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Clinical report

O-glucuronidation, a newly identified metabolic pathway for topotecan and *N*-desmethyl topotecan

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During topotecan analysis of clinical urine samples, an additional peak eluting just after the solvent front was observed. This potential metabolite was isolated by chromatographic methods. Mass spectrometry data along with chromatographic retention data and fluorescence characteristics showed that the isolated fractions contained two compounds, i.e. topotecan-*O*-glucuronide and *N*-desmethyl topotecan-*O*-glucuronide. The concentrations of the metabolites in human urine were relatively low. When topotecan was given as a 30 min infusion at a dosage of 1.5 mg/m^2 daily for five consecutive days every 3 weeks, the maximal metabolite concentrations in a 24 h urine sample were approximately 10% of topotecan-*O*-glucuronide and 3.5% of *N*-desmethyl topotecan-*O*-glucuronide with respect to the concentration of topotecan in the urine. This is the first report demonstrating that glucuronide metabolites of topotecan are present in the urine of treated patients. [© 1998 Lippincott Williams & Wilkins.]

Key words: Hycamtin™, N-desmethyl topotecan-O-glucuronide, topotecan, topotecan-O-glucuronide.

Introduction

Topotecan (Hycamtin™, SK&F 104864, [*S*]-9-dimethylaminomethyl-10-hydroxycamptothecin, NSC 609669, Figure 1), is a semisynthetic derivative of camptothecin. The lactone structure, which is in a pH-dependent equilibrium with the open-ring hydroxycarboxylate form (SK&F 105992; Figure 1),[1-3] is essential to inhibit the intranuclear enzyme topoisomerase I.[4,5] Antitumor activity has been shown in phase I studies,[6-14] and the drug is currently undergoing evaluation in phase II and III clinical trials.[15-18] Thus far, only demethylation to form *N*-desmethyl topotecan has been identified as a metabolic pathway for topotecan.[19] The maximal concentrations of *N*-desmethyl topotecan detected in human plasma and urine were very low, and the clinical relevance of the metabolite may therefore be limited. When topotecan was given as a 30 min infusion at 1.0 mg/m^2, *N*-desmethyl topotecan levels (lactone and carboxylate forms) of about 0.7% of the maximal concentration of topotecan were found in plasma. The average amount of metabolite excreted in the 24 h urine samples was $2.5 \pm 1.0\%$ (n=20) of the delivered dose.[19]

During topotecan high-performance liquid chromatography (HPLC) analysis of urine samples from treated patients an unidentified peak was observed in the chromatograms. This peak was not present in the chromatograms of pre-dose samples, calibration samples, quality controls or samples originating from stability studies and was thus indicated as a potential metabolite. To investigate the physicochemical properties of the metabolite and to perform structural analysis we isolated the compound from human urine. This paper describes the isolation and stuctural elucidation of this potential metabolite.

Materials and methods

Chemicals

Topotecan (hydrochloride salt, SKF 104864-A, lot MM-15906-194, purity 89.2%, Figure 1) and *N*-desmethyl topotecan reference standard (hydrochloride salt, SB 209780-A, lot JW-19178-221A1, purity 86.5%, Figure 1) originated from SmithKline Beecham Pharmaceuticals (King of Prussia, PA).[20] β-Glucuronidase (Type IX-A from *Escherichia coli*) was purchased from Sigma (St

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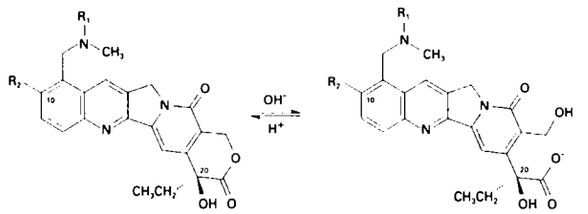


A B

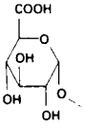
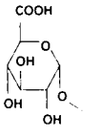
Compound	R_1	R_2
Topotecan	CH_3	OH
N-desmethyl topotecan	H	OH
Topotecan-*O*-glucuronide	CH_3	
N-desmethl topotecan-O-glucuronide	H	

Figure 1. Chemical structures of topotecan and its metabolites. The compounds can undergo a pH-dependent hydrolysis to the ring-opened hydroxycarboxylate form (B) and the proton catalyzed reverse reaction to yield the lactone form (A).

