Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVIII, No. 8

Stabilities of ^{3}H- and ^{2}H-labelled Camptothecins.

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SUMMARY

Commercially available [^{3}H]-camptothecin, labeled mainly at the C-5 position, had partially lost the tritium label after recovery from the plasma of a patient injected with this drug or after incubation in plasma at 38°C for 72 h. Camptothecin dissolved in $CH_3O^2H/^2H_2O$, incorporated deuterium at the C-5 position with a 50% uptake after one day at pH 11.0 or after 10-11 days at pH 7.4. At pH 2.0, the deuterium uptake was negligible. Camptothecin, dissolved in deuterated sulfuric acid, incorporated 37 or 80% deuterium at C-14 when heated to 65 or 80°C, respectively. Commercially available [^{3}H]-camptothecin, labeled mainly at the C-5 position, is thus not useful for *in vivo* metabolism or pharmacokinetic studies due to rapid loss of tritium in plasma. In contrast, [^{3}H]-camptothecin prepared as described in this paper for [^{2}H]-camptothecin is expected to be useful.

Key Words: camptothecin, tritiated camptothecin, deuterium-labeled camptothecin

INTRODUCTION

The alkaloid 20(S)-camptothecin (CPT), isolated from the Chinese tree *Camptotheca acuminata,* is currently under investigation as a cancer

CCC 0362–4803/96/080733–10

Received 19 December 1995
Revised 14 March 1996

chemotherapeutic agent (1). It has shown antitumor activity *in vitro* and *in vivo*, presumably, because it inhibits topoisomerase I, a key enzyme in the replication of DNA (2). For the evaluation of its pharmacokinetic characteristics, radiolabeled CPT is required. Ronman et al. described the synthesis of [12-^{3}H]-CPT by reduction of 12-bromocamptothecin with tritium gas in the presence of palladium on carbon (3). In the same paper, a simpler labeling procedure had also been reported, which involved ^{1}H/^{2}H exchange at the C-5 and, to some extent, at the C-7 positions by deuterium gas in the presence of palladium on carbon. CPT labeled at the C-5 position has been considered useful, because deuterium or tritium was introduced by an exchange reaction rather than by a specific synthesis. Moreover, this label was considered stable under *in vivo* conditions due to its synthesis under strongly alkaline conditions.

In our investigation, we have examined the stability of ^{3}H- and ^{2}H-labeled CPT *in vivo* and *in vitro* prior to pharmacokinetic studies with this chemotherapeutic agent. We have also carried out exchange reactions *in vitro* to optimize the reaction conditions for the preparation of a stably labeled drug.

RESULTS and DISCUSSION

Stability of ^{3}H-labeled CPT *in vivo* and *in vitro*. Differing CPT concentrations were recorded in plasma of a patient dosed with commercially available ^{3}H-labeled CPT when different modes of detection were used for the HPLC analysis (Fig. 1). Plasma CPT levels were comparable with either detection system up to 7 h post injection. However, 13 h after dosing of the patient or later, drug levels in identical plasma samples determined by fluorescence detection remained at a plateau value of approximately 63 ng/mL, whereas they declined with a half-life of 16.3 h when analyzed by a radiodetector placed in series. The decrease in radioactivity content of CPT was not due to a decrease in concentration as evidenced by the concomitant fluorescence intensities, but must have been due to a loss of ^{3}H-label from the drug.

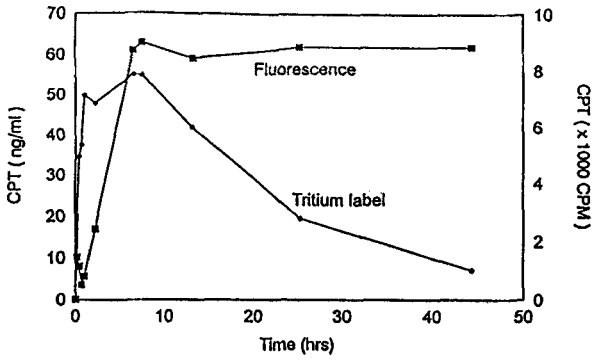


Figure 1: CPT concentrations in plasma of a patient treated with a single oral dose of 15.4 mg/m^2 CPT containing 2 µCi [^{3}H]-CPT. Drug concentrations of each sample were determined by HPLC using both fluorescence detector (▪) and radiodetector (•) placed in series.

