IDENTIFICATION OF THE METABOLITES OF 9-NITRO-20(S)-CAMPTOTHECIN IN RATS

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ABSTRACT:

9-Nitro-20(S)-camptothecin is a novel anticancer drug. In this study, metabolites of 9-nitro-20(S)-camptothecin in rats were identified. Rats were dosed with the drug, and the metabolites in the bile were isolated and collected by high-performance liquid chromatography using a gradient elution. By LC/MS\(^n\) (n = 1–3), the biliary metabolites in addition to the unchanged drug were identified as 9-amino-20(S)-camptothecin (M3), 9-acetamido-20(S)-camptothecin (M4), and the glucuronide of 9-hydroxy-20(S)-camptothecin (M1) with the aid of the reference substances and the enzymatic hydrolysis. The accurate mass data for metabolites M2 and M5 were determined by a quadrupole time-of-flight mass spectrometer. Metabolite M5 was suggested to be the glutathione conjugate of 10-hydroxy-20(S)-camptothecin, and M2 may arise from the loss of glutamic acid from M5. Metabolites M1 and M3 were also found in the urine, and M4 in the feces. Fecal metabolite M7 was confirmed to be 9-hydroxy-20(S)-camptothecin (the aglycon of M1) by being compared with the reference standard. The mercapturic acid conjugate of 10-hydroxy-20(S)-camptothecin (M6) was detected in the urine and feces by LC/MS\(^n\).

20(S)-Camptothecin (CPT\(^1\)), a natural alkaloid extracted from the leaves and fruit of *Camptotheca acuminata*, is an inhibitor of DNA synthesis. It results in single-strand DNA break and finally in cell death, by reversibly stabilizing the cleavable complex between topoisomerase I and DNA (Hsiang et al., 1989; Schneider et al., 1990).

In past years, 9-nitro-20(S)-camptothecin (9NC), an analog of CPT, has been a focus of attention in cancer research and has been in early clinical trials (Verschraegen et al., 1998, 1999). Pharmacological studies have shown that the antitumor activity of 9NC is superior to the activity of CPT in human tumors xenografted in nude mice (Giovanella et al., 1991). 9NC is partially metabolized into 9-amino-20(S)-camptothecin (9AC) in vitro and in vivo (Peterslund and Boesen, 1994; Schoemaker et al., 2002). Pharmacokinetics of 9NC in dogs, mice, and humans have been reported, and the results of the studies on the conversion of 9NC to 9AC in different species were described (Hinz et al., 1994). In the present study, more metabolites of 9NC in rat bile, urine, and feces were identified after i.v. administration.

Materials and Methods

Chemicals and Drugs. 9-Nitro-20(S)-camptothecin was provided by Dr. Dafang Zhong, Laboratory of Drug Metabolism and Pharmacokinetics, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, P. R. China. E-mail: zhongdf@china.com

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Abbreviations used are: CPT, 20(S)-camptothecin; 9NC, 9-nitro-20(S)-camptothecin; 9AC, 9-amino-20(S)-camptothecin; 9-oh-CPT, 9-hydroxy-20(S)-camptothecin; 9-aa-CPT, 9-acetamido-20(S)-camptothecin; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; LC/MS\(^n\), liquid chromatography–ion trap mass spectrometry (n = number); HPLC, high-performance liquid chromatography; Q-TOF, quadrupole time-of-flight.

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Sample Preparation. The urine or bile samples (0.1 ml) were diluted with 0.2 ml of water and then were applied to preconditioned 1.5-ml Sep-Pak C18 cartridges (J. T. Baker, Phillipsburg, NJ). The columns were washed with water, and the metabolites were eluted with methanol. Fecal specimens (0.3 g) were initially homogenized in 1 ml of water, and the suspension was centrifuged at 3000 g for 10 min after the ultrasonic vibration for 10 min. The supernatant was collected and filtered through precut membranes (0.45 μm). The filter was applied to a preconditioned cartridge in the same fashion as in the preparation of the urine and bile.

