ELSEVIER

Journal of Chromatography B, 707 (1998) 227–233

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic quantitation of total and lactone 20(S)camptothecin in patients receiving oral 20(S)camptothecin

Farheena Ahmed[a,b], Viral Vyas[a], Ahamed Saleem[a], Xi-Guang Li[a], Rita Zamek[a], Adam Cornfield[a], Paul Haluska[a], Nageatte Ibrahim[a], Eric H. Rubin[a], Elora Gupta[a,b,*]

[a]*UMDNJ – The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901, USA*
[b]*Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA*

Received 25 August 1997; received in revised form 7 November 1997; accepted 2 December 1997

Abstract

We have developed a sensitive high-performance liquid chromatography (HPLC) assay to quantitate total and lactone forms of 20(S)camptothecin (CPT) in human plasma. Lactone and total CPT were extracted using solid-phase extraction and liquid–liquid extraction, respectively. The extracted lactone samples could be stored without immediate HPLC analysis. The two forms of CPT were quantitated by reversed-phase HPLC with fluorescence detection. The extraction efficiencies were about 100% and 92% for the total and lactone forms, respectively. The lower limit of quantitation was 5.74 n*M* for the two forms. The method was reproducible with a mean interday and intraday variability of 6% for total CPT and 4% and 6%, respectively, for lactone CPT. The assay could effectively quantitate lactone and total CPT in patients receiving single dose and multiple doses of oral CPT. © 1998 Elsevier Science B.V.

Keywords: Lactone 20(S)camptothecin; 20(S)Camptothecin

1. Introduction

20(S)Camptothecin (CPT), derived from the Chinese plant *Camptotheca acuminata*, is an alkaloid having antineoplastic activity [1]. The mechanism of action involves poisoning of topoisomerase I, an enzyme essential for DNA transcription and overall cellular replication [2–5]. Since the drug has been shown to have maximal activity in the S-phase of the cell cycle [6], a prolonged exposure is required for optimal efficacy. Due to improved patient compliance, oral administration is preferred over continuous intravenous administration for achieving prolonged drug exposure. We have completed a phase I trial of sequential oral CPT and etoposide [7,8]. Patients received oral CPT for 14 days followed by intravenous etoposide. One of the objectives of the trial was to describe drug disposition following single and multiple oral doses of CPT. Hence, we have developed a reproducible, sensitive high-per-

*Corresponding author. Fax: +1 732 2357493.

0378-4347/98/$19.00
PII S0378-4347(97)00615-4

formance liquid chromatography (HPLC) assay to rapidly quantitate CPT in patient plasma.

CPT is a pentacyclic indole alkaloid with the terminal α-hydroxy ring being extremely labile (Fig. 1). Opening of the ring to the carboxylate form under alkaline conditions results in loss of activity [9,10]. Hydrolysis of the lactone form involves a sequential conversion to the corresponding acid which is ionized to the carboxylate form at pH values greater than the pK_a of the acid [11]. CPT is primarily in the lactone form at pH<5 and converts predominantly to the ionized carboxylate form at pH>8.0. Since the pH of human plasma is about 7.58 [12], a substantial fraction of the CPT dose should be converted to the inactive carboxylate. In addition, the carboxylate form shows preferential binding with the plasma albumin leading to stabilization of this form [13]. Since inhibition of topoisomerase I is mediated only by the closed ring lactone [14], it is important to determine the relative concentrations of lactone and total (lactone plus carboxylate) CPT in patients.

There have been previous reports of assay development of the two forms of CPT. Scott et al. and Loh and Ahmed have validated the quantitation of the lactone and carboxylate forms of the sodium salt of CPT in rat plasma [15–17]. Supko and Malspeis have also developed a reproducible and sensitive quantitation method of the two forms [18]. However, due to the instability of the lactone, these methods require immediate extraction and HPLC analysis for lactone quantitation. These procedures would be inconvenient for our study which involved a 23 day sampling schedule with some sample collections occurring during the evening. Therefore, we developed a convenient and reproducible method which would allow storage of collected samples until HPLC analysis.

