Rapid Quantification of Topotecan in Biological Samples by Liquid Chromatography/Tandem Mass Spectrometry

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ABSTRACT – A rapid liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay method was developed for the determination of topotecan levels in rat serum. The assay utilized a single liquid-liquid extraction with a mixture of ethyl acetate and acetonitrile (6:1 v/v) and isocratic elution. The multiple reaction monitoring was based on the transition of m/z 422.0→376.5 for topotecan and 315.1→226.6 for clomipramine (internal standard). The developed assay was validated to demonstrate the specificity, recovery, lower limit of quantification (LLOQ), accuracy and precision. The assay was linear over a concentration range from 0.5-100 ng/mL, with LLOQ being 0.5 ng/mL using a small volume of rat serum (0.1 mL). The mean intra- and inter-day assay accuracy was 87.7-111.0% and 97.8-108.3, respectively, and the mean intra- and inter-day precision was between 1.6-4.3% and 3.8-10.3, respectively. The developed assay was applied to a pharmacokinetic study after a bolus i.v. injection of topotecan in rats.

Key words – Topotecan, LC/MS/MS, Clomipramine, Pharmacokinetics

Topotecan is an analog of camptothecin and is classified as a topoisomerase I inhibitor. Topoisomerase I inhibitors break the single-strand DNA and terminate the DNA replication, leading to cell death.[1-4] Compared with camptothecin, topotecan is more water soluble, less toxic and exhibits higher selectivity in solid tumors.[5] Topotecan shows the antitumor activity in several tumor models.[6,7] It is commercially available under a trade name Hycamtin for the treatment of ovarian and lung cancers. Recent clinical phase I studies have shown that i.p. chemotherapy is also effective for the treatment of metastatic peritoneal cancers.[8,9] Topotecan undergoes pH-dependent, reversible conversion between lactone and carboxylate forms. The lactone form is known to possess higher topoisomerase activity than the carboxylate form.[10] The lactone form may be more easily influenced by the route of administration and the biological pH.[11] The systemically administered topotecan is eliminated mainly by the liver and kidney, with 60-70% excreted in urine as unchanged drug in children.[11] Topotecan is known to be a substrate of the ATP-binding cassette transporter. A number of pharmacokinetic studies have been conducted to relate the topotecan pharmacokinetics with the action of transporters.[12-16] To date, a number of high-performance liquid chromatographic assay methods are available for the quantification of topotecan in biological samples utilizing liquid-liquid extraction[17-19] and protein precipitation.[20] These assays, however, require relatively long chromatographic running times. The present study for the first time reports a rapid and sensitive LC/MS/MS assay method for the quantification of topotecan in rat serum. The developed assay was utilized in a pharmacokinetic study in rats after i.v. injection.

Materials and Methods

Chemicals

Topotecan hydrochloride was purchased from Geneary (Shanghai, China), and clomipramine hydrochloride (internal standard), formic acid and phosphoric acid were purchased from Sigma (St. Louis, MO, USA). Potassium monophosphate and potassium diphosphate were obtained from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile, methanol and ethyl acetate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was de-ionized prior to use using a Millipore Milli-Q water purification system (Milford, MA, USA).

Preparation of Standard and Quality Control Samples

The stock solutions of topotecan and clomipramine (internal standard) were prepared by separately dissolving 10 mg each in 100 mL of methanol. The standard working solutions of topotecan were prepared by serial dilution with methanol,

yielding concentrations of 1000, 500, 100, 50, 10 and 5 ng/mL. The standard calibrator solution was prepared by spiking the standard working solutions (10 μL each) to blank rat serum (90 μL), yielding drug concentrations of 100, 50, 10, 5, 1 and 0.5 ng/mL. The internal standard working solution was prepared by dilution with methanol, yielding a concentration of 250 ng/mL. The quality control (QC) samples were prepared by spiking the working drug solutions to blank rat serum to provide high concentration QC (80 ng/mL), medium concentration QC (20 ng/mL), low concentration QC (8 ng/mL), and lower limit of quantification (LLOQ) QC (0.5 ng/mL). Each QC sample was prepared once, and aliquots (100 μL) were placed in borosilicate glass tubes. QC samples, stock and standard working solutions were stored at -20°C.