HPLC Analyses of the Bile. A reversed-phase HPLC method was used to separate 9NC and its metabolites. The chromatographic system included an HP 1100 system (Hewlett Packard) equipped with a G1314A UV detector, a G1316A column oven (set at 25°C), a G1313A autosampler, a vacuum degasser unit, and a G1311 quaternary pump. Separation of 9NC and its metabolites was carried out using a Hypersil BDS-C18 column (150 mm x 4.6 mm i.d., 5 μm; Elite, Dalian, China) preceded by a Hypersil BDS-C18 precolumn (10 mm x 4.6 mm i.d., 5 μm) at a flow rate of 1.2 ml/min. The UV detector was set at 370 nm. The mobile phase consisted of a gradient mixed from acetonitrile and water containing 2% formic acid (pH 2). The column was equilibrated with 10% acetonitrile at time 0; after injection of the bile sample (50 μl), the acetonitrile content was increased linearly to 30% at 15 min and then decreased within 3 min to 10% to equilibrate the column for 3 min before application of the next sample.

Mass Spectrometry. Liquid chromatography-ion trap mass spectrometry

**TABLE 1**

<table>
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<th></th>
<th>9NC</th>
<th>9AC</th>
<th>9-OH-CPT</th>
<th>9-AA-CPT</th>
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<td>7.81, 1H, m</td>
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<td>9-NH</td>
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<td>9-OH</td>
<td></td>
<td></td>
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<td>2.21, 3H, s</td>
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</table>

-COCH

a The data from 13C NMR and heteronuclear multiple quantum coherence spectroscopy indicated that the signals for 10-H and 12-H were overlapped in 1H NMR spectra.

**FIG. 1.** HPLC chromatograms of the blank bile (A) and the bile sample after 8 mg/kg i.v. dose (B).
LC/MS$^n$ assay technique was used in this study. The analyses of 9NC and its metabolites were performed using an HPLC system fitted with a Shimadzu LC-10AD pump (Shimadzu, Kyoto, Japan), and the system was coupled in line to a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) via an electrospray ionization interface. The instrument was operated in the positive mode. Following the optimization of the setting, the source voltage was maintained at 4.25 kV; the N$_2$ sheath gas flow was set at 0.7 l/min, the auxiliary gas flow at 0.15 l/min, and the capillary temperature at 200°C. The mobile phase consisted of a mixture of acetonitrile/water/formic acid (30:70:2, v/v) and was delivered at a flow rate of 0.5 ml/min. Data were collected and analyzed by Navigator software (version 1.2; Thermo Finnigan).

Fig. 2. Full scan MS$^2$ chromatograms of the synthesized reference substances (I), the urinary metabolites (II), and the fecal metabolites (III) after 8 mg/kg i.v. dose.

Fig. 3. MS$^2$ spectra of [M + H]$^+$ ion (m/z 394) of 9-nitro-20(S)-camptothecin.
Accurate mass data were obtained for M2, M5, and 9-OH-CPT using an ABI QSTAR Pulsar quadrupole time-of-flight (Q-TOF) mass spectrometer (ABI Canada, Toronto, ON, Canada) equipped with a TurboIonSpray interface operated in the positive ion mode. The ion spray voltage was maintained at 4.5 kV; the ion source gas (N2) flow rate was set at 25 units, the curtain gas flow at 33 units, and the declustering potential at 60 eV. Acquisition and analysis of data were performed with the Analyst QS software.

NMR Analysis. 1H NMR spectra of reference compounds were recorded at 300 MHz on a Bruker AMX 300 NMR spectrometer (Bruker, Faellanden, Switzerland). All compounds were dissolved in DMSO. All chemical shifts (δH) are reported in parts per million relative to tetramethylsilane as an internal standard. The essential data are summarized in Table 1.