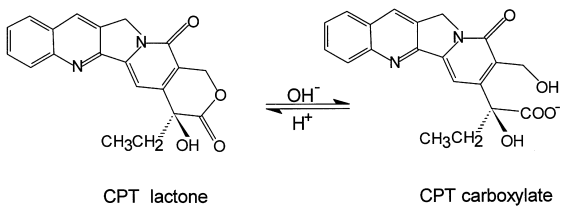

Fig. 1. Chemical structures of CPT lactone and carboxylate forms.

2. Experimental

2.1. Reagents

CPT lactone and internal standard SN-38 (7-ethyl-10-hydroxycamptothecin), were gifts from the Stehlin Foundation for Cancer Research (Houston, TX, USA) and Pharmacia and Upjohn (Kalamazoo, MI, USA), respectively. The acetonitrile and methanol used were of HPLC grade (Fisher, Springfield, NJ, USA). Potassium dihydrogenphosphate (KH_2PO_4) and sodium heptanesulphonate were purchased from Sigma (St. Louis, MO, USA). All reagents were used as received. Fresh frozen human plasma was obtained from healthy volunteers.

2.2. Preparation of standards

A 1 mg quantity of CPT lactone was dissolved in 1 ml dimethyl sulphoxide (DMSO) to produce a 2.874 m*M* stock solution. Plasma calibration standards were prepared by adding diluted stock solutions to fresh human plasma to give final concentrations of 5.74 n*M*, 11.48 n*M*, 57.4 n*M*, 114.8 n*M*, 574 n*M*, and 1148 n*M* of total CPT. Stock solutions of SN-38 were prepared in DMSO and were subsequently diluted in water to produce 20 n*M* SN-38. All stock solutions and plasma calibration standards were stored at −70°C.

2.3. Plasma collection for pharmacokinetics

Patients received a 6 mg/m^2/day dose of CPT for a period of 14 days. Heparinized blood samples were collected at predose, at 1, 2, 3, 4, 6, 8, and 10 h after the first dose of CPT. A single sample was also collected on days 2, 4, 6, 8, 15, 16, 17, 18, 19, 22, and 23 during the dosing schedule. In addition, three samples were obtained on day 14 (last dose of CPT). Once the blood was drawn, it was immediately placed on ice and transferred to the laboratory. The samples were centrifuged at 400 *g* for 10 min at 4°C and the plasma was used for quantitation. For the assessment of total CPT, plasma samples were frozen at −70°C until further extraction and HPLC analysis. For the quantitation of lactone CPT, extraction of the lactone from plasma was performed

immediately and the extracted samples were stored at −70°C.

2.4. Extraction of lactone and total CPT from plasma

Lactone CPT was extracted from plasma using a modification of the solid-phase extraction described by Takimoto et al. [12]. C_{18} solid-phase extraction columns (Waters, Milford, MA, USA) were equilibrated with 1 ml of methanol followed by 1 ml of filtered water. A volume of 100 μl of plasma was combined with 10 μl of SN-38, vortexed for 5 s and then loaded onto the column. The columns were then washed twice with 1 ml of filtered distilled water followed by 1 ml of 20% methanol. The 20% methanol wash is effective in completely removing the carboxylate form [12]. Lactone CPT and SN-38 were eluted with 1 ml of 75% methanol and 25 m*M* KH_2PO_4 at pH 2.55. The extracted samples were frozen at −70°C until HPLC analysis.

Total CPT was quantitated using a modification of the procedure described by Supko et al. [18]. A 100 μl volume of plasma was combined with 10 μl of SN-38 and deproteinized with 300 μl of methanol. The mixture was vortexed for 5 s and centrifuged at 5800 *g* for 3 min. The clear supernatant was acidified with 50 μl of 0.5 *M* HCl before HPLC analysis.

2.5. HPLC

Separation of the compounds of interest was carried out at room temperature on a μBondapak, 10 μm, 3.9 mm×300 mm C_{18} column preceded by a μBondapak precolumn (Waters). The solvent delivery system (L-7100; Hitachi, Danbury, CT, USA) was used to deliver an isocratic mobile phase of 35% acetonitrile and 65% 25 m*M* KH_2PO_4 containing 1 m*M* sodium heptanesulphonate, pH 4.8. The flow-rate was maintained at 0.85 ml/min. Plasma extracts were injected onto the column using an autosampler (L-7200; Hitachi). The detection of the CPT and SN-38 peaks were achieved using a scanning fluorescence detector (474; Waters) at λ_{ex} 360 nm and λ_{em} 440 nm. Chromatographic peak height data acquisition and integration was achieved using Powerchrom (AD Instruments, Castle Hill, Australia).

