Total plasma 9AC measurement

Total plasma 9AC was defined as the sum of the 9AC lactone plus the 9AC carboxylate level in each plasma sample. The total plasma 9AC level was measured by a slight modification of

the 9AC lactone extraction procedure. Plasma aliquots (0.1 ml) were acidified with 0.9 ml of 8.5% phosphoric acid and incubated at room temperature for 15 min to allow for the complete conversion of 9AC carboxylate to 9AC lactone. The sample was extracted and analyzed as described previously for 9AC lactone. The 9AC carboxylate plasma level could be calculated by subtracting the concentration of 9AC lactone from the total plasma level. 9AC carboxylate plasma standards were used to construct total plasma 9AC standard curves over the concentration range 2.5–100 n*M*.

2.10. Analysis of patient samples

Steady-state plasma samples of 9AC lactone and total plasma 9AC were obtained from 3 patients who were treated at the initial dose level of 0.36 mg/m^2 of 9AC given as a 72-h continuous infusion (5 μg/m^2/h) in a Phase I clinical trial. Samples were obtained at 24, 48, and 72 h after the start of the infusion and the values were averaged to give the mean steady-state plasma level (C_{ss}) for each patient. Total body clearance (Cl_{TB}) was calculated by dividing the infusion rate by C_{ss}. Clinical samples were drawn at approximately the same time of the day for all patients.

3. Results

3.1. Solid-phase extraction

The accuracy of this assay depends upon the rapid and complete separation of 9AC lactone from 9AC carboxylate by solid-phase extraction with no closure of the carboxylate ring during sample processing. In order to validate this point, plasma samples were spiked in triplicate with 5, 10, and 50 n*M* of 9AC carboxylate and analyzed by solid-phase extraction. Even at the highest plasma concentration of 50 n*M* 9AC carboxylate, no 9AC lactone was detected in the final elution step, demonstrating the effectiveness of solid-phase extraction in separating the two species.

The absolute recovery of 9AC from 1 ml plasma was compared with unextracted standards made by directly diluting 9AC lactone into 1 ml of elution buffer. The absolute recoveries of 0.25, 2.5, and 10.0 n*M* 9AC lactone from plasma in the final elution fraction were (mean ± S.D.) 87.8 ± 3.7%, 83.6 ± 6.4%, and 87.1 ± 16.9%, respectively. When the wash fractions were analyzed, most of the remaining drug could be accounted for, demonstrating a high overall efficiency of extraction of 9AC lactone from plasma. The total cumulative recovery of drug in all the fractions was 87.8 ± 3.7%, 103.6 ± 8.5%, and 103.7 ± 8.1%, for the 0.25, 2.5, and 10 n*M* 9AC lactone concentrations, respectively. The absolute recovery of 1 n*M* camptothecin, the internal standard, in the final elution step was 90.9 ± 6.6%, and, when the elution and wash fractions were combined, the total amount of camptothecin extracted was 96.7 ± 6.2%.

3.2. HPLC analysis

High-performance liquid chromatography effectively resolved 9AC lactone and the internal standard, camptothecin, with retention times of 9.1 ± 0.1 min and 24.5 ± 0.9 min, respectively (Fig. 2). The 45% methanol concentration used in the mobile phase allowed for sufficient resolution of 9AC lactone from interfering endogenous substances in the plasma without excessively prolonging the retention time of camptothecin. Each run took approximately 30 min to complete; thus, a complete calibration curve (6 points) and 3 unknown samples in triplicate could be analyzed in about an 8-h period. The acidic mobile phase (pH 2.55) was required to enhance the fluorescence of 9AC lactone, thereby increasing the sensitivity of detection [7]. Lowering the pH of the mobile phase from 3.30 to 2.55 increased the relative fluorescence of 9AC lactone by 3.4-fold (data not shown), while still maintaining peak integrity and reproducibility. A greater reduction in the pH further enhanced 9AC lactone fluorescence but broadened the camptothecin internal standard peak and also approached the lower limit of pH tolerance of the reversed-phase column (pH 2.50). Despite

