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Determination of Mertansine in Rat Plasma Using Liquid Chromatography-Tandem Mass Spectrometry and Pharmacokinetics of Mertansine in Rats

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Abstract : Mertansine, a thiol-containing maytansinoid, is a tubulin inhibitor used as the cytotoxic component of antibody-drug conjugates for the treatment of cancer. Liquid chromatography-tandem mass spectrometry was described for the determination of mertansine in rat plasma. 50-μL rat plasma sample was pretreated with 25 μL of 20 mM tris-(2-carboxyethyl)-phosphine, a reducing reagent, and further vortex-mixing with 50 μL of 50 mM *N*-ethylmaleimide for 3 min resulted in the alkylation of thiol group in mertansine. Alkylation reaction was stopped by addition of 100 μL of sildenafil in acetonitrile (200 ng/mL), and following centrifugation, aliquot of the supernatant was analyzed by the selected reaction monitoring mode. The standard curve was linear over the range of 1–1000 ng/mL in rat plasma with the lower limit of quantification level at 1 ng/mL. The intra- and inter-day accuracies and coefficient variations for mertansine at four quality control concentrations were 96.7–113.1% and 2.6–15.0%, respectively. Using this method, the pharmacokinetics of mertansine were evaluated after intravenous administration of mertansine at doses of 0.2, 0.5, and 1 mg/kg to female Sprague Dawley rats.

Keywords : Mertansine, LC-MS/MS, Pharmacokinetics, Rat plasma

Introduction

Mertansine (Figure 1A, called DM1), a thiol-containing maytansinoid, has anticancer property by binding to tubulin and blocking microtubule assembly[1-3] and used clinically and studied as the cytotoxic component of antibody-drug conjugates (ADCs) to reduce side effects due to severe toxicity and lack of tumor specificity and increase treatment effectiveness.[3-7] Several ADCs containing mertansine have been developed, including bivatuzumab mertansine, cantuzumab mertansine, lorvotuzummab mertansine, and trastuzumab emtansine (T-DM1, Kadcyla®).[3-7]

After an intravenous injection of [^{3}H]-mertansine in the rats, mertansine showed rapid clearance from the blood, extensive distribution to highly perfused organs such as liver, kidney, spleen, lungs, heart, and gastrointestinal tract, extensive metabolism to multiple metabolites, and predominant biliary and fecal excretion.[8,9] Mertansine was metabolized to 11 metabolites by *S*-oxidation, *S*-methylation, hydrolysis, and glutathione conjugation and CYP3A4/5 and CYP2D6 enzymes were involved.[8-10] Mertansine inhibited CYP2C8-mediated paclitaxel 6α-hydroxylation, CYP2D6-mediated dextromethorphan *O*-demethylation, UGT1A1-mediated SN-38 glucuronidation, UGT1A3-mediated chenodeoxycholic acid 24-acyl-β-glucuronidation, and UGT1A4-mediated trifluoperazine *N*-β-D-glucuronidation with K_i values of 11, 14, 13.5, 4.3, and 21.2 μM, respectively, in human liver microsomes; it inhibited midazolam 1′-hydroxylation in recombinant human CYP3A4 supersomes with a K_i of 3.4 μM and a k_{inact} of 0.058 min^{-1}.[10-12] Treatment of mertansine in human hepatocytes for 2 days suppressed the mRNA levels of CYP1A2, CYP2B6, CYP3A4, CYP2C8, CYP2C9, CYP2C19, UGT1A1, and UGT1A9 with IC_{50} values of 93.7, 36.8, 160.6, 32.1, 578.4, 539.5, 856.7, and 54.1 nM, respectively.[12] The maximal plasma concentration of the catabolite mertansine is low (≤ 7.2 ± 2.7 nM) in T-DM1 treated cancer patients.[13-15] However, there was no report on the pharmacokinetics of mertansine after intravenous administration of mertansine itself in the experimental animals and humans.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods have been used to analyze mertansine and its catabolites of T-DM1 in biological fluids[8,13-16] or *in vitro* investigations.[17] Because mertansine