2.6. Determination of extraction efficiency, precision, and accuracy

The extraction efficiency (recovery) was determined by comparison of peak height ratios (PHR) of lactone or total CPT in plasma extracts versus external standards prepared in DMSO and subsequently diluted in methanol (for total CPT) or mobile phase (for lactone CPT) (described in Section 2.2). All the external standards were acidified by 50 μl of 0.5 *M* HCl prior to HPLC analysis. Plasma stocks of 1148, 574 and 114.8 n*M* were extracted using the two methods. SN-38 was added to the extracted samples before HPLC analysis. The extraction efficiency was calculated by comparison of the PHR of extracted samples and external standards.

The plasma standard solutions of lactone and total CPT were extracted and analyzed by HPLC for three consecutive days. Standard curves were generated by plotting the PHR as a function of the plasma standard concentration and fitting the data using least squares linear regression. The mean PHR and standard deviation was obtained for all the standards. The interday assay variability (precision) was determined as the percent coefficient of variation. The intraday assay precision was based on extractions and HPLC analyses performed three times during the same day.

The accuracy of the assay methodology was determined by fitting the lactone or total CPT PHR to the standard curve equation obtained on the same day and comparing the predicted and actual concentrations.

2.7. Stability of CPT

The reduction of total CPT concentrations in donor plasma was determined at −70°C, 4°C and at 37°C. Chilled plasma was spiked with CPT to give final concentrations of 5.74 n*M*, 57.4 n*M* and 574 n*M* and equilibrated at the respective temperatures before the initiation of the stability studies. Aliquots of 100 μl were removed at 1 h, 2 h, 4 h and 24 h for liquid–liquid extraction and HPLC analysis as described in Section 2.5.

The stability of CPT in patient plasma samples stored at −70°C was evaluated in five different

patients. Using 100 μl aliquots of plasma, the initial liquid–liquid extractions for total CPT were performed within 24 h after receiving the samples. After a period of 30 days, 100 μl aliquots of the stored plasma were extracted again and the concentrations were compared to those obtained after the initial extraction. In a similar manner, we determined the stability of extracts of lactone CPT obtained from patient plasma samples which were stored at −70°C over a period of 30 days. Following solid-phase extraction, aliquots of the extracts were analyzed by HPLC within 24 h and after 30 days of storage at −70°C.

2.8. Determination of pharmacokinetic parameters

The plasma concentration–time profiles of lactone and total CPT were analyzed by noncompartmental methods using PCNONLIN (v4.2; SCI, Lexington, KY, USA). The maximal plasma concentrations (C_{max}) and the corresponding times (t_{max}) after the first dose were determined by visually inspecting the profiles. The elimination halflife ($t_{1/2}$) was the ratio of 0.693 and λ_z, the slope obtained by loglinear regression of the profiles. AUC (area under plasma concentration–time curve) was estimated by the trapezoidal rule [19]. Following multiple CPT dosing, the average steady state concentrations (C_{ss}) were obtained by averaging concentrations obtained on days 4, 6 and 8.

