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A sensitive LC–MS/MS method for the determination of free maytansinoid DM4 concentrations—Method development, validation, and application to the nonclinical studies of antitumor agent DM4 conjugated hu-anti-Cripto MAb B3F6 (B3F6-DM4) in rats and monkeys

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ABSTRACT

A sensitive, specific, and high throughput method was developed and validated for the quantitation of free maytansinoid DM4 in cynomolgus monkey and Sprague-Dawley rat plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The Sciex API 5000 mass spectrometer was operated in multiple-reaction monitoring (MRM) and electrospray positive ionization mode. An MS/MS transition of 780 → 216 was used to monitor the analyte DM4 and 635 → 547 for the internal standard (IS) ansamitocin P-3. A liquid–liquid extraction was utilized for sample pre-treatment with a volume of 100 μL plasma. A Thermo Hypersil Gold PFP column was used for chromatographic separation with a 2.0 min HPLC gradient. The quantitation range of the method was 0.500–100 ng/mL with a lower limit of quantitation of 0.500 ng/mL. The method was validated in monkey and rat plasma according to FDA guidance on Bioanalytical Method Validation (2001). The intra- and inter-day precision and accuracy were found to be within the FDA recommended acceptance criteria. The validated method was employed to monitor the free DM4 levels in plasma in the IND-enabling toxicology studies of antitumor agent DM4 conjugated hu-anti-Cripto MAb B3F6 (B3F6-DM4).

1. Introduction

Human Cripto-1 (CR-1) is a cell membrane protein that is expressed at high levels in certain types of human tumors (breast, colon, ovarian, etc.) but not in normal tissues [1]. CR-1 has been probed as a potential biomarker for disease diagnosis [2], as well as a novel target for molecular target-based cancer therapies [3–6]. DM4 Conjugated hu-anti-Cripto MAb B3F6 (B3F6-DM4), like other antibody-maytansinoid conjugates [7–10], is constructed by covalently linking highly cytotoxic maytansinoid molecules with humanized anti-Cripto antibody by disulfide bonds (Fig. 1). The therapeutic hypothesis is that once this antibody-maytansinoid conjugate enters into blood circulation, it will find and bind to tumor cells expressing the CR-1 antigen. The conjugate will then internalize, release the cytotoxic payload and kill the tumor cells [10,11]. B3F6-DM4 is currently in clinical study for relapsed/refractory solid tumors. Currently there are several other antibody-maytansinoid conjugates that are under clinical development [8,10,12], including BT062 against multiple myeloma (Biotest), trastuzumab-DM1 against breast cancer (Genentech), IMGN901 for CD56+ solid tumors and multiple myeloma, IMGN388 (DM4) against solid tumors (ImmunoGen), and SAR3419 (DM4) against Non-Hodgkin's lymphoma (Sanofi-Aventis).

One of the challenges in the nonclinical and clinical development of antibody-maytansinoid conjugates is to assess the exposure levels of the free maytansinoid components *in vivo*. Animals dosed with a high level of maytansine linked antibody showed typical signs of maytansinoid toxicity which may be caused by the dissociation of the conjugate *in vivo*. Competition ELISA assays are commonly used to determine free maytansinoids in plasma or red blood cells from *in vitro* and *in vivo* studies with a limit of detection around 1 ng/mL [11,13–15]. However ELISA assays are time consuming and require the development of an antimaytansinoid antibody. Also, ELISA assays are indirect assays and may lack specificity depending on the quality of the antimaytansinoid anti-