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has a free thiol group, plasma samples were pretreated with a reducing agent, tris-(2-carboxyethyl)-phosphine (TCEP) and an alkylating agent, *N*-ethylmaleimide (NEM) to prevent the reaction of thiol group with proteins.[8,13-16]

We have developed a rapid and reproducible LC-MS/MS method for the quantification of mertansine in rat plasma using alkylation of thiol group and protein precipitation as sample preparation procedures. Using this method, the pharmacokinetics of mertansine were evaluated after intravenous injection of mertansine at 0.2, 0.5, and 1 mg/kg doses to female Sprague Dawley (SD) rats.

Experimental

Materials

Mertansine (96.9% purity) was obtained from BrightGene Biomedical Technology (Jiangsu, China). Dimethyl sulfoxide, TECP, NEM, and formic acid were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Water, dichloromethane, and methanol (LC-MS grade) were supplied by Fisher Scientific Co. (Fair Lawn, NJ, USA). Mertansine-NEM (Figure 1B) was synthetized in-house. All other chemicals used were of the highest quality available.

Preparation of calibration standards, quality control samples, and derivatization reagent

Standard stock solution was prepared separately by dissolving mertansine (1 mg) in 1 mL of dimethyl sulfoxide and was diluted with acetonitrile for the preparation of standard solutions (0.02 to 20 μg/mL). The internal standard working solution (sildenafil, 200 ng/mL) was prepared by diluting an aliquot of the stock solution with acetonitrile. All standard solutions were stored at 4°C in darkness for 4 weeks.

Rat plasma calibration standards for mertansine were prepared at eight concentration levels: 1, 2, 5, 10, 50, 250, 750, and 1000 ng/mL. QC samples for mertansine were prepared at the concentrations of 1, 3, 150, and 800 ng/mL in drug-free rat plasma and stored at –80°C until analyzed.

For the alkylation of mertansine, 50 mM NEM was prepared immediately before use by diluting 1 M NEM in acetonitrile with the mixture of 0.1 M HCl and acetonitrile (2:3, v/v).

Sample preparation

50-μL aliquot of blank rat plasma, calibration standards, and QC samples were vortex-mixed with 25 μL of 2 mM TCEP in water for 3 min; then, 50 μL of 50 mM NEM was added to the mixture of TCEP and plasma and vortex-mixed for 3 min at room temperature. Reactions were stopped by addition of 100 μL of sildenafil in acetonitrile (200 ng/mL), followed by centrifugation at 13000 × g for 8 min. An aliquot (10 μL) of the supernatant was injected onto LC-MS/MS system for analysis.

LC-MS/MS analysis

An ultra-performance liquid chromatograph (Agilent infinity 1290; Agilent Technologies, Wilmington, DE, USA) coupled with a tandem mass spectrometer (Agilent 6495) was used for the LC-MS/MS analysis. Chromatographic separation was performed on a ZORBAX SB-C18 column (1.8 μm; 2.1 mm i.d. × 50 mm, Agilent Technologies, Wilmington, DE, USA) using a gradient elution of 0.1% formic acid in 10 mM ammonium formate [mobile phase (MP) A] and 0.1% formic acid in acetonitrile (MP B) at a flow rate of 0.3 mL/min as follows: 20% MP B for 0.5 min, 20% to 85% MP B for 0.3 min, 85% to 90% MP B for 1.2 min, 90% MP B for 3.0 min, 90% to 20% MP B for 0.1 min, and 20% MP B for 2.4 min. The column and autosampler tray were maintained at 40°C and 4°C, respectively. The electrospray source settings for ionization of the analytes in positive mode were as follows: gas temperature, 200°C; gas flow, 14 L/min; nebulizer, 45 psi; sheath gas temperature, 350°C; sheath gas flow, 12 L/min; and capillary voltage, 4000 V. Nitrogen gas was used as the collision gas at a pressure of 2 bar on the instrument. The collision energies for the fragmentation of mertansine, mertansine-NEM, and sildenafil (IS) were 24, 32 and 45 eV, respectively. The selective reaction monitoring (SRM) transitions for the quantification were as follows: *m/z* 863.2 → 547.1, *m/z* 738.2 → 547.1, and *m/z* 475.1 → 283.0 for mertansine-NEM, mertansine, and sildenafil, respectively. Mass Hunter software (Agilent Technologies) was used for LC-MS/MS system control and data processing.