Louis, MO). All other reagents were of analytical grade and double-distilled water was used throughout.

Liquid chromatography (LC) with fluorescence detection

The chromatography system consisted of a type P100 solvent delivery system and a model AS300 automatic sample injection device (Thermo Quest, Fremont, CA). LC System I consisted of a Zorbax SB-C18 column (150 × 4.6 mm i.d., particle size 3.5 μm; Rockland Technologies, Newport, DE) protected with a guard column (3 × 10 mm) packed with reversed-phase material (Chrompack, Middelburg, The Netherlands). The eluent contained a citric acid (10 mM)/phosphate (20 mM) buffer, pH 3.0-methanol (75:25, v/v). The flow rate was 1.0 ml/min and volumes of 25 μl were injected onto the column which was thermostated at approximately 34°C. The same chromatographic conditions were used for LC System II, albeit with another mobile phase comprising 20 mM ammonium acetate, pH 5.0-methanol (80:20, v/v). For LC System III column and eluent from System I were used at a temperature of approximately 40°C and a flow-rate of 1.5 ml/min. In all systems, detection was performed fluorimetrically with a FP920 Intelligent Fluorescence Detector (Jasco International, Tokyo, Japan); the excitation wavelength was 380 nm and the emission wavelength was 527 nm with a 40 nm bandwidth and a digital filter set at 10 s. The capacity of the flow cell of the fluorescence detector was 16 μl. A Data Jet integrator was coupled to a WINner data system (both from Thermo Quest).

Clinical samples

Urine samples were obtained from patients with ovarian cancer who participated in a pharmacokinetic study in our clinic. In this study, topotecan was given daily for 5 days every 3 weeks as a 30 min infusion at 1.5 mg/m^2 and oral at 2.3 mg/m^2. All patients gave informed consent.

Before treatment a sample of each patient's urine was collected and stored at −30°C. This sample was used as a blank. During treatment, 24 h urine collections were done for 2 days. The total volume was recorded and approximately 20 ml of each 24 h sample was stored at −30°C. Before LC analysis the urine samples were diluted 25 times in methanol and acidified (1:1, v/v) with 25 mM phosphoric acid to convert any ring-opened form to the lactone form. LC System I was used to determine the total levels of topotecan in the clinical samples.

Isolation from human urine

A 10 ml urine sample containing 472 ng/ml topotecan was acidified with 10 ml of 5% (v/v) acetic acid. From the diluted sample 100 μl aliquots were injected onto the column. LC System II with an eluent containing ammonium acetate was used for the isolation. In this system the potential metabolite eluted after 4.3 min and topotecan after 35.0 min (capacity factors of 2.0 and 13.3, respectively). Fractions of 1.8 ml were collected from 3.6 to 5.4 min after the start of the injection. The fractions (120 in total) were evaporated to dryness (TurboVap LV Evaporator; Zymark, Hopkinton, MA) under a nitrogen stream at 40°C and used for further analysis.

Reaction with β-glucuronidase

To investigate whether the isolated compound is a substrate for β-glucuronidase, a solution of the enzyme (0.4 mg=7000 units) was prepared in 175 μl of 25 mM phosphate buffer, pH 6.8. The residues of four metabolite fractions were dissolved in separate distilled water volumes of 100 μl. To 175 μl of the pooled sample 25 μl of the β-glucuronidase solution was added. A control sample was prepared by adding 25 μl of 25 mM phosphate buffer pH 6.8 to 175 μl of the pooled sample. Both samples were incubated at 37°C for 2 h. Before assaying the samples, 800 μl of eluent (LC System III) was added. Volumes of 25 μl were injected and analyzed using LC System III. To quantify the amount of isolated compound per fraction, a topotecan/*N*-desmethyl topotecan solution in the eluent, containing 10 ng/ml of both compounds was also injected.