Enzymatic Hydrolysis. Five hundred microliters of the bile samples was mixed with 500 μl of 0.5 M acetate buffer (pH 4.5), incubated with β-glucuronidase (500 U; Sigma-Aldrich, St. Louis, MO) at 37°C for 24 h. The samples were centrifuged at 3000 g for 10 min, and 50 μl of the supernatant was analyzed by the LC/MSn method as described above.

Results and Discussion

In the HPLC chromatogram of the bile sample, large peaks were detected at 8.5 min (M1), 9.0 min (M2), 9.3 min (M3), 9.7 min (M4), 10.2 min (M5), and 17.7 min (M0) by comparing them with the blank bile chromatogram (Fig. 1). The structures of the fractions collected

Fig. 4. Selected reaction monitoring chromatograms of M1 (m/z 541→m/z 365→m/z 321) and 9-hydroxy-20(S)-camptothecin (m/z 365→m/z 321) in the bile sample (A) and the bile sample after treatment with β-glucuronidase (B).
from HPLC were elucidated by the analysis of their MS\(^n\) spectra, as well as chromatographic and mass spectral comparison to the synthesized reference substances. The metabolites in the urine and feces were also confirmed by LC/MS\(^n\) (Fig. 2).

**Parent Drug.** Single-stage full scan mass spectrum of 9NC gave abundant protonated molecule at \(m/z\) 394. The MS\(^2\) spectrum of the \([M + H]\)^+ provided the characteristic fragment ions at \(m/z\) 350 and 365, suggesting the loss of CO\(_2\) (−44 Da) and the loss of CH\(_2\)NH (−29 Da), respectively (Fig. 3). Metabolite M0 and 9NC displayed the same protonated molecule and MS\(^2\) fragment ions (Table 2). It was thus identified as 9NC, further confirmed by comparison of its retention time on LC/MS with the synthesized reference substance. 9NC was found in the urine, bile, and feces.

**Metabolite M3.** M3 was present in the urine and bile. The mass spectrum of M3 displayed the protonated molecule at \(m/z\) 364, being the same as the \([M + H]\)^+ ion of the reference compound 9AC. Product ions of \(m/z\) 364 appeared at \(m/z\) 320 (−44 Da) and \(m/z\) 335 (−29 Da) in the MS\(^2\) spectrum, which also appeared in the case of 9AC (Table 2, Fig. 2). By the identical MS\(^2\) product ions and the retention time, M3 was identified as 9AC, which was a reduction product of 9NC.

**Metabolite M4.** The mass spectrum of M4 revealed the protonated molecule at \(m/z\) 406. A mass shift of 42 Da compared with 9AC implied acetylation of the molecule, and M4 showed the identical MS\(^2\) product ions at \(m/z\) 362 (−44 Da) and \(m/z\) 377 (−29 Da) and the retention time with the reference substance 9-AA-CPT (Table 2, Fig. 2). Consequently, M4 was identified as 9-AA-CPT, a secondary metabolite of 9NC in the bile and feces.

**Metabolite M1.** M1 was detected in the bile and urine. The mass spectrum of M1 showed the protonated molecule at \(m/z\) 541. By a mass shift of 176 Da (characteristic loss of glucuronic acid), the base peak at \(m/z\) 365 in the MS\(^2\) product-ion spectrum corresponds to the protonated molecule of monohydroxy-CPT. For the identification of the glucuronic acid conjugate, a comparison was made between the LC/MS\(^2\) chromatograms of samples before and after enzymatic hydrolysis with \(-\text{glucuronidase. When the bile sample was incubated with } \beta\text{-glucuronidase, peak M1 disappeared with a concomitant increase in the peak of the monohydroxy-CPT (Fig. 4). The aglycon of}

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**Fig. 5. Proposed metabolic pathways of 9-nitro-20(S) camptothecin in rats.**
M1 had the same retention as the reference substance 9-OH-CPT and produced the identical product ions at \( m/z \) 321 (−44 Da) and \( m/z \) 336 (−29 Da) in MS\(^2\) spectrum with 9-OH-CPT (Table 2, Fig. 2). Based on the data above, M1 was identified as the glucuronide of 9-OH-CPT. As shown in Fig. 5, during the formation of 9-OH-CPT, the aren ring oxide was proposed to be an intermediate product according to the literature about the bioactivation of the arenes (Mabic et al., 1999).