Method validation

Method validation was performed according to the methods set out in the FDA Guidance on Bioanalytical Method Validation. To evaluate intra- and inter-day precisions and accuracies, we analyzed batches of calibration standards and QC samples (1, 3, 150, and 800 ng/mL) in six replicates on three different days. Accuracy was defined as the RE (%) of the measured mean value deviating from the nominal value, and precision was defined as the CV (%) of the measured concentration. The LLOQ value was defined as the lowest amount of mertansine in a rat plasma sample that could be quantified as follows: signal-to-noise ratio, > 5; CV, < 20%; accuracy, 80-120%.

The stability of mertansine in rat plasma was evaluated by analyzing low and high QC samples in triplicate: post-preparation sample stability in the autosampler at 4 °C for 12 h; short-term storage stability following storage of plasma samples at room temperature for 2 h; and three freeze–thaw cycles.

The recovery of mertansine were determined by comparing the peak areas of the derivatized extract of mertansine-spiked plasma with those of mertansine-NEM spiked post-extraction into six different blank plasma

extracts at 3, 150, and 800 ng/mL levels.

Pharmacokinetic study of mertansine in rats

This validated method was applied to the pharmacokinetic study of mertansine after an intravenous injection of mertansine at 0.2, 0.5, and 1 mg/kg doses to female SD rats (n = 5/dose; body weight, 198-210 g; 8 weeks; Orient Bio, Seongnam, Korea). The study protocol was approved by the Institutional Animal Care and Use Committee at The Catholic University of Korea (Approval No. 2016-033-01). The room was maintained at a temperature of 22-24°C with a 12 h light/dark cycle and relative humidity of 50 ± 10%. Rats were anesthetized by isoflurane and were cannulated with polyethylene tubing (PE-50, Natsume Co., Tokyo, Japan) in the jugular vein for blood sampling and in the femoral vein for intravenous injection. Each rat was housed individually in a rat metabolic cage and allowed to recover from anesthesia for 1 day prior to the start of the study. Rats were not restrained at any time during the study. Heparinized isotonic saline (10 IU/mL) was used to flush the catheters to prevent blood clotting. Mertansine was dissolved in dimethyl sulfoxide:propylene glycol:water (1:7:2, v/v) and administered to the femoral vein of the rats at 0.2, 0.5, and 1 mg/kg doses. Blood sample (approximately 200 μL) was collected from the jugular vein before (control) and at 1, 5, 15, 30 min and at 1, 2, 4, 6, 8, 24, 30, and 48 h after drug administration. Plasma samples were harvested by centrifugation at 13,000 × g for 5 min at 4°C; plasma samples were immediately collected in 1.5-mL amber polypropylene microcentrifuge tubes and stored at –80°C until LC-MS/MS analysis.

Pharmacokinetic parameters, including the area under the plasma concentration–time curve during the period of observation (AUC_{last}), the area under the plasma concentration–time curve to infinite time (AUC_{inf}), the terminal half-life ($t_{1/2}$), clearance (CL), volume of distribution at steady state (V_{ss}), and mean residence time (MRT), were evaluated using noncompartmental analysis (WinNonlin; Pharsight, Mountain View, CA, USA). Each value is expressed as the mean ± standard deviation (SD).