Fluorescence spectra

The residues of four metabolite fractions were dissolved in separate methanol volumes of 150 μl. The fractions were pooled and evaporated to dryness under nitrogen at 40°C. The combined fractions were re-dissolved in 1800 μl of eluent (LC System I). To the latter solution 200 μl of a stock solution of topotecan (100 ng/ml methanol) was added to obtain a mixture of both compounds. From this solution, 25 μl volumes were injected on LC System III with the emission wavelength set to 527 nm and the excitation wavelength rising from 300 to 420 nm in steps of 10 nm. This procedure was repeated with the emission wavelength set to 450 nm and the excitation wavelength was increased from 250 to 400 nm. To record the emission spectra, the excitation wavelength was set to 380 nm and the emission wavelength was increased from 420 to 610 nm. The peak areas of topotecan and the metabolite were recorded.

LC with mass spectrometric (MS) detection

The LC system consisted of a Perkin-Elmer 200 series pump and an ISS 200 autosampler (Perkin-Elmer, Norwalk, CT). A methanol-0.1% acetic acid (20:80, v/v) mixture was used as mobile phase. At a flow-rate of 200 μl/min, the mobile phase was pumped through a Zorbax Rx-C18 column (15 × 2.1 mm i.d., particle size 5 μm; Rockland Technologies). The column outlet was directly connected to the Turbo IonSpray™ sample inlet (Sciex, Thornhill, Canada) without splitting. Ions were created at atmospheric pressure and were transferred into an API 365 triple quadrupole mass spectrometer (Sciex). Major parameters used in this study are listed in Table 1. Q1 scans were acquired with a dwell time of 10 ms at *m/z* 380-620 with a stepsize of 0.5 a.m.u.

The residues of 30 metabolite fractions were dissolved in separate methanol volumes of 100 μl. The fractions were pooled and evaporated to dryness under nitrogen at 40°C. The combined fractions were re-dissolved in 100 μl of methanol-0.1% acetic acid (20:80, v/v). A volume of 5 μl was injected onto the column.

Results

During LC analysis of topotecan in human urine (Figure 2), an additional peak was detected in the chromatograms. This unknown peak was not present in the chromatograms of pre-dose samples, calibration samples, quality controls or samples originating from stability studies. After isolation, a part of the sample was incubated with β-glucuronidase. This resulted in the complete disappearance of the metabolite peak and the appearance of two additional peaks in a ratio of 3:1 (Figure 3). The capacity factors noted for these peaks were identical to topotecan and *N*-desmethyl topotecan. This observation is indicative of the presence of topotecan glucuronide and *N*-desmethyl topotecan glucuronide in the isolated fractions. Under the assay conditions the glucuronide mixture had only approximately 50% of the molar fluorescence yield of topotecan plus *N*-desmethyl topotecan.

The calculated yields of the metabolites after isolation were 3.4 ng (5.7 pmol) of topotecan glucuronide and 1.15 ng (2.0 pmol) of *N*-desmethyl topotecan glucuronide per fraction. The urine sample which

Table 1. MS settings

Parameter	Setting
Electrospray voltage (positive ion mode)	+5500 V
Orifice voltage	+30 V
Ring voltage	+200 V
Quad 0	−2 V
Inter quad 1	−6 V
Stubbies	−7 V
Rod offset 1	−3 V
Inter quad 2	−12.5 V
Nebulizer gas (compressed air) flow rate	1.8 l/min
Curtain gas (N_2 5.0) flow rate	1.8 l/min
Turbo gas (N_2 5.0) flow rate	6 l/min
Temperature	450°C

was used for the isolation contained 472 ng/ml topotecan. Per injection, 23.6 ng (56.0 pmol) of topotecan was brought onto the LC column. So it can be concluded that the original urine sample contained approximately 10% of topotecan glucuronide and 3.5% of *N*-desmethyl topotecan glucuronide with respect to the topotecan concentration.