**Metabolite M2 and M5.** M2 and M5 were found in the bile. In mass spectrum of M2, [M + H]\(^+\) ion was observed at \( m/z \) 541. The MS\(^2\) product-ion spectrum of M2 displayed the base peak at \( m/z \) 397 (Table 2). The [M + H]\(^+\) ion of M5 was observed at \( m/z \) 670, which produced a fragment ion \( m/z \) 541 in the MS\(^3\) spectrum. In the MS\(^3\) spectrum, the fragment ion \( m/z \) 541 produced the same product ion as the [M + H]\(^+\) ion of M2 (\( m/z \) 541 → \( m/z \) 397). Based on these data, M5 was identified as a conjugate of M2. The accurate masses of M2, M5, and a monohydroxylation product of CPT, 9-OH-CPT, were determined by a Q-TOF mass spectrometer. Table 3 summarizes the observed and expected masses for M2, M5, and 9-OH-CPT. With respect to M5, a mass shift of 305 Da of the molecular weight of the monohydroxy-CPT implied a glutathione conjugate of the molecule, and M2 may arise from the loss of glutamic acid from M5. As shown in Fig. 5, the aren ring oxide is proposed to be an intermediate product during the biotransformation of 9NC, which may undergo a reaction with glutathione to form the corresponding conjugate, with attack taking place on the more electrophilic carbon atom (C-9) bonded with the nitro group. Therefore, M5 was suggested to be the glutathione conjugate of 10-hydroxy-20(S)-camptothecin, which derived from the replacement of the nitro in the arene oxide by the glutathione.

**Metabolite M6.** M6 was found in the urine and feces. For \( m/z \) 526, one peak was monitored at retention time of about 7.3 min (Fig. 2), and its product ions appeared at \( m/z \) 397 and 438 in the MS\(^2\) spectrum. Based on the molecular weight and the characteristics of the metabolism of the glutathione conjugate, metabolite M6 was suggested to be the mercapturic acid conjugate of 10-hydroxy-20(S)-camptothecin, which was the further metabolite of M5 in the urine.

**Metabolite M7.** For \( m/z \) 365, one peak was found at about 8.1 min (Fig. 2), and its product ions appeared at \( m/z \) 321 (−44 Da) and \( m/z \) 336 (−29 Da) in MS\(^2\) spectrum, which showed a good agreement in mass fragmentation with the reference substance 9-OH-CPT. In the feces, a great deal of 9-OH-CPT was found instead of the glucuronide, a main biliary metabolite, perhaps caused by the hydrolysis of the conjugate in the intestines.

In addition, we investigated the metabolism of 9AC, and the N-acetylation product was the major metabolite of 9AC in the rat bile and urine. An isolated perfused rat liver system had been used to investigate the metabolism and biliary excretion of CPT (Platzer et al., 2000). Structure identification of the biotransformation products by LC/MS analysis revealed monohydroxylation and dihydroxylation of the CPT core.

In summary, seven metabolites of 9NC were found in rats in vivo, including 9-AC (M3), 9-AA-CPT (M4), the glucuronide of 9-OH-CPT (M1), the glutathione conjugate of 10-OH-CPT (M5), a conjugate arising from the loss of a glutamic acid molecular from M5 (M2), the mercapturic acid conjugate of 10-hydroxy-20(S)-camptothecin (M6), and the parent drug (M0).

**References**


**TABLE 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elemental Composition</th>
<th>Observed Mass</th>
<th>Theoretical Mass</th>
<th>Difference</th>
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<tr>
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<td>540.1401</td>
<td>540.1293</td>
<td>19.9952</td>
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<tr>
<td>M5</td>
<td>C₂₉H₂₅N₉O₂S</td>
<td>669.1769</td>
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<td>7.4719</td>
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