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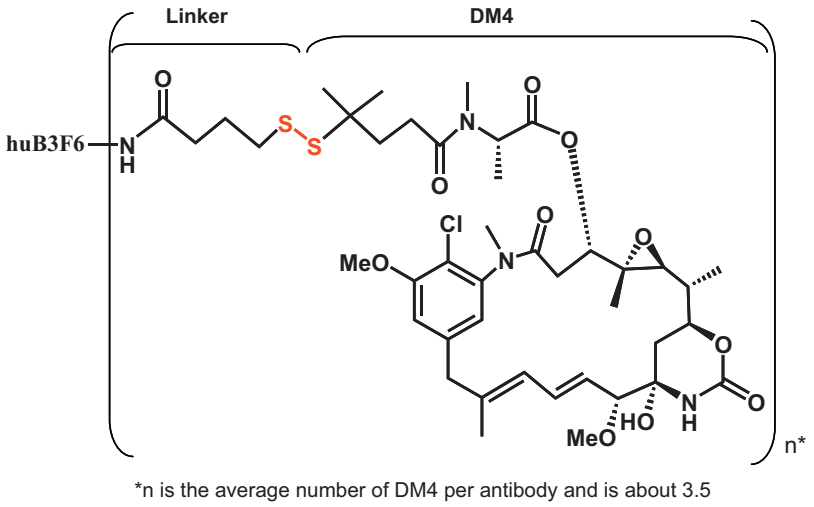

Fig. 1. A diagram of conjugated hu-anti-Cripto MAb B3F6 (B3F6-DM4). The attached DM4 can be released by reduction of the disulfide bond or disulfide exchange.

Table 1
Extraction recovery of DM4 and ansamitocin from monkey (top) and rat (bottom) plasma.

Statistics	DM4 QC (ng/mL, monkey plasma)			Ansamitocin
	0.500 ng/mL	5.00 ng/mL	100 ng/mL	25.0 ng/mL
Extracted peak area (mean)	7545	75,735	1,232,187	1,455,655
Non-extracted peak area (mean)	8524	81,810	1,390,297	1,489,988
n	3	3	3	9
Recovery (%)	88.5	92.6	88.6	97.7
Statistics	DM4 QC (ng/mL, rat plasma)			Ansamitocin
	0.500 ng/mL	5.00 ng/mL	100 ng/mL	25.0 ng/mL
Extracted peak area (mean)	3219	28,524	620,611	508,041
Non-extracted peak area (mean)	3463	38,437	650,386	583,102
n	3	3	3	9
Recovery (%)	93.0	74.2	95.4	87.1

body available. For instance, antimaytansinoid antibody may cross react to both the maytansinoid (e.g., free DM4) and its metabolites (e.g., S-methyl-DM4) [16–18], which results in compromised selectivity of the ELISA assay. Recently an LC–MS/MS method procedure was developed to determine ansamitocin P-3 (an analog of DM4) in rat plasma with a calibration range of 1–500 ng/mL [19]. In comparison to ELISA assays, LC–MS/MS methods are direct methods and offer two dimensional separations for better selectivity of the free maytansinoid from its potential metabolites: first by elution time difference from the LC column, then by the different multiple-reaction monitoring (MRM) transitions with tandem mass spectrometer detector. In this study, our objective was to develop a sensitive and selective LC–MS/MS method for the quantitation of free DM4, a maytansinoid derivative with a thiol group (thiol-DM4), in Cynomolgus monkey and Sprague-Dawley rat plasma. This free DM4 method utilized liquid–liquid extraction for sample processing with a volume of 100 μL plasma. A Thermo Hypersil Gold PFP column was used for chromatographic separation with a 2.0 min HPLC gradient. The quantitation range of the method was 0.500–100 ng/mL with a lower limit of quantitation of 0.500 ng/mL. The method was validated in monkey and rat plasma per FDA guidelines. The validated free DM4 method was used to support GLP nonclinical studies of the antibody-maytansinoid conjugate B3F6-DM4.

Table 2
Matrix effect of monkey (top) and rat (bottom) plasma to DM4 measurement.

Monkey plasma	Lot DM4 (5.00 ng/mL)	% Bias
1	5.11	2.2
2	4.91	−1.8
3	4.46	−10.8
4	4.60	−8.0
5	4.87	−2.6
6	4.85	−3.0
Rat Plasma	Lot DM4 (5.00 ng/mL)	% Bias
1	5.41	8.2
2	5.66	13.2
3	4.74	−5.2
4	4.62	−7.6
5	4.94	−1.2
6	5.05	1.0

Table 3
Calibration curve parameters for DM4 in monkey and rat plasma.