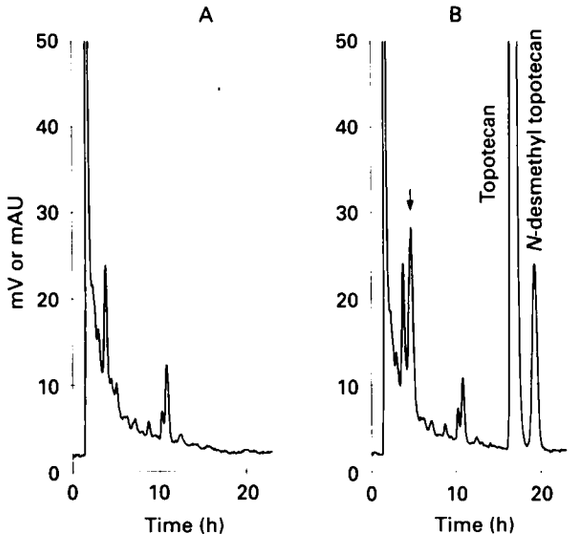


Figure 2. LC chromatograms for the analysis of topotecan in human urine: a blank (A) and a 24 h urine sample (B) with the latter containing topotecan concentrations of 472 ng/ml. Topotecan is eluting after 17.0 min, N-desmethyl topotecan just after topotecan (19.3 min) and the unknown peak is indicated by an arrow (4.8 min). The experimental conditions are described in the text.

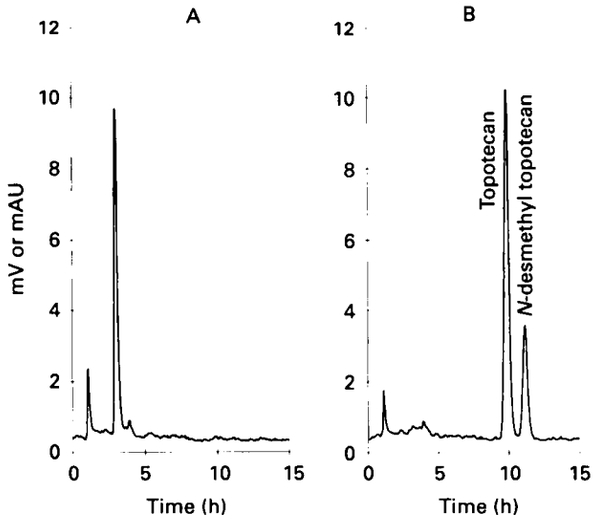


Figure 3. LC chromatograms of the isolated sample before (A) and after (B) incubation with β-glucuronidase: the original peak (3.0 min) disappeared and two additional peaks were distinguished being topotecan and N-desmethyl topotecan. The experimental conditions are described in the text.

The fluorescence spectra of topotecan and the glucuronide mixture are depicted in Figure 4. The optimal excitation wavelengths for topotecan and the metabolite mixture in the eluent were 380 and 370 nm, respectively. There was a significant difference in the emission maxima: the fluorescence intensity for topotecan was maximal at 530 nm, whereas the emission maximum of the metabolite mixture was shifted to a lower wavelength (about 455 nm).

Selected ion chromatograms at *m/z* 598.5 and 584.5, including the recorded LC-MS spectrum, are shown in Table 5. The protonated molecular ions ($M+H^+$) are consistent with the molecular weights of topotecan glucuronide (M_W=597.58 a.m.u.) and *N*-desmethyl topotecan glucuronide (M_W=583.58 a.m.u).

Discussion

To our knowledge no data have been published on the glucuronidation of topotecan, although for SN-38, the pro-drug of irinotecan, it has been demonstrated that glucuronidation is a major metabolic pathway.[21] This may be explained by the hydrophilic character of the glucuronides, resulting in the absence of retention in most reversed-phase HPLC systems.

LC-MS data (Figure 5) of the isolated sample showed the protonated molecular ions ($M+H^+$) of the metabolites at *m/z* 598.5 and 584.5, corresponding with the molecular weights of topotecan-*O*-glucuronide and *N*-desmethyl topotecan-*O*-glucuronide, respectively. The 14 mass units of difference indicate that a CH_2 moiety has been lost from the parent, consistent with the

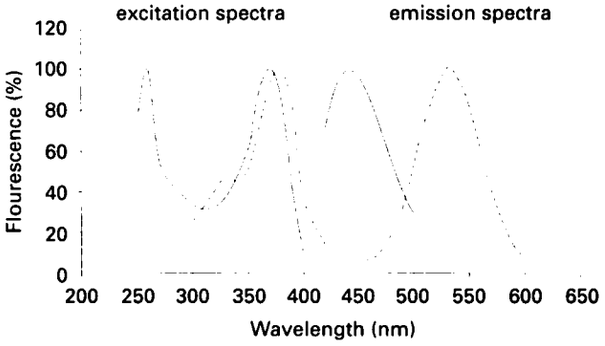


Figure 4. Normalized excitation and emission spectra of topotecan reference (- - -) and the isolated sample (—). The experimental conditions are described in the text.