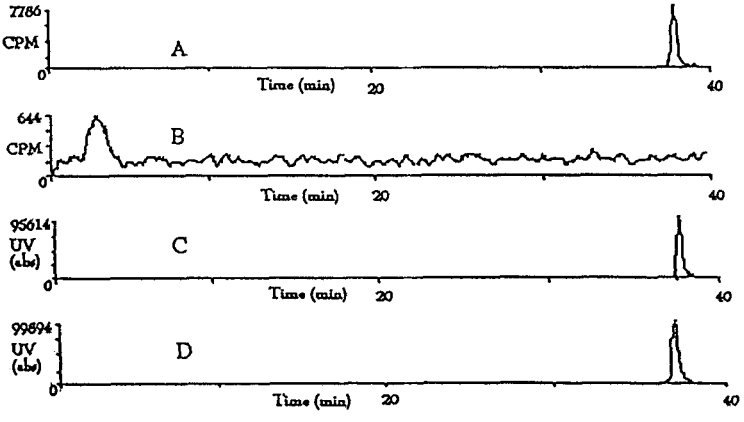
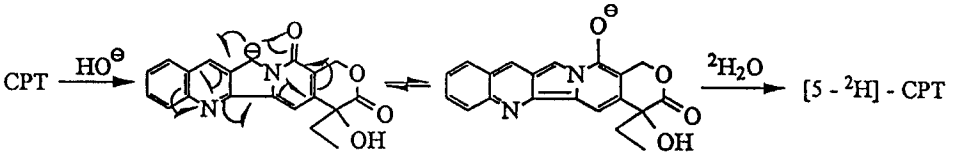


Figure 2: Loss of tritium label from CPT incubated with human blood for 4 or 72 h (traces A and C or B and D, respectively). Plasma was prepared and analyzed by HPLC using both fluorescence detector (traces C and D) and radiodetector placed in series (traces A and B). Retention time of CPT: 35 min.

This suspected loss of ^{3}H-label was also investigated *in vitro* by incubating this [^{3}H]-CPT preparation with plasma at 38°C for 4 or 72 h. After 4 h of incubation, CPT was detectable by both fluorescence and radiodetection (Fig. 2, traces C and A, respectively). In contrast, after 72 h of incubation, CPT was detected only by fluorescence detection (Fig. 2, trace D), whereas the radioactivity of this sample eluted in the solvent front (Fig. 2, trace B). These data clearly demonstrate a loss of

^{3}H-label from the commercially obtained ^{3}H-labeled CPT. They also demonstrate that this uniformly ^{3}H-labeled CPT carries radioactivity mainly at the labile C-5 position.

Synthesis of [5-^{2}H]-CPT. The facile elimination of tritium from [^{3}H]-CPT *in vivo* or in plasma *in vitro* prompted us to examine by NMR the uptake of deuterium from $CH_3O^2H/^2H_2O$ solutions of unlabeled CPT into its C-5 position. At room temperature, hydrogen at C-5 was eliminated with a half-life of 1 day at pH 11.0 and 10-11 days at pH 7.4, whereas under acidic conditions there was little $^1H/^2H$ exchange (Fig. 3). A mechanism of deuterium uptake at position C-5 is proposed as shown below.



The driving force for the facile removal of a proton from C-5 of CPT may be the enlarging of the resonance system to encompass four of the five fused rings of CPT as shown above. In this process, the five-membered ring is converted into a much more stable heteroaromatic imidazol system.

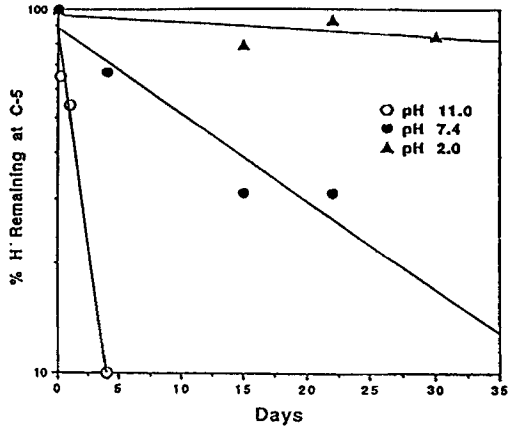
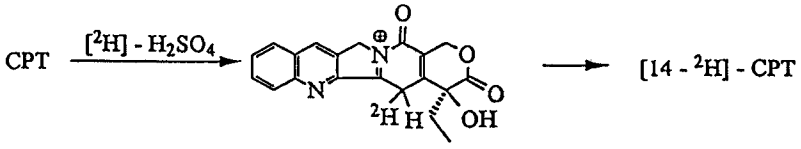


Figure 3: $^1H/^2H$ exchange at C-5 of CPT. CPT was dissolved in mixtures of $CH_3O^2H/^2H_2O$ at neutral pH (•), in this solvent system made alkaline (pH 11.0) by addition of potassium carbonate (O), or made acidic (pH 2.0) by addition of ^{2}HCl (▲). These solutions were kept at room temperature for the indicated periods of time. CPT was isolated from these mixtures and analyzed by NMR for deuterium uptake. Data are expressed as %^{1}H remaining at C-5 of CPT as a function of time.