Results and Discussion

LC-MS/MS optimization

The molecular ions ($[M+H]^+$) of mertansine and mertansine-NEM were observed at m/z 738.2 and 863.2, respectively, and SRM transitions of the precursor ion ($[M+H]^+$) to the intense product ion at m/z 547.1 were selected based on MS/MS spectra (Figure 1) for data acquisition due to the high selectivity and sensitivity (Figure 1).

ZORBAX SB-C18 column exhibited excellent peak shape, better separation, and good sensitivity for mertansine and mertansine-NEM using a gradient elution of 0.1% formic acid in acetonitrile and 0.1% formic acid in 10 mM ammonium formate compared to the Luna C18 (3 μm; 2 mm i.d. × 50 mm; Phenomenex, Torrance, CA, USA), Luna phenyl-hexyl (3 μm; 2 mm i.d. × 50 mm; Phenomenex,

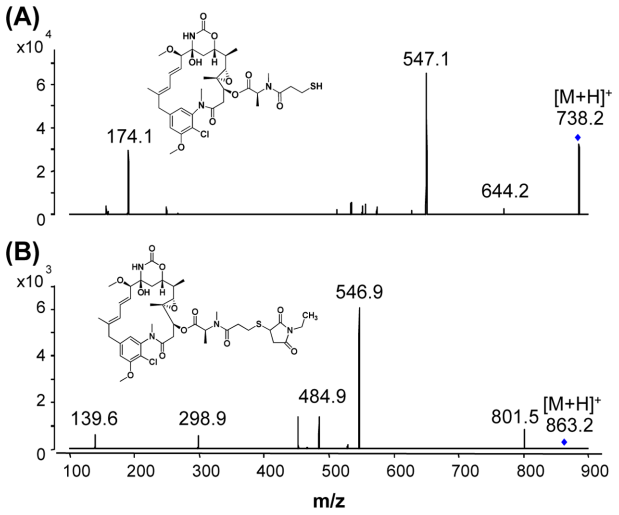

Figure 1. Product ion spectra of (A) mertansine and (B) mertansine-NEM.

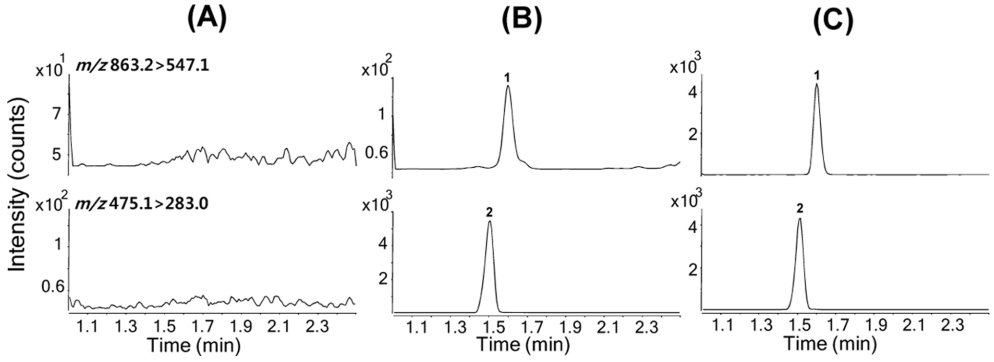

Figure 2. Selected reaction monitoring chromatograms of (A) rat blank plasma; (B) rat plasma spiked with mertansine at the LLOQ level (1 ng/mL); and (C) rat plasma obtained 15 min after intravenous injection of mertansine at a dose of 0.2 mg/kg to a female SD rat. 1, Mertansine-NEM; 2, sildenafil (internal standard).

Torrance, CA, USA), Halo C18 (2.7 μm; 2.1 mm i.d. × 50 mm; Advanced Materials Technology, Wilmington, DE, USA), and Atlantis dC_{18} (1.7 μm; 2.1 mm i.d. × 50 mm; Waters Co., Milford, MA, USA) columns.