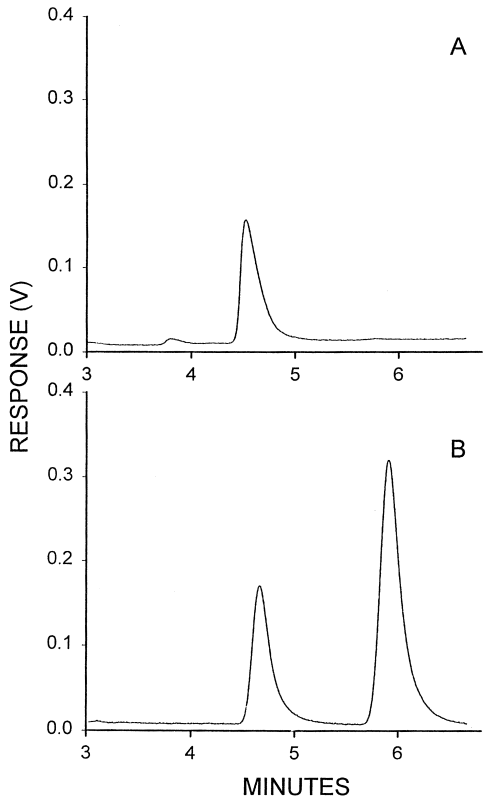

Fig. 2. Representative chromatograms of plasma samples. (A) Predose sample spiked with 20 n*M* SN-38. (B) Sample from the same patient after receiving an oral dose of CPT. Chromatographic peaks: SN-38, 4.7 min; CPT, 6 min.

3. Results and discussion

We have developed a sensitive and convenient method to quantitate both total and lactone forms of CPT in human plasma. Unlike other methods requiring immediate extraction and HPLC analysis of the lactone form, our method involves solid-phase extraction which separates carboxylate from lactone CPT. This method, therefore, allows extracted lactone samples to be stored until HPLC analysis.

Representative chromatograms of plasma extracts from a patient receiving CPT are shown in Fig. 2. Use of an ion-pairing agent (sodium heptanesulfonate) and a mobile phase pH of 4.5 enhanced sensitivity as well as peak symmetry (data not shown). Mobile phase consisting of 25% acetonitrile in buffer pumped at a flow-rate of 0.85 ml/min resulted in an acceptable column pressure of 1200 p.s.i.. The retention times of SN-38 and CPT were 4.7 and 6.0 min, respectively, and a run time of about 7.5 min was sufficient to quantitate both compounds. Interference due to endogenous compounds was not observed. A typical patient profile included 12 standards (six each for total and lactone, respectively) and 42 samples (21 each for total and lactone, respectively) collected over 23 days. Due to the short run time all 54 samples could be analyzed within 10 h thus ensuring rapid analysis of patient samples.

The extraction efficiency for total CPT was 107%, 105% and 112% for the concentrations of 1154, 574 and 114.8 n*M*, respectively. The recoveries for the

lactone form at the same concentrations were 95%, 80% and 102%, respectively. The lower recovery of the lactone form could be attributed to the instability of this form. The halflife of the lactone in plasma is about 341 min at 0°C and 6.98 min at 37°C [18]. Thus even for samples placed on ice, a delay of 5 min before extraction would result in hydrolysis of 8% of the initial concentration. Therefore, for the lactone quantitation, immediate placement of samples on ice followed by rapid extraction would aid in minimizing hydrolysis.

The precision of the assays (Table 1) is established by the low mean inter-day variability of 6% (total) and 10% (lactone) and mean intra-day variability of 5.7% (total) and 6.1% (lactone). The standard curves for the total and lactone forms were linear within the range of 5.74 n*M*–1148 n*M*. The average correlation coefficient was 0.998. Based on the degree of accuracy at the lowest concentrations (Table 2), the lower limit of quantitation was 5.74 n*M* for CPT. These limits of quantitation enabled CPT estimation in the terminal sampling time points. Thus a relatively small blood volume of 2–3 ml (1–1.5 ml plasma) would be sufficient for quantitation of the two forms.

CPT was stable in spiked plasma at −70°C, 4°C and 37°C during the 24 h evaluation period. The percent of drug recovered at the end of 24 h was ≥99% for all the three concentrations of plasma standards that were evaluated. CPT was also stable in patient plasma samples stored at −70°C. There was ≤10% variability in total CPT quantitation between samples that were processed within 24 h or 30 days after storing at −70°C. The plasma extracts for the quantitation of lactone CPT that were analyzed with 24 h and after 30 days of storage at −70°C also exhibited a similar stability profile (≤12% variability). Since all patient samples were extracted and analyzed within 30 days after receiving them, there were no alterations in the lower limit of quantitation (5.74 n*M*), precision or accuracy of the quantitations.