Synthesis of [14-^{2}H]-CPT. The negligible $^1H/^2H$ exchange at C-5 under acidic conditions led us to investigate exchange reactions under acidic conditions with the goal of preparing a stably labeled CPT without concomitant label incorporation at the labile C-5 position. CPT, dissolved in concentrated [^{2}H]-sulfuric acid and heated to 65°C or 92°C, incorporated 37% or 80% deuterium, respectively, at the C-14 position as determined by NMR analysis (Table 1). An analysis of deuterium uptake by mass spectrometry of the product formed at 65°C revealed 24% ^{2}H incorporation at C-14. At temperatures >100°C, the CPT was charred and destroyed. A mechanism of deuterium uptake at C-14 is proposed as shown below.



The rapid decrease in yield with decreasing reaction temperatures demonstrates the stability of this CPT labeled with deuterium at C-14.

Table 1: Synthesis of [14-^{2}H]-CPT by deuteration of CPT with 98% deuterated sulfuric acid at indicated temperatures for three days. The deuterium uptake at C-14 of CPT was determined by NMR as described below.

Experiment #	Reaction Temperature (°C)	% ^{2}H at C-14	Remarks
I	50	<5	
II	130	N.D.	decomposition
III	65	37	
IV	92	80	

CONCLUSION

Our experiments identify a very labile (C-5) and a very stable position (C-14) of the CPT ring system. The facile $^{1}H/^{2}H$ exchange reaction at C-5 under neutral or alkaline conditions demonstrates that CPT labeled at this position is poorly suited for pharmacological studies. The discrepancy between human plasma concentrations of "uniformly" ^{3}H-labeled CPT determined by radioactivity *vs.* fluorescence detection likely has been caused by $^{3}H/^{1}H$ exchange at this position under *in vivo* or *in vitro* conditions. Thus the "uniformly" labeled [^{3}H]-CPT, available commercially, mainly contains ^{3}H-label at the C-5 position and may thus not be useful for accurate determinations of CPT concentrations unless labile tritium is removed by an initial back-exchange reaction using reaction conditions outlined in this report. In contrast, the $^{2}H/^{1}H$ exchange at C-14 of CPT under strongly acidic conditions and at elevated temperatures provides a pathway for the facile formation of stably labeled CPT.

EXPERIMENTAL

Chemicals. CPT was purchased from Good Land Enterprises, Ltd. (3213 Coleridge Avenue, Vancouver, British Columbia, Canada) or purchased from Sigma Biochemicals, lot number 80H0075 (St. Louis, MO) and purified prior to use to 99% as described elsewhere (4). Deuterium oxide, 99.9 atom %, deuterium chloride, 37 wt % solution in $^{2}H_2O$ (99.5 atom % ^{2}H), and [^{2}H]-sulfuric acid (98 wt %) solution in $^{2}H_2O$ were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetic acid, acetonitrile, acetone, chloroform and methanol were ACS quality reagents and obtained from Aldrich Chemical Co. (Milwaukee, WI). Perchloric acid was from Fisher Scientific (Pittsburgh, PA). HPLC buffers were filtered through a 0.45 μm nylon filter from Schleicher and Schuell (Keene, NH) before use. The scintillation fluid Ready Gel and Ready Organic were from Beckman Instruments (Fullerton, CA). Uniformly tritiated CPT with a specific activity of 160 mCi/mmole or 20 Ci/mmole were

purchased from Moravek Biochemicals Inc. (Brea, CA) or from American Radiochemicals (St. Louis, MO), respectively. Their respective purities were greater than 95% and 98%, as assessed by HPLC in our laboratory.

Instrumentation. The HPLC system consisted of a Beckman 421 gradient controller with two 110A pumps and a 200 µl injection loop. The UV and the fluorescence detectors were from Shimadzu (Kyoto, Japan) models SPD-110AV and a RF 551, respectively, and the radioisotope detector was from Radiomatic Instruments (Meridian, CT). The HPLC detectors were connected in series and set to monitor the UV absorbance at 220 nm. The fluorescence detector excitation was set to 347 nm and the emission was monitored at 418 nm. Samples were analyzed by reverse phase HPLC using isocratic conditions with 30% aqueous acetonitrile containing 0.1% aqueous acetic acid or 40% acetonitrile with 0.1% acetic acid at room temperature with a flow rate of 1 mL/min. The integrating software used was from EZChrome (Shimadzu) and FLO-ONE\Beta (Radiomatic Instruments). A dual channel model BD112 flatbed recorder was from Kipp and Zonen (Bohemia, NY). The samples were analyzed on a C_8 Microsorb MV HPLC column (Rainin Instruments; Woburn, MA).