Sample Extraction

To rat serum (100 μL), 100 μL of formic acid (0.5%) was added for conversion of carboxylate form to lactone form. One hour later, the internal standard working solution (10 μL, clomipramine 250 ng/mL in methanol) and the mixture of ethyl acetate and acetonitrile (6:1, v/v) (3.5 mL) were added and vortex-mixed for 10 min. The upper organic layer was transferred to a test tube and evaporated on a heating block (Dry Thermobath MG-2100, Tokyo Rikakikai, Tokyo, Japan) at 40°C under N_2. The residue was reconstituted with the mobile phase (300 μL) on the vortex mixer for 10 min and a portion (20 μL) was injected into LC/MS/MS.

Instrumentation and LC/MS/MS Conditions

HPLC was performed by a Shimadzu 10Avp system (Kyoto, Japan) consisting of SCL-10Avp system controller, LC-10ADvp pump, SIL-10Avp auto sampler, CTO-10Avp column oven and DGU-14A degasser. Compounds were separated on a Gemini C_{18} column (150×2.1 mm i.d., 5 μm) with a Security Guard column (4×2 mm i.d.) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile and deionized water containing formic acid (0.1%) at a ratio of 80:20 (v/v). The flow rate of the mobile phase and the column oven temperature were set at 0.2 mL/min and 40°C, respectively. The HPLC system was coupled to an API 2000 triple-quadrupole mass spectrometer equipped with a turbo ion spray ionization source (AB MDS Sciex, Toronto, Canada). The turbo ion spray ionization source was operated in a positive mode. The curtain, nebulizer, and turbo gas (nitrogen) were set at 25, 60, and 60 psi, respectively. The turbo gas temperature was 400°C. The mass spectrometer was operated at a unit resolution for both Q1 and Q3 in the multiple reaction monitoring (MRM) mode with a dwell time of 300 ms in each transition.

The transition of the precursor to the product ion was monitored at 422.0→376.5 for topotecan and 315.1→226.6 for clomipramine. The collision energy was set at 27 and 57 eV for topotecan and clomipramine, respectively. Data acquisition was preformed with the Analyst 1.4 software (AB MSD Sciex, Toronto, Canada).

Calibration

Calibration curves ranging from 0.5-100 ng/mL were prepared by spiking the blank rat serum (100 μL) at concentrations of 0.5, 1, 5, 10, 50 and 100 ng/mL. The calibration curves were constructed by the weighted regression method (1/x) of peak area ratios of topotecan to internal standard vs. actual concentration. The lower limit of quantification (LLOQ) was defined as the lowest serum topotecan concentration that yielded a S/N ratio >10, with acceptable accuracy and precision (<15%). The precision was expressed as the coefficient of variation (%CV) at each concentration, and the accuracy was expressed as the percentage of mean calculated vs. actual concentrations. Intra- and inter-day assay variability of topotecan was determined by assaying LLOQ (0.5 ng/mL), low (8 ng/mL), medium (20 ng/mL) and high QC (80 ng/mL) samples on 3 consecutive days with 3 replicate samples each day, respectively.

Stability

The stability of topotecan was examined under 3 different conditions described below using 3 replicates of low and high QC samples. To assess the topotecan stability in rat serum at room temperature (RT), low and high rat serum QC samples left at RT for 6 hr were analyzed. To assess the auto sampler stability, extracted low and high QC samples of topotecan dissolved in the mobile phase left in the auto sampler rack at RT for 24 h were compared with the immediate work-up concentrations. To assess the freeze-thaw stability, serum samples spiked with topotecan at 8 and 80 ng/mL stored at -20°C were subject to 3 freeze-thaw cycles, and remaining drug concentrations were determined. The results were expressed as the percentage of mean deviation over actual concentrations.