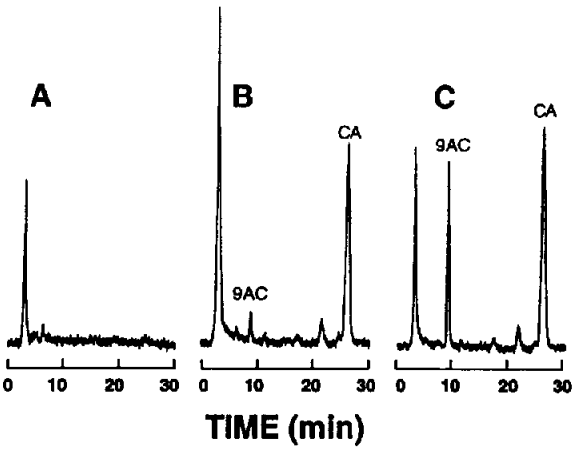

Fig. 2. Chromatograms of plasma extracts of (A) normal human plasma; (B) 0.25 n*M* 9-aminocamptothecin (9AC) plasma standard; and (C) plasma from a patient receiving a 72-h infusion of 9AC with a measured 9AC lactone plasma level of 1.57 n*M*. The concentration of the camptothecin (CA) internal standard is 1 n*M*. Plasma samples were processed and analyzed as described in Experimental.

the low pH of the mobile phase, the retention times were highly stable. A comparison of the calculated theoretical plates for a single HPLC column after over 200 injections over a 2-month period revealed no significant change; the initial value was 32 384 ± 1948 plates/meter, and, after over 200 injections, it was 28 328 ± 6745 plates/meter ($p = 0.3$).

3.3. Standard curves

The standard curve constructed from 6 concentrations of 9AC lactone in plasma (0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 n*M*) was linear over this range. The lowest quantifiable 9AC lactone concentration was 0.25 n*M* from 1 ml of plasma. At this drug level, the 9AC lactone peak height was approximately 3.5 times greater than the baseline signal noise. Using an HPLC injection volume of 125 μl, this corresponded to a minimal quantifiable amount of 9AC lactone of 0.031 picomoles.

Assay reproducibility was examined by analyzing standard curves from 10 different experiments performed over a 3-month period. The correlation coefficient (r) for these curves was (mean ± S.D., $n = 10$) 0.9995 ± 0.0006, with a slope of 0.308 ± 0.060, and a y-intercept of 0.003 ± 0.012. Table 1 lists the actual measured values and the coefficients of variation for each concentration in all 10 of these standard curves. The assay was accurate and reproducible with coefficients of variation ranging from 1.8% to 7.6% over the concentration range of 0.25 n*M* to 10 n*M*.

Table 1
Accuracy and reproducibility of the 9AC assay

Amount of 9AC lactone added (n*M*)	Amount of 9AC lactone measured (mean ± S.D., $n = 10$) (n*M*)	Coefficient of variation (%)
0.25	0.253 ± 0.011	4.5
0.50	0.497 ± 0.038	7.6
1.0	1.03 ± 0.06	6.0
2.5	2.46 ± 0.09	3.5
5.0	5.01 ± 0.17	3.4
10	10.0 ± 0.2	1.8

Ten separate standard curves were analyzed on different days over a 3-month period. The measured amount of 9AC lactone quantitated in each concentration standard in the calibration curve was determined as described in Experimental.

3.4. Stability of stored samples

The stability of plasma extracts stored at −70°C was examined by repeatedly analyzing the same stored standards and three previously extracted, unknown patient samples on 4 different days over a 2-month period. Reproducibility was high with a correlation coefficient of (mean ± S.D, $n = 4$) 0.9993 ± 0.0004, a slope of 0.333 ± 0.018, and a y-intercept of − 0.004 ± 0.014. The coefficients of variation ranged from 1.7% to 3.9% for the plasma standards ($n = 4$) and from 3.6% to 7.6% for the unknown samples ($n = 3$) despite prolonged storage and repeated freeze-thawing (Table 2).