Spectroscopic Analysis. Spectra were obtained using a JEOL GX270WB Fourier transform NMR spectrometer operating at 270MHz for ^1H, with an internal deuterium lock supplied by [$^2H_6$]-DMSO as solvent. Signals of 512 acquisitions were averaged with an interpulse delay of 20 seconds to give an excellent signal to noise ratio and to allow for complete T1 relaxation for accurate peak area integration. Amounts of ^2H incorporation were determined using C-18 methyl (at 0.877 ppm) as an internal integration standard. Chemical shift assignments were as reported previously (5).

Mass Spectra were recorded by direct inlet probe using a Finnigan Inco 50 gas chromatograph-mass spectrometer and electron impact ionization. The CPT standard contained the following characteristic ions [m/z (% rel. intensity)]: 348 (89) M^+; 319 (37) M-C_2H_5; 304 (38) M-CO_2; 291 (34) M-C_2H_5-CO; 289 (45); 275 (41)

M-C_2H_5-CO_2; 248 (79); 247 (74) M-C_2H_5-CO_2-CO; 219 (73) M-C_2H_5-CO_2-CO-CO. The deuterium-enriched sample was recorded using identical instrument conditions. The deuterium content was determined by subtracting ion intensities of the standard in the molecular ion region (m/z 348, 349, 350) from those of the enriched sample and expressing the difference as percent of the total ion intensity for that region.

Blood Plasma. The blood of a patient was collected into tubes containing sodium heparin from Becton Dickinson Vacutainer Systems (Rutherford, NJ), mixed and the plasma was separated by centrifugation. Plasma samples were stored at -80°C.

HPLC Analysis. Plasma (200 µL) was added to 20 µL 1.5 M perchloric acid. The mixture was vortexed for one min and allowed to equilibrate at room temperature for 10 min. Then, 1000 µL of acetone were added, the mixture was vortexed for 10 sec and incubated at room temperature for 5 min. Hereafter, the solution was centrifuged at 13,000xg for 2 min. The clarified supernatant was removed and dried under a flow of nitrogen. The dry material was dissolved in 1000 µL of a mixture consisting of 300 µL of methanol, 1000 µL of water and 100 µL 1.5 M perchloric acid. This solution was kept at room temperature for five min, centrifuged at 13,000xg for two min and then analyzed by HPLC.

Stability of [^{3}H]-CPT in Plasma. Plasma (5 mL) was incubated at 38°C with 10 µL of a solution containing 1.1 µCi of tritiated CPT and 5.0 µg of unlabeled CPT in a mixture of ethanol and cottonseed oil (1:3). After 4 and 72 h of incubation, the mixture was centrifuged, and the plasma was collected and analyzed by HPLC as described above.

The peak areas for CPT were linear in the range of 1 to 256 ng/mL. (correlation coefficient = 0.98; R^2 = 0.975).

pH Dependence of ^{1}H/^{2}H exchange at C-5 of CPT. Solutions of approximately 0.25 mg CPT in 0.9 mL CH_3O^2H each were combined with two drops of either

a) ^2H_2O, pH 7.4; b) ^2H_2O, pH 2.0, acidified by addition of ^2HCl in ^2H_2O; or c) ^2H_2O, pH 11.0, made alkaline by addition of anhydrous potassium carbonate. These mixtures were allowed to stand in the dark at room temperature for the indicated number of days. The reactants were evaporated in a stream of nitrogen gas. These residues were then lyophilized for 1 h, dissolved in deuterated dimethylsulfoxide and analyzed by NMR.

$^1H/^2H$ exchange at C-14 of CPT. Solutions of 1.0 - 1.8 mg CPT in 0.4 mL of 98% $^2H_2SO_4$, covered with a nitrogen blanket and protected from light, were heated to the indicated temperatures. After three days, the solutions were cooled to room temperature, poured onto ice, and extracted three times with chloroform. The chloroform extracts were combined, dried with anhydrous sodium sulfate and concentrated in a stream of nitrogen gas. The residues were then lyophilized for 1 h, dissolved in deuterated dimethylsulfoxide and analyzed by NMR.

ACKNOWLEDGEMENTS

This work was supported in part by funds from the Stehlin Foundation for Cancer Research and the Friends of the Stehlin Foundation. The authors gratefully acknowledge the Sealy-Smith Foundation for funds for the purchase of an NMR spectrometer, the preparation of the schemes by Dr. Zhisong Cao, Stehlin Foundation for Cancer Research, and the recording of mass spectra by Dr. Shen Nan Lin, Analytical Chemistry Center, University of Texas Medical School, Houston, TX.

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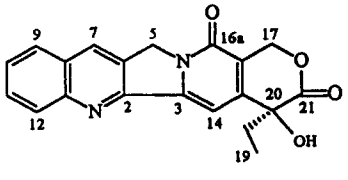

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