Matrix	Run number	Slope	Intercept	*R*-Squared
Monkey plasma	1	1.15E−02	1.10E−03	0.997
	2	9.52E−03	4.39E−04	0.997
	3	6.31E−03	6.93E−04	0.999
Rat plasma	1	6.63E−03	4.42E−04	0.997
	2	1.21E−02	−5.21E−04	0.996
	3	1.01E−02	−5.84E−04	0.997

Table 4
Quality control sample concentrations of DM4 in monkey (top) and rat (bottom) plasma (between-run and within-run precision and accuracy).

Run code	QC LOQ0.500 ng/mL	QC low1.50 ng/mL	QC medium20.0 ng/mL	QC high75.0 ng/mL	QC dilution500 ng/mL
Monkey					
Mean	0.476	1.54	19.9	72.1	513
%CV	7.2	4.9	5.0	3.3	3.4
n	6	6	6	6	6
Mean %Diff	−4.8	2.7	−0.5	−3.9	2.6
Mean	0.495	1.59	20.7	74.0	
%CV	6.3	2.4	1.6	2.2	
n	6	6	6	6	
Mean %Diff	−1.0	6.0	3.5	−1.3	
Mean	0.420	1.51	20.7	73.5	
%CV	3.7	4.7	0.8	1.8	
n	6	6	6	6	
Mean %Diff	−16.0	0.7	3.5	−2.0	
Between-run mean	0.464	1.55	20.4	73.2	513
Between-run %CV	9.1	4.4	3.5	2.6	3.4
n	18	18	18	18	6
Between-run mean %Diff	−7.2	3.3	2.0	−2.4	2.6
Rat					
Mean	0.439	1.44	20.9	74.1	471
%CV	16.4	7.2	3.0	2.6	4.4
n	6	6	6	6	6
Mean %Diff	−12.2	−4.0	4.5	−1.2	−5.8
Mean	0.508	1.49	19.0	78.8	
%CV	6.6	12.4	12.8	1.4	
n	6	6	5	6	
Mean %Diff	1.6	−0.7	−5.0	5.1	
Mean	0.542	1.40	22.3	82.7	
%CV	6.9	6.6	4.8	7.0	
n	6	6	6	6	
Mean %Diff	8.4	−6.7	11.5	10.3	
Between-run mean	0.496	1.44	20.8	78.5	471
Between-run %CV	13.1	9.1	9.4	6.3	4.4
n	18	18	17	18	6
Between-run mean %Diff	−0.8	−4.0	4.0	4.7	−5.8

2. Experimental

2.1. Materials

DM4 (N-methyl-N-[4-mercapto-4-methyl-1-oxopentyl]-L-alanine ester of maytansinol, free base, Formula: $C_{38}H_{54}ClN_3O_{10}S$), the reference standard of the analyte, was acquired from ImmunoGen (Waltham, MA) in accurately pre-weighed vials. The internal standard (IS), ansamitocin P-3, was purchased from Sigma–Aldrich Inc. (St. Louis, MO). All solvents were HPLC grade. Acetonitrile, ethyl acetate, methanol, acetic acid, ethyl alcohol, and ammonium hydroxide were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Methyl t-butyl ether was from Honeywell Burdick and Jackson (Morristown, NJ). Formic acid and ammonium formate were from Sigma–Aldrich (St. Louis, MO). Water that was used was purified using an Elga Lab Water Purelab Ultra Analytic water purification system. Blank Cynomolgus monkey and Sprague-Dawley rat plasma with K_2EDTA as the anticoagulant was from Bioreclamation (Hicksville, NY).

2.2. Preparation of standard and QC samples

The DM4 stock solution (500 μg/mL) was prepared by pipetting the appropriate volume of acetonitrile/water (1:1) directly into vials containing accurately pre-weighed reference material (1.000 ± 0.002 mg) and vortexing for approximately 2 min. DM4 working solutions were made in acetonitrile/water (1:1) and diluted to appropriate concentrations. The IS stock solution (200 μg/mL) was prepared by accurately weighing of the ansamitocin P-3 reference compound and dissolving into an appropriate volume of acetonitrile/water (1:1). The IS working solution was prepared by diluting the IS stock solution with acetonitrile/water (1:1) to a concentration of 250 ng/mL. All stock solutions and working solutions were stored at −20 °C for up to 5 weeks.