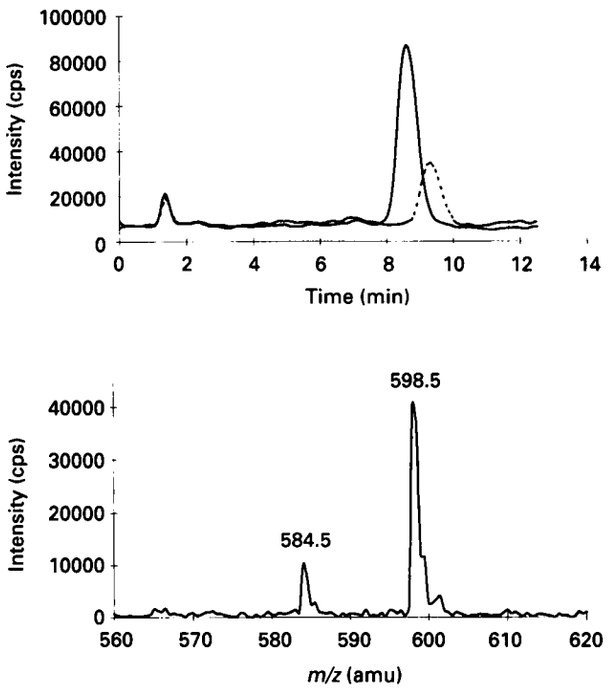


Figure 5. Selected ion chromatograms at m/z 598.5 (—) and 584.5 (- - -), underneath the recorded LC-MS spectrum. Conditions are described in the text.

proposed *N*-demethylation of topotecan.[19]

From the fluorescence spectra it is obvious that the glucuronides have completely different fluorescence properties compared to the parent drug: the emission maximum of the glucuronides was shifted from 530 to 445 nm (Figure 4).

The β-glucuronidase experiment (Figure 3) confirms the presence of *O*-glucuronides in the isolated sample, because the parent compounds are formed after incubation with the enzyme and only *O*-glucuronides are a substrate for β-glucuronidase. There are three hydroxyl groups present in the closed or open forms of the drug at which glucuronidation theoretically may occur. When the pH of the isolated sample was adjusted to 13, a front peak was observed. The original glucuronide peak reappeared when the sample was acidified (data not shown). This observation is indicative of the presence of an intact lactone function in the structure of the metabolites, excluding that acyl-glucuronides of the hydroxy-carboxylate forms were isolated. So either the phenolic (C-10) or alcoholic (C-20) functional group of topotecan is available as a center for conjugation. As the isolated glucuronides have significantly different fluorescence properties compared to topotecan (Figure 4), it is most likely that the $\pi \rightarrow \pi^*$ transition levels of the aromatic system are influenced by the introduction of the glucuronic acid at the adjacent C-10. Besides, the reactivity of a phenolic moiety is much greater than a tertiary alcohol group. SN-38 glucuronidation occurs at the same C-10 phenolic group. Therefore it is assumed that the glucuronic acid conjugation takes place at the phenolic hydroxyl group (C-10, Figure 1).

The maximal concentrations of the glucuronides detected in human urine samples (N=71) were approximately 10% of topotecan glucuronide and 3.5% of *N*-desmethyl topotecan glucuronide with respect to the topotecan concentration. After topotecan administration (1.5 mg/m^2 daily × 5) the amounts of topotecan, *N*-desmethyl topotecan, topotecan-*O*-glucuronide and *N*-desmethyl topotecan-*O*-glucuronide in the urine of this one subject were 18.2, 2.0, 1.9 and 0.7% of the given dose, respectively. To investigate the clinical relevance of these metabolites, an assay for the determination of the glucuronides will be required to quantify the metabolites in urine samples from pharmacology studies. Potential factors may be identified (hepatic and renal functions) influencing the topotecan glucuronide disposition.

Conclusion

In conclusion, we isolated unreported metabolites of topotecan from human urine. Mass spectrometry data along with LC and fluorescence data were consistent with the metabolites being topotecan-*O*-glucuronide and *N*-desmethyl topotecan-*O*-glucuronide. This study demonstates *O*-glucuronidation as being a newly identified metabolic pathway for topotecan and *N*-desmethyl topotecan.

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