3.5. Hydrolysis of the 9AC lactone

The rate of hydrolysis of 9AC lactone was measured *in vitro* in fresh human plasma at 0°C,

Table 2
Accuracy and reproducibility of stored samples

Sample (n*M*)	Amount of 9AC lactone added (n*M*)	Amount of 9AC lactone measured (mean ± S.D.)(n*M*)	Coefficient of variation (%)
Plasma standards (n = 4)			
0.25	0.25	0.248 ± 0.006	2.3
0.50	0.50	0.508 ± 0.019	3.7
1.0	1.0	1.04 ± 0.04	3.7
2.5	2.5	2.43 ± 0.08	3.2
5.0	5.0	4.90 ± 0.19	3.9
10	10	10.2 ± 0.2	1.7
Clinical samples (n = 3)			
Unknown 1	–	1.26 ± 0.10	7.6
Unknown 2	–	0.557 ± 0.020	3.6
Unknown 3	–	2.00 ± 0.10	5.2

A standard curve and three previously extracted patient plasma samples were analyzed on repeated occasions over a 2-month period and the amount of 9AC lactone was determined as described in Experimental. The samples were stored at −70°C and repeatedly thawed prior to each HPLC analysis.

25°C, and 37°C (Fig. 3). The mean pH of fresh human plasma was 7.58 ± 0.08 ($n = 3$) and the half-lives of 9AC lactone were 12.15 h, 0.76 h, and 0.34 h, at 0°C, 25°C, and 37°C, respectively. Frozen and thawed human plasma was not used because it had a higher mean pH of 8.10 ± 0.15 and resulted in a shorter half-life of 9AC lactone. When the samples were allowed to incubate in plasma until no further changes in the 9AC lactone concentration were observed, the 9AC carboxylate species accounted for over 99% of the total plasma 9AC. The reversibility of the hydrolysis was demonstrated by measuring the total plasma 9AC at the end of the incubation; essentially 100% of the total drug added to the plasma could be recovered (data not shown).

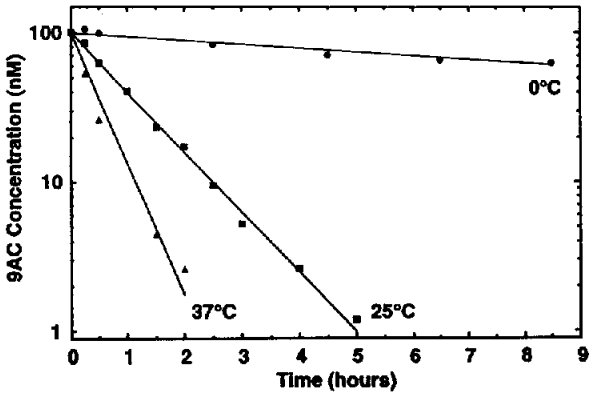

Fig. 3. Stability of 9-aminocamptothecin (9AC) lactone in fresh human plasma. 9AC lactone was added to fresh human plasma at a concentration of 100 n*M* and incubated at 0°C, 25°C, and 37°C. Aliquots were removed at various time points and the amount of 9AC lactone was measured as described in Experimental. The half-life of 9AC lactone in fresh human plasma was 12.15 h, 0.76 h, and 0.34 h, at 0°C, 25°C, and 37°C, respectively.

3.6. Total plasma 9AC measurement

Acidification of plasma samples prior to solid-phase extraction allowed for the determination of total plasma 9AC levels. Because the total plasma 9AC levels were much higher than the 9AC lactone levels, the total plasma standard curves were constructed over the concentration range of 2.5 n*M* to 100 n*M* using six 9AC carboxylate standards. The correlation coefficient for these curves was (mean ± S.D., $n = 6$) 0.9990 ± 0.0006, the slope was 0.028 ± 0.006, and the *y*-intercept was 0.005 ± 0.014. The coefficients of variation were 4.6%, 9.7%, 5.5%, 4.8%, 2.7%, and 3.7% for the 2.5, 5.0, 10.0, 25, 50, and 100 n*M* standards, respectively.

Table 3
Steady-state plasma levels and clearance of 9-AC lactone and total plasma 9AC in patients

Parameter	Value (mean ± S.D., $n = 3$)		
	Patient 1	Patient 2	Patient 3
9-Aminocamptothecin lactone			
C_{ss} (nM)	0.38 ± .07	1.40 ± 0.37	0.69 ± 0.19
Cl_{TB} (l/h/m^2)	37.0 ± 6.3	10.4 ± 3.8	21.2 ± 6.7
Total plasma 9-aminocamptothecin			
C_{ss} (nM)	6.83 ± 1.61	20.5 ± 2.3	23.8 ± 3.8
Cl_{TB} (l/h/m^2)	2.09 ± 0.45	0.68 ± 0.08	0.59 ± 0.09

Clinical samples were obtained at 24, 48, and 72 h after the start of the infusion in three patients treated at the initial dose of 0.36 mg/m^2 of 9AC given as a 72-h infusion (5 μg/m^2/h) in a Phase I clinical trial. 9AC lactone and total plasma 9AC levels were determined as described in Experimental.