Analysis of blank plasma samples obtained from 20 rats revealed no significant interference peaks in the retention times of the analytes, indicating good selectivity of the present method (Figure 2A). Figure 2B presents typical SRM chromatograms of a rat plasma sample spiked with mertansine at 1 ng/mL. Figure 2C presents representative SRM chromatograms of a plasma sample obtained 15 min after intravenous administration of mertansine at a dose of 0.2 mg/kg in a rat.

The concentration and reaction time of TCEP for the reduction of disulfide bond between mertansine and proteins were optimized: 25 μL of 2 mM TCEP was vortex-mixed with 50 μL of rat plasma sample for 3 min in the sample preparation procedures. The concentration of NEM, reaction time, and solution pH for thiol-specfic alkylation of mertansine with NEM were optimized[18,19]: 50 μL of 50 mM NEM in 0.1 M HCl and acetonitrile (2:3, v/v) was vortex-mixed with TCEP-treated plasma for 3 min at room temperature.

Method validation

Calibration curve was linear over the concentration ranges of 1–1000 ng/mL of mertansine in rat plasma with the coefficient of determination of 0.9926 from linear regression analysis with a weighting of 1/concentration^2 (Table 1). The accuracy and CV of the calculated concentrations were 90.0% to 110.0% and 0.8% to 9.8%, respectively, for eight calibration points. The CV value for the regression line slope was 6.6%, indicating good method repeatability.

The intra- and inter-day accuracy and CV values for mertansine in LLOQ, low, medium, and high QC samples ranged from 96.7% to 113.1% and from 2.6% to 15.0%, respectively (Table 2), suggesting that the accuracy and precision of this method are acceptable.

Using pretreatment of plasma sample with TCEP, thiol alkylation with NEM, and protein precipitation with acetonitrile as sample preparation procedures, the recoveries of mertansine at 3, 150, and 800 ng/mL plasma levels were 56.5 ± 6.2%, 52.6 ± 1.1%, and 52.3 ± 2.7%, respectively.

Table 3 presents three freeze–thaw, short-term (2 h) storage at room temperature, and post-preparation stabilities of mertansine; these processes had negligible effects on sample stability.

Pharmacokinetics of mertansine in rats

After intravenous administration of mertansine at 0.2, 0.5, and 1 mg/kg doses to female SD rats, the plasma concentrations declined in a polyexponential mode (Figure 3). Large V_{ss} (20.1-47.2 L/kg) and systemic clearance (*CL*) (15.9-33.6 mL/min/kg) values (Table 4) support that mertansine showed the extensive distribution to highly perfused organs; the extensive metabolism after an

Table 1. Calculated concentrations of mertansine in calibration standards prepared with rat plasma (n = 3).

Variables	Theoretical concentrations of mertansine (ng/mL)								slope	r^2
	1	2	5	10	50	250	750	1000		
Mean (ng/mL)	1.1	1.8	5.1	10.7	50.8	262.3	705.1	964.7	0.00356	0.9926
Accuracy (%)	110.0	90.0	102.0	107.0	101.6	104.9	94.0	96.5	-	-
CV (%)	2.7	3.7	5.6	7.5	9.8	3.3	2.2	0.8	6.6	0.1

Table 2. Accuracy and precision (CV, %) of mertansine in rat plasma QC samples.

Variables	Intra-day (n = 6)				Inter-day (n = 18)			
QC (ng/mL)	1.0	3.0	150	800	1.0	3.0	150	800
Mean (ng/mL)	1.0	3.0	165.7	904.9	1.0	2.9	159.5	831.8
Accuracy (%)	100.0	100.0	110.5	113.1	100.0	96.7	106.3	104.0
CV (%)	15.0	7.1	2.6	4.1	14.9	10.6	7.7	9.2

Table 3. Post-preparation, short-term, and freeze–thaw stabilities of mertansine in rat plasma QC samples (n = 3).