Recovery

The matrix effect, extraction recovery and process efficiency were evaluated for topotecan and the internal standard (80 ng/mL for topotecan and 25 ng/ml for internal standard, n=3 each). The matrix effect was evaluated by comparing the peak areas between the QC and internal standard solutions added to the extracted blank serum and the QC and internal standard solutions prepared in mobile phase. The extraction recovery

was evaluated by comparing the peak areas between the extracted QC samples and the extracted blank serum added with the QC and internal standard solutions. The process efficiency was evaluated by comparing the peak areas between extracted QC samples and standard samples prepared in mobile phase.

Application

Male Sprague-Dawley rats (8–10 weeks old, body weight 257 ± 26 g) (Hyochang Science, Daegu, Korea) were kept in plastic cages with free access to standard rat diet (Samyang, Seoul, Korea) and water. The animals were maintained at a temperature of 22-24°C with a 12 h light-dark cycle and relative humidity of $50\pm10\%$. The rats were anaesthetized by i.p. injection of zoletil 50 (20 mg/kg) (Virbac, Carros, France) and cannulated with polyethylene tubing (0.58 mm i.d., 0.96 mm o.d, Natsume, Tokyo, Japan) in the right jugular and femoral veins. After a 2-day recovery, topotecan dissolved in a mixture of PEG 400:KH_2PO_4 (pH 3) (1:3 v/v) was i.v. injected at a dose of 5 mg/kg into the femoral vein (n = 4). Venous blood samples (0.3 mL each) were collected in eppendorf tubes prior to and 5, 10, 15, 30 min, 1, 2, 4, 6, 12 and 24 h after injection, and the same volumes of saline were replaced. Serum samples were harvested by centrifugation at 1,500 g for 10 min and stored at -20°C until analysis. The serum concentration-time data were analyzed by a non-compartmental method using the nonlinear least-squares regression program WinNonlin (Pharsight, Mountain View, CA, USA).