Calibration samples were prepared in chilled K_2-EDTA monkey or rat plasma and stored at approximately −20 °C until use. Calibration standards were prepared at 0.500, 1.00, 2.00, 5.00, 20.0, 50.0, 90.0, and 100 ng/mL in monkey or rat plasma. Quality control (QC) samples were prepared by fortifying blank plasma with spiking solutions. QC samples were prepared at 0.500, 1.50, 20.0, and 75.0 ng/mL in plasma, pipetted into 0.100 mL aliquots and stored at −70 °C until analysis. Additionally, a dilution QC was prepared at 500 ng/mL.

2.3. Sample extraction

Samples were prepared using a liquid–liquid extraction procedure in an ice water bath. Frozen samples were thawed in an ice water bath prior to use. When the QC dilution samples were used, they were diluted 10-fold by chilled blank plasma. One hundred microlitres of the plasma samples were transferred into new 12 mm × 75 mm glass test tubes. A volume of 10.0 μL of IS working solution (ansamitocin P-3, 250 ng/mL in acetonitrile/water (1:1)) was added to each sample except the blank, to which 10.0 μL of solvent was added. To this mixture, 0.400 mL of a chilled ammonium formate solution (0.1 M, pH 6.4) was added. Additionally, a

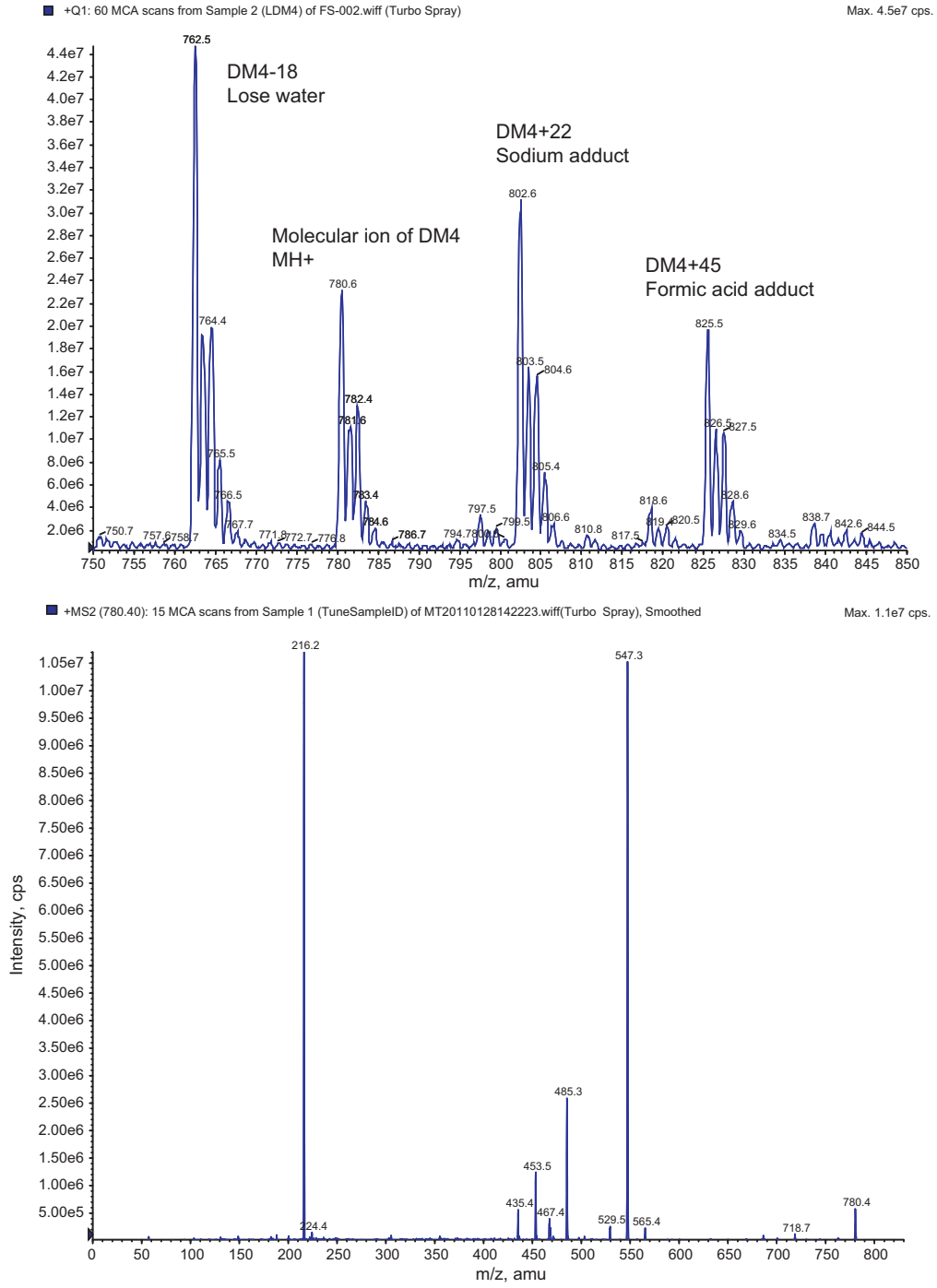

Fig. 2. DM4 mass spectra: (a) full scan mass spectrum of DM4 (top) and (b) product ion spectrum of *m*/*z* 780 (bottom).

volume of 75.0 μL of chilled ethanol was added in the rat plasma method, to improve the release of the analyte from protein binding. To perform the liquid–liquid extraction, 1.50 mL of the extraction solvent (ethyl acetate/methyl t-butyl ether (1:1)) was added into each test tube. The tubes were capped securely, inverted several times and vortex mixed thoroughly for 2 min. After vortexing, the samples were centrifuged at 3000 RPM for 5 min at approximately 4 °C to separate the two liquid layers. The upper organic layer containing DM4 and its IS was removed and evaporated to dryness under nitrogen gas at room temperature. The dried residue was reconstituted in 0.100 mL of ethanol/acetonitrile/water/acetic acid/ammonium formate (700:600:100:0.4:1.0; v:v:v:v:w), transferred into autosampler vials, crimp-capped, and was ready for injection. A volume of 4 μL was injected onto the LC–MS/MS system.