Table 1
Precision of HPLC quantitation of total and lactone CPT in human plasma

Concentration (n*M*)	Total CPT	Lactone CPT
Inter-day variability		
5.74	5.3%	21.7%
11.48	16.8%	7.8%
57.4	1.6%	6.5%
114.8	4.7%	13.1%
574	2.9%	6.7%
1148	4.9%	5.6%
Intra-day variability		
5.74	10.5%	17.3%
11.48	11.8%	4.2%
57.4	3.2%	6.3%
114.8	3.6%	3.7%
574	3.4%	4.2%
1148	1.9%	1.0%

Plasma standards were extracted and analyzed for three consecutive days for estimation of inter-day variability. Three separate standard curves were extracted and analyzed the same day for quantitation of intra-day variability.

Table 2
Accuracy of HPLC quantitation of total and lactone CPT in human plasma

Concentration (n*M*)	Total CPT	Lactone CPT
5.74	92%	115%
11.48	118%	109%
57.4	103%	96%
114.8	100%	95%
574	100%	102%
1148	100%	99%

The calculated concentration was expressed as percentage of the actual CPT concentration.

Fig. 3 shows a representative total and lactone CPT profile in a patient after a single dose (A) and multiple doses (B). The average lactone to total ratio in the samples was about 9.6%. The two forms appeared to have similar disposition profiles having a slow and sustained absorption with steady state being attained within 2–3 days of dosing. Pharmacokinetic parameters obtained in three patients after single and multiple doses of CPT are listed in Table 3. The drug appeared to be absorbed slowly and peaked at 2 to 3 h after administration. A prominent feature of the parameter estimates was the substantial interpatient variability in the C_{max} and AUC estimates. This finding suggests that the bioavailability of CPT is variable. Substantial variability was also noted in the C_{ss} of CPT following multiple doses. To our knowledge this is the first description of the oral absorption of CPT. Since camptothecins have a narrow therapeutic index, the variability in bioavailability may be a critical determinant of the therapeutic efficacy and

Table 3
Pharmacokinetic parameter estimates of total and lactone CPT in patients after a single and multiple doses of oral CPT (6 mg/m^2)

Parameter	Patient 1	Patient 2	Patient 3
Total CPT			
C_{max} (n*M*)	283	127	482
T_{max} (h)	2	2	4
AUC (n*M*·h)	2080	1659	3052
$t_{1/2}$ (h)	20.9	23.7	10.5
C_{ss} (n*M*)[a]	299	82.3	491
Lactone CPT			
C_{max} (n*M*)	23.9	12.5	23.5
T_{max} (h)	3	4	3
AUC (n*M*·h)	173	111	152
$t_{1/2}$ (h)	21.4	17.8	12.1
C_{ss} (n*M*)[a]	28.2	5.9	51.5

Plasma samples were obtained at predetermined points during the dosing schedule. Total and lactone CPT in the samples were quantitated as described in Section 2. Pharmacokinetic parameters were estimated using noncompartmental methods.
[a] Obtained from multiple dosing profiles.

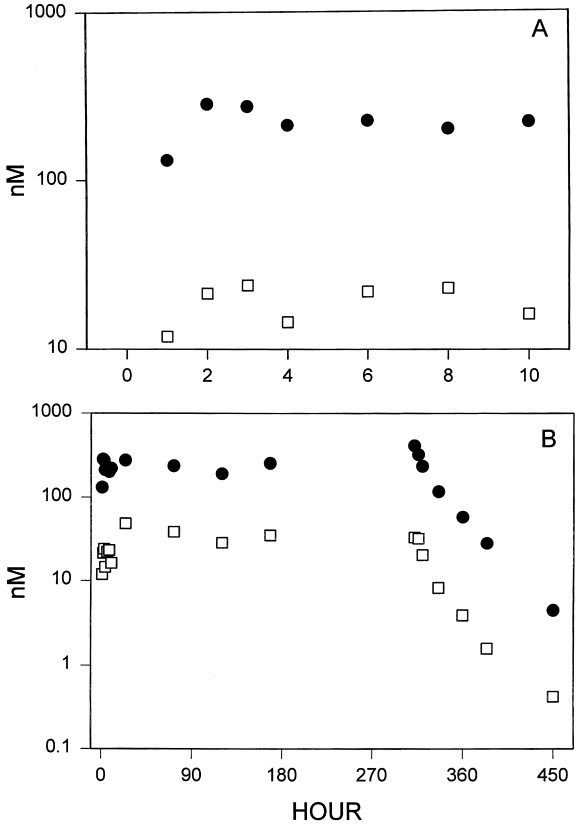

Fig. 3. CPT total (●) and lactone (□) concentrations in a patient after a single CPT dose (A) and during multiple CPT doses (B).

toxicity of oral CPT. Mechanistic factors affecting CPT intestinal absorption are presently being investigated.