Results and Discussion

Mass Spectrometry and Chromatography

A rapid and sensitive LC/MS/MS assay was developed for the determination of topotecan levels in rat serum. Serum samples were processed by a simple liquid-liquid extraction, and the assay was validated over the linear concentration range from 0.5-100 ng/mL. The positive Q1 mass spectrum of topotecan and product ion mass spectrum of protonated topotecan (m/z = 422.0) are shown in Fig. 1. In the full scan Q1 mass spectrum, the most abundant $[M+H]^+$ was found at m/z = 422.0, while other adduct peaks including sodium adduct $[M+Na]^+$ were not detected. The most abundant ion in the product ion scan of topotecan was found at m/z = 376.5. Fig. 2 shows the positive Q1 mass spectrum of clomipramine and the product ion mass spectrum of protonated clomipramine (m/z = 315.1). The Q1 mass spectrum was dominated by the protonated molecular ion $[M+H]^+$ at m/z = 314.6 and the most prominent product ion of the protonated clomipramine was
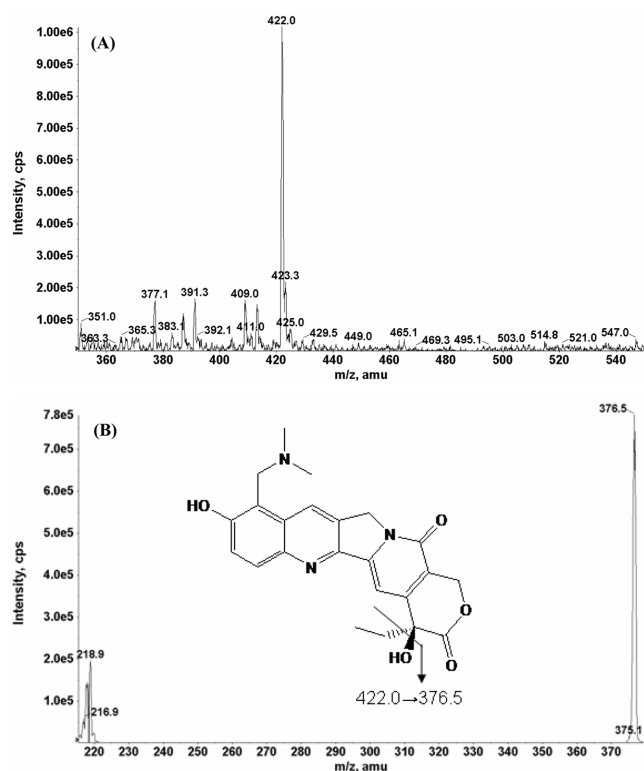
Figure 1−(A) Positive ion Q1 mass spectrum of topotecan and (B) product ion mass of protonated topotecan ($[M + H]^+$, m/z 422.0).
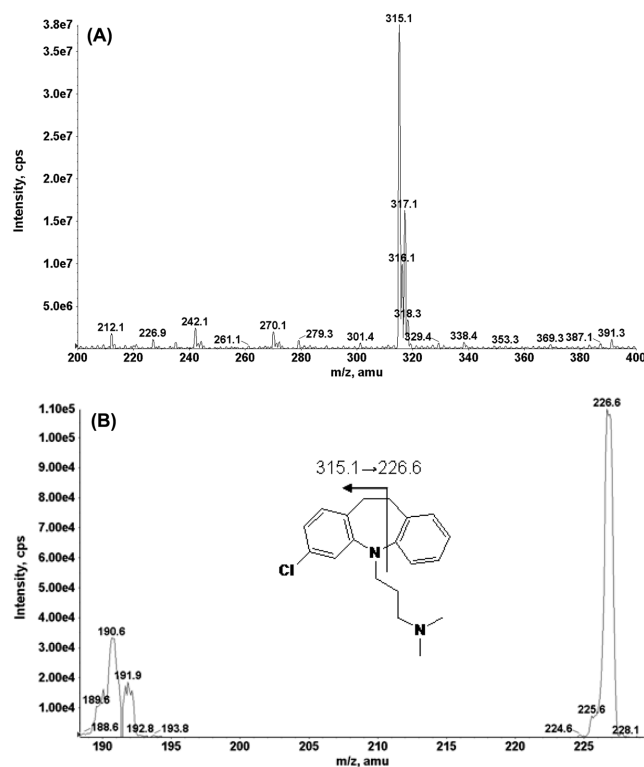
Figure 2−(A) Positive ion Q1 mass spectrum of clomipramine and (B) product ion mass of protonated clomipramine ($[M + H]^+$, m/z 315.1).
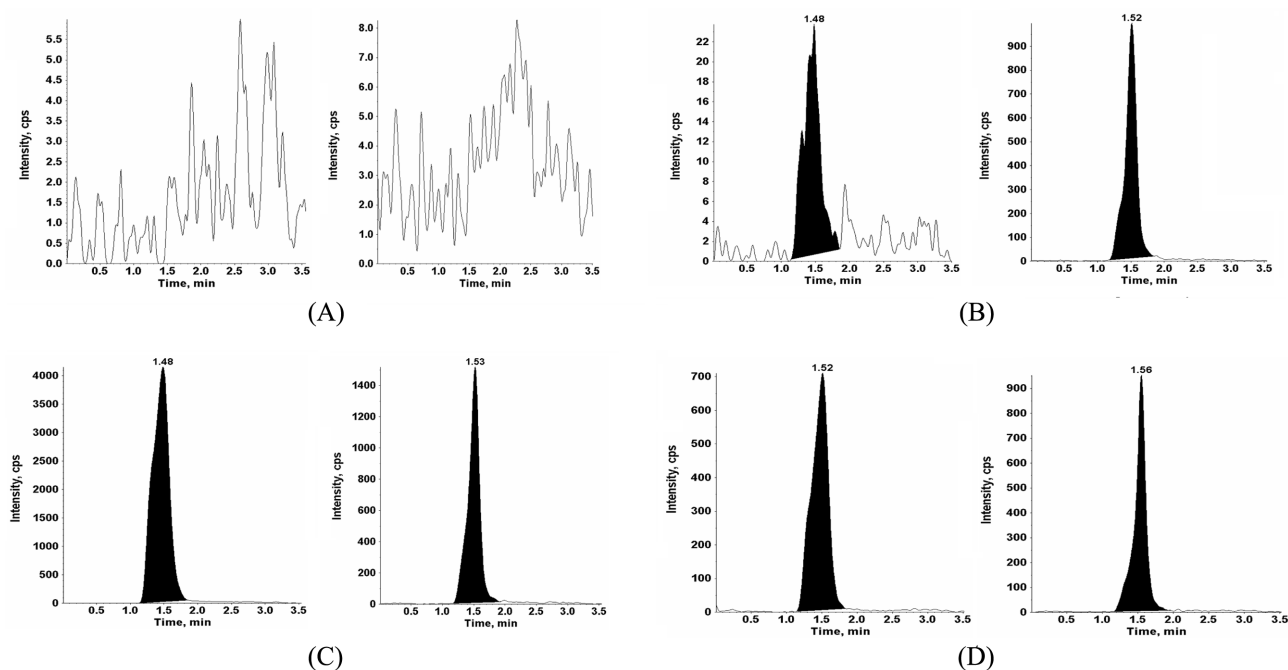
Figure 3–MRM chromatograms of topotecan (left) at 422.0→367.5 and clomipramine (right) at 315.1→226.6 obtained by extraction of (A) blank rat serum, (B) serum spiked with topotecan (0.5 ng/mL) and clomipramine (25 ng/mL), (C) serum spiked with topotecan (100 ng/mL) and clomipramine (25 ng/mL), and (D) serum obtained 2 hr (20.1 ng/mL) after i.v. injection of topotecan to rat (5 mg/kg).