Stability conditions	Concentrations of mertansine (ng/mL)			
	3		800	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Post-preparative (12 h at 4°C)	98.3	2.3	100.5	0.5
Short-term (2 h at room temperature)	92.5	7.1	85.9	0.8
Freeze–thaw (three cycles of –80°C to room temperature)	91.3	7.5	102.1	4.2

Table 4. Pharmacokinetic parameters of mertansine after a single intravenous injection of mertansine at 0.2, 0.5, and 1 mg/kg doses to female SD rats. Data are shown as mean ± SD (n = 5).

Pharmacokinetic parameters	0.2 mg/kg	0.5 mg/kg	1 mg/kg
AUC_{last} (ng·h/mL)	96.1 ± 18.7	206.9 ± 50.4	1044.1 ± 167.9*
AUC_{inf} (ng·h/mL)	129.6 ± 20.4	275.5 ± 44.5	1172.2 ± 156.3*
CL (mL/min/kg)	27.7 ± 5.8	33.6 ± 6.4	13.4 ± 2.6*
V_{ss} (L/kg)	46.2 ± 17.4	47.8 ± 15.2	18.7 ± 5.0*
$t_{1/2}$ (h)	16.7± 13.6	22.8 ± 0.6	18.4 ± 4.3
MRT (h)	11.4 ± 2.2	10.3 ± 1.4	12.3 ± 2.6

* Significantly different from 0.2 and 0.5 mg/kg doses

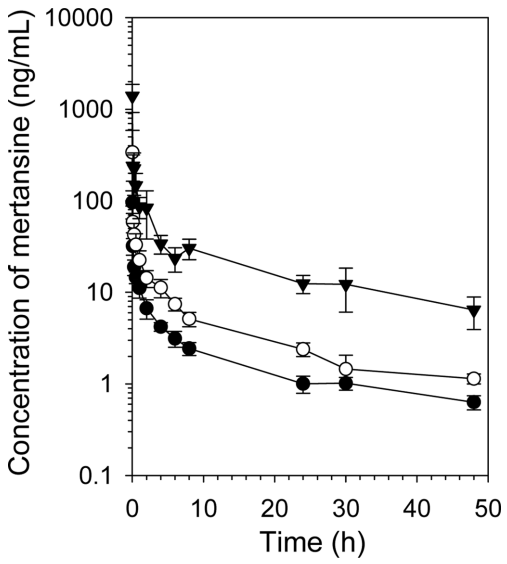

Figure 3. Mean plasma concentration–time profiles of mertansine after a single intravenous injection of mertansine at doses of 0.2 (●), 0.5 (○), or 1 (▼) mg/kg to female SD rats. Points represent mean ± SD (n = 5).

intravenous injection of [^{3}H]-mertansine in the rats.[6] The CL and V_{ss} values were significantly reduced at 1 mg/kg dose compared to 0.2 and 0.5 mg/kg, and AUC_{last} of mertansine at 1 mg/kg showed significant increase (Table 4). The dose normalized (based on 0.2 mg/kg) AUC values were calculated as 96.1 ± 18.7, 82.8 ± 20.2, 208.8 ± 33.9 ng·h/mL for 0.2, 0.5, and 1 mg/kg doses, respectively. MRT and $t_{1/2}$ values of mertansine were comparable among three doses studied (Table 4).

Conclusions

A sensitive and reproducible LC-MS/MS method using thiol alkylation and protein precipitation as a sample clean-up procedure was developed for the determination of mertansine with LLOQ level of 1 ng/mL in 50 μL of rat plasma. We evaluated the plasma concentrations of mertansine using this method and the pharmacokinetic parameters of mertansine after intravenous administration of mertansine at 0.2, 0.5, and 1 mg/kg doses to female SD rats.

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