In summary, we have established an accurate and convenient assay for lactone and total CPT that enables pharmacokinetic evaluation of the two forms in patient plasma.

Acknowledgements

We are thankful to Drs. Beppino Giovanella, Ethan Natelson and Takis Pantazis of the Stehlin Foundation for Cancer Research (Houston, TX, USA) for providing CPT. We are grateful to Dr. Deborah Toppmeyer and Judy Much of The Cancer Institute of New Jersey, for their valuable input in the phase I trial.

References

[1] M.E. Wall, M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail, G.H. Sim, J. Am. Chem. Soc. 88 (1966) 3888.
[2] Y.H. Hsiang, R.P. Hertzberg, S.M. Hecht, L.F. Liu, J. Biol. Chem. 260 (1983) 14873.
[3] R.P. Hertzberg, M.J. Caranfa, S.M. Hecht, Biochemistry 28 (1989) 4629.
[4] Y.H. Hsiang, L.F. Liu, Cancer Res. 48 (1988) 1722.
[5] Y.H. Hsiang, L.F. Liu, M.E. Wall, M.C. Wani, S. Kirshenbaum, R. Silber, M. Potmesil, Cancer Res. 49 (1989) 4385.
[6] G.D. Del Bino, P. Lassota, Z. Darynkiewicz, Exp. Cell Res. 193 (1991) 27.
[7] E. Gupta, P. Sinko, A. Saleem, X.-G. Li, R. Zamek, S. Goodin, D. Toppmeyer, E. Nathelson, B. Giovanella, E. Rubin, Proc. Am. Assoc. Cancer Res. 38 (1997) 75.
[8] D. Toppmeyer, E. Gupta, A. Saleem, R. Zamek, J. Much, N. Ibrahim, M. Bindi, P. Haluska, X.-G. Li, S. Goodin, J. Aisner, E. Natelson, B. Giovanella, E. Rubin, Proc. Am. Soc. Clin. Oncol. 16 (1997) 203a.
[9] M.C. Wani, P.E. Ronman, J.T. Lindley, M.E. Wall, J. Med. Chem. 23 (1980) 554.
[10] R.P. Hertzberg, M.J. Caranfa, K.G. Holden, D.R. Jakas, G. Gallagher, M.R. Mattern, S.M. Mong, J.O. Bartus, R.K. Johnson, W.D. Kingsbury, J. Med. Chem. 32 (1989) 715.
[11] J. Fassberg, V.J. Stella, J. Pharm. Sci. 81 (1992) 876.
[12] C.H. Takimoto, R.W. Klecker, W.L. Dahut, L.K. Yee, J.M. Strong, C.J. Allegra, J.L. Grem, J. Chromatogr. B 655 (1994) 97.
[13] T.G. Burke, C.B. Munshi, Z. Mi, Y. Jiang, J. Pharm. Sci. 84 (1995) 518.
[14] C. Jaxel, K.W. Kohn, M.C. Wani, M.E. Wall, Y. Pommier, Cancer Res. 49 (1989) 5077.

[15] D.O. Scott, D.S. Bindra, V.J. Stella, Pharm. Res. 10 (1993) 1451.
[16] D.O. Scott, D.S. Bindra, S.C. Sutton, V.J. Stella, Drug Metab. Dispos. 22 (1994) 438.
[17] J.-P. Loh, A.E. Ahmed, J. Chromatogr. 530 (1990) 367.
[18] J.G. Supko, L. Malspeis, J. Liq. Chromatogr. 14 (1991) 1779.
[19] M. Gibaldi, Biopharmaceutics and Clinical Pharmacokinetics, fourth ed., Lea and Febiger, PA, 1991.