found at m/z = 226.6. The mass transition was monitored at 422.0→376.5 for topotecan and 315.1→226.6 for clomipramine, and the optimum MS/MS parameters were selected for the assay. The MRM chromatograms of topotecan and clomipramine obtained by extraction of rat serum are shown in Fig. 3. Topotecan and clomipramine were eluted at 1.4 and 1.5 min, respectively, and the total assay run time was ≤3 min, allowing for improved sample throughput times over other assay methods reported previously[17-20]. Without adjustment of the mobile phase pH, the shape of topotecan peaks was broad. Under the acidic mobile phase condition (0.1% formic acid), a suitable peak shape was obtained, and no endogenous or extraneous peaks interfered with the analytes.

Method Validation

The assay was linear over the concentration range from 0.5-100 ng/mL, with a typical correlation coefficient ≥0.999. The LLOQ of this assay was 0.5 ng/mL, using 0.1 mL of rat serum. The intra- and inter-day accuracy and precision determined at 0.5, 8, 20 and 80 ng/mL are shown in Table 1. The intra- and inter-day accuracy ranged from 87.7-111.0% and 97.8-108.3%, respectively, with %CV less than 4.3% and 10.3%, respectively. Results of the auto sampler stability (24 h), freeze–thaw stability (3 cycles) and short-term stability in serum (6 h at RT) are shown in Table 2. The absolute mean percentages of devi-

Table I–*Average intra- and inter-day Accuracy (mean±SD) and Precision of Topotecan Assay in rat serum*

Topotecan conc. (ng/mL)	Intra-day (n=3)		Inter-day (n=3)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.5	87.7±3.8	4.3	104.4±10.7	10.3
8	106.3±2.1	2.0	102.8±5.3	5.2
20	111.0±2.6	2.4	108.3±4.2	3.8
80	88.2±1.4	1.6	97.8±5.4	5.6

Table II–*Stability of Topotecan*

Topotecan Conc. (ng/mL)	Deviation of mean from theoretical (%)		
	Auto sampler (24 h)	Freeze-thaw (3 cycles)	Serum at RT (6 h)
8	-7.9	9.4	-9.3
80	4.3	4.8	-4.8

ation of calculated vs. theoretical concentrations were ≤7.9% for auto sampler stability, ≤9.4% for freeze-thaw stability and ≤9.3% for short-term stability in serum. The extraction recoveries determined at high QC concentrations were 68.0±5.7% for topotecan and 58.9±3.4% for the internal standard. The extraction recovery was not high, probably due to the hydrophilic proprieties of topotecan. Nevertheless, the variance was