2.4. Instrumentation and LC–MS/MS conditions

A Shimadzu LC-10AT LC pump (Shimadzu Scientific Instruments, Columbia, MD) and a refrigerated (4 °C) PerkinElmer AS-200 autosampler (Waltham, MA) was used as the liquid chromatographic-autosampler system. A Thermo Hypersil

Gold PFP column (4.6 mm × 50 mm, 3 μm, ThermoFisher Scientific, Waltham, MA) was utilized at ambient temperature to perform chromatographic separation. An isocratic condition was adopted. The mobile phase for the rat plasma method was acetonitrile/methanol/water/formic acid/ammonium formate (500:300:200:1.00:2.00; v:v:v:v:w). For the monkey plasma method, the mobile phase was acetonitrile/water/acetic acid/ammonium formate (600:100:0.400:1.00; v:v:v:w). The flow rate was 1.00 mL/min with a 2.0 min run time. Methanol was used for needle wash for each injection cycle. Four microliters of reconstituted extracts were injected. A Sciex API 5000 Triple Quad mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo spray interface was used as the detector. The analyte and internal standard were monitored using multiple-reaction monitoring (MRM) and electrospray positive ionization mode. The mass transition for DM4 was 780 → 216 with collision energy of 38 eV. The mass transition for ansamitosin P-3 was 635 → 547 with the same deflector potential and collision energy used for the analyte. The turbo ionspray probe temperature was set at 550 °C with the ionization potential at 4500 V. The curtain, auxiliary, nebulizing and collision gases were set at 10, 70, 70, and 8.00, respectively. The declustering potential (DP), entrance potential (EP), and collision exit potential (CXP) were set at 120, 10.5 and 15 eV respectively. The dwell times were 200 and 100 ms for DM4 and ansamitosin P-3 with a pause time of 5 ms. The chromatographic peaks were detected at approximately 0.8 min for the internal standard and DM4.