3.7. Determination of plasma levels of 9AC lactone

In a Phase I clinical trial of 9AC lactone administered as a 72-h continuous infusion, the steady-state 9AC lactone plasma levels were successfully measured in the first 3 patients receiving the initial dose of 0.36 mg/m^2 (5 μg/m^2/h) (Table 3). The measured 9AC lactone levels ranged from 0.38 to 1.40 nM, demonstrating the sensitivity of this assay. Total plasma 9AC levels ranged from 6.83 to 23.8 nM (Table 3).

4. Discussion

The accurate measurement of the active, lactone forms of the camptothecin derivatives has previously required the immediate processing and HPLC analysis of plasma samples because of the instability of the lactone ring [7–9]. This has made the pharmacokinetic monitoring of the active form of these agents logistically difficult. Our new analytic procedure used solid-phase extraction to rapidly separate 9AC lactone from 9AC carboxylate, thereby eliminating the need for immediate HPLC analysis. Solid-phase extraction also enhanced assay sensitivity by removing interfering endogenous plasma compounds and by deproteinating the extract without diluting the sample. Cleaner sample extracts permitted larger volumes of plasma to be processed for HPLC analysis. This resulted in an over 50-fold lower limit of quantitation of 9AC lactone in plasma compared to a previously published assay [7]. Clinical specimens were generally analyzed within 2 to 3 days after being drawn; however, sample extracts were stable for up to 2 months when stored at −70°C. Acidifying the isocratic mobile phase simplified the HPLC methodology by maximizing drug fluorescence without the need for post-column acidification.

The rapid hydrolysis of the 9AC lactone ring in fresh human plasma at 37°C ($t_{1/2} = 0.34$ h) demonstrated the need for rapid blood sample processing. The solid-phase extraction procedure required only *ca.* 10 min per time point, after which the sample extracts could be stored for at least 2 months for later analysis. In order to insure the most accurate measurement of 9AC lactone, we recommend that blood samples be drawn and immediately placed on ice, followed by plasma separation and extraction. Samples can then be stored and batched for later HPLC analysis.

Our assay successfully measured the steady-state plasma concentration of 9AC lactone in the first 3 patients treated at the lowest dose level in our Phase I clinical trial. A slight modification of the assay also allowed for the determination of the total plasma 9AC levels in these patients. This measurement was facilitated by the finding that the steady-state total plasma 9AC levels were substantially higher than the 9AC lactone levels during a 72-h continuous infusion. The 9AC carboxylate level could be determined by subtracting 9AC lactone concentration from the total plasma 9AC level. In these first 3 patients, the 9AC carboxylate levels were 14- to 33-fold higher than the 9AC lactone concentrations in each individual patient. No toxicity or drug effects of 9AC were observed at this initial dose. We are currently escalating the dose of 9AC and

continuing our pharmacokinetic monitoring. Because the pharmacodynamic characteristics of the camptothecin analogues are still incompletely defined, both the active lactone and the total plasma levels of these drugs should be measured in order to provide a complete pharmacokinetic profile of these agents.

Further improvements in the sensitivity of this assay may still be possible. Because the processed plasma samples were relatively free from interfering endogenous substance, larger volumes of plasma may be processed or larger injections volumes used for HPLC analysis. In addition, the stability of 9AC lactone in the acidic elution buffer may allow for sample concentration or derivatization to further enhance 9AC fluorescence. The solid-phase extraction technique used to separate the 9AC lactone from 9AC carboxylate is theoretically applicable to other camptothecin analogues. Preliminary data suggest that the lactone forms of camptothecin, topotecan, CPT-11, and SN-38 can also be separated from their corresponding carboxylate forms by solid-phase extraction (data not shown). Development of similar assays will hopefully facilitate the pharmacokinetic monitoring of these agents.

5. Acknowledgement

The authors wish to thank Dr. Jerry M. Collins, Office of Research Resources, Center for Drug Evaluation and Research, Food and Drug Administration for his assistance, guidance and support.

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