Table 3–*Recovery, Matrix Effect and Process Efficiency of Topotecan (mean±SD)*

Recovery (%)		Matrix effect (%)		Process efficiency (%)	
Analyte	I.S.	Analyte	I.S.	Analyte	I.S.
68.0±5.7	58.9±3.4	90.6±4.5	79.4±5.8	61.5±4.2	46.7±4.2

relatively low (8.3% for topotecan and 5.8% for internal standard). To optimize the extraction recovery, various solvents including *t*-butyl methyl ether, pentane and ethyl acetate were examined. The extraction recovery was low (<10%) for pentane and *t*-butyl methyl ether. The highest efficiency was obtained for the ethyl acetate and acetonitrile mixture (6:1, v/v). It is possible that co-eluting endogenous components may affect the signal via suppression of the ESI efficiency of analytes. In this assay, the retention times of topotecan and internal standard were less 2 min, resulting in high suppression of ESI due to co-elution of endogenous interference. The matrix effect appears acceptable for topotecan (90.6±4.5%) and the internal standard (79.4±5.8%), with relatively low variance. It is possible that the liquid–liquid extraction used in this assay may have effectively removed co-eluting endogenous components, resulting in insignificant matrix effects for the analytes. The overall process efficiency was 61.5±4.2% for topotecan and 46.7±4.2% for the internal standard.

Application

The developed LC/MS/MS assay was applied to a pharmacokinetic study in rats after i.v. injection of topotecan at a dose of 5 mg/kg. The LLOQ of the assay was sufficient to
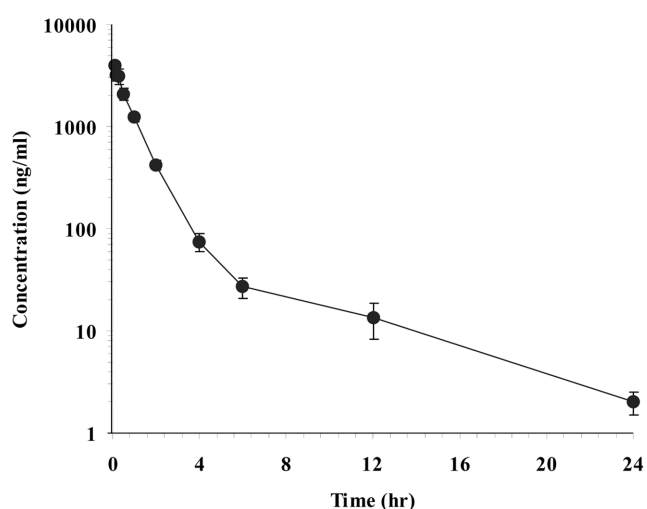
Figure 4–Average serum concentration-time curves (mean±SD) obtained in rats after intravenous injection administration of topotecan (5 mg/kg, n = 4).

Table 4–*Pharmacokinetic Parameters of Topotecan in rats (n = 4) obtained after i.v. injection at a dose of 5 mg/kg*

Parameters	Mean±S.D.
$t_{1/2}$ (hr)	4.6±0.4
AUC_{all} (ng·hr/mL)	4029±224
AUC_{inf} (ng·hr/mL)	4060±250
Cl_s (mL/min)	20.6±1.3
V_{ss} (L/kg)	1.9±0.1

characterize the pharmacokinetics of topotecan. The mean concentration of topotecan vs. time profile is shown in Fig. 4. Pharmacokinetic parameters of topotecan are summarized in Table 4. The terminal elimination half-life ($t_{1/2, \, lz}$), systemic clearance (Cl_s), and steady-state volume of distribution (V_{ss}) were 4.6±0.4 h, 20.6±1.3 mL/min/kg, and 1.9±0.1 L/kg, respectively.

Conclusions

A rapid and sensitive LC/MS/MS assay was developed for the determination of topotecan levels in rat serum. The assay showed a linear dynamic range from 0.5-100 ng/mL, with acceptable intra- and inter-day accuracy and precision. The developed assay was applied to a pharmacokinetic study in rats after i.v. injection of topotecan (5 mg/kg dose).

Acknowledgements

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