2.5. Nonclinical studies

The studies were reviewed and approved by the Testing Facility's IACUC prior to the initiation, and conducted in accordance with the regulations of the USDA Animal Welfare Act and in compliance with the Testing Facility's Animal Welfare Assurance. Studies were performed in Cynomolgus monkeys and Sprague-Dawley rats. Animals were dosed via bolus intravenous injection of the B3F6-DM4 once every 3 weeks. The doses were 0, 5, 10, and 20 mg/kg in rat study, 0, 1.26, 3.34 and 8.34 mg/kg for the monkey study. Plasma samples were collected via venipuncture obtained from the femoral vein with K_2EDTA as the anticoagulant, immediately chilled on wet ice, centrifuged under refrigerated condition, and stored at −70 °C.

3. Results and discussion

3.1. Liquid chromatography and tandem mass spectrometry

The full scan and product ion mass spectra were obtained for DM4 (Fig. 2a and b) and ansamitocin P-3 using electrospray positive ion mode. The full scan spectrum of DM4 contained the signal of the protonated molecular ion (780), as well as the signals of molecular ion minus a water molecule (762), the sodium adduct (802), and the formic acid adduct (825). The protonated ion (780) was chosen as the parent ion of DM4. The transition of 780 → 216 was found to be the most predominant ion pair with the least interference from the matrix and was therefore chosen for the DM4 detection. Similarly, the transition of 635 → 547 was selected for the internal standard ansamitocin P-3. The presence of the other adducts diluted the parent ion intensity and adversely affected the limit of quantitation. Both API 4000 and API 5000 mass spectrometers were tested as the detector during method development. API 5000 was found to provide greater S/N ratio and therefore was adopted for the methods.

A Hypersil Gold PFP column and isocratic conditions were used for chromatographic separation. In the monkey plasma method, the mobile phase was adjusted slightly from those of rat method (Section 2.4) in order to eliminate several interference peaks present in this matrix. Figs. 3 and 4 show the representative chromatograms of DM4 and ansamitocin P-3 from monkey and rat plasma respectively. The retention times for DM4 and the internal standard were 0.96 and 0.94 min for monkey method, 0.86 and 0.82 for rat method respectively. Although the mobile phase for the rat method is slightly different, the retention times were very similar. Representative chromatograms of study samples from monkey and rat studies are also shown in Figs. 3 and 4.

Table 5
Stability experiment results of DM4 in monkey (top) and rat (bottom) plasma.

Stability	Statistics	QC low (1.50 ng/mL)	QC high (75.0 ng/mL)
Monkey			
Ice water bath (6 h)	Mean	1.28	63.5
	%CV	2.3	3.7
	n	3	3
	Mean %Diff	−14.7	−15.2
Freeze/thaw (4 cycles)	Mean	1.53	72.2
	%CV	3.0	1.7
	n	3	3
	Mean %Diff	2.0	−3.7
Storage at −70 °C (129 days)	Mean	1.48	73
	%CV	3.5	1.4
	n	3	3
	Mean %Diff	−1.3	−2.7
Processed sample at room temperature (20 h)	Mean	1.56	76.4
	%CV	8.8	1.0
	n	3	3
	Mean %Diff	4.0	1.9
Rat			
Ice water bath (12 h)	Mean	1.47	76.0
	%CV	4.8	1.0
	n	3	3
	Mean %Diff	−2.0	1.4
Freeze/thaw (4 cycles)	Mean	1.52	73.6
	%CV	6.2	6.7
	n	3	3
	Mean %Diff	1.6	−1.8
Storage at −70 °C (29 days)	Mean	1.44	78.8
	%CV	1.4	1.1
	n	3	3
	Mean %Diff	−4	5.1
Processed sample at 4 °C (27 h)	Mean	1.34	76
	%CV	6.4	1.0
	n	3	3
	Mean %Diff	−10.7	1.4

3.2. Sample preparation and extraction

When developing the free DM4 method, it is essential to apply experimental conditions that maintain stabilities for both the free DM4 and conjugate antibody. The free DM4 and conjugate antibody were found stable in animal plasma in ice bath. Therefore reagent solutions and blank plasma were chilled before use. The preparation of standard and QC samples, and the extraction procedures were all performed in an ice bath. Protein precipitation, solid phase extraction, and liquid–liquid extraction methods were tested for the extraction of free DM4 from plasma. A liquid–liquid extraction method was found to give good recovery of free DM4 with minimal matrix effects and maximal DM4 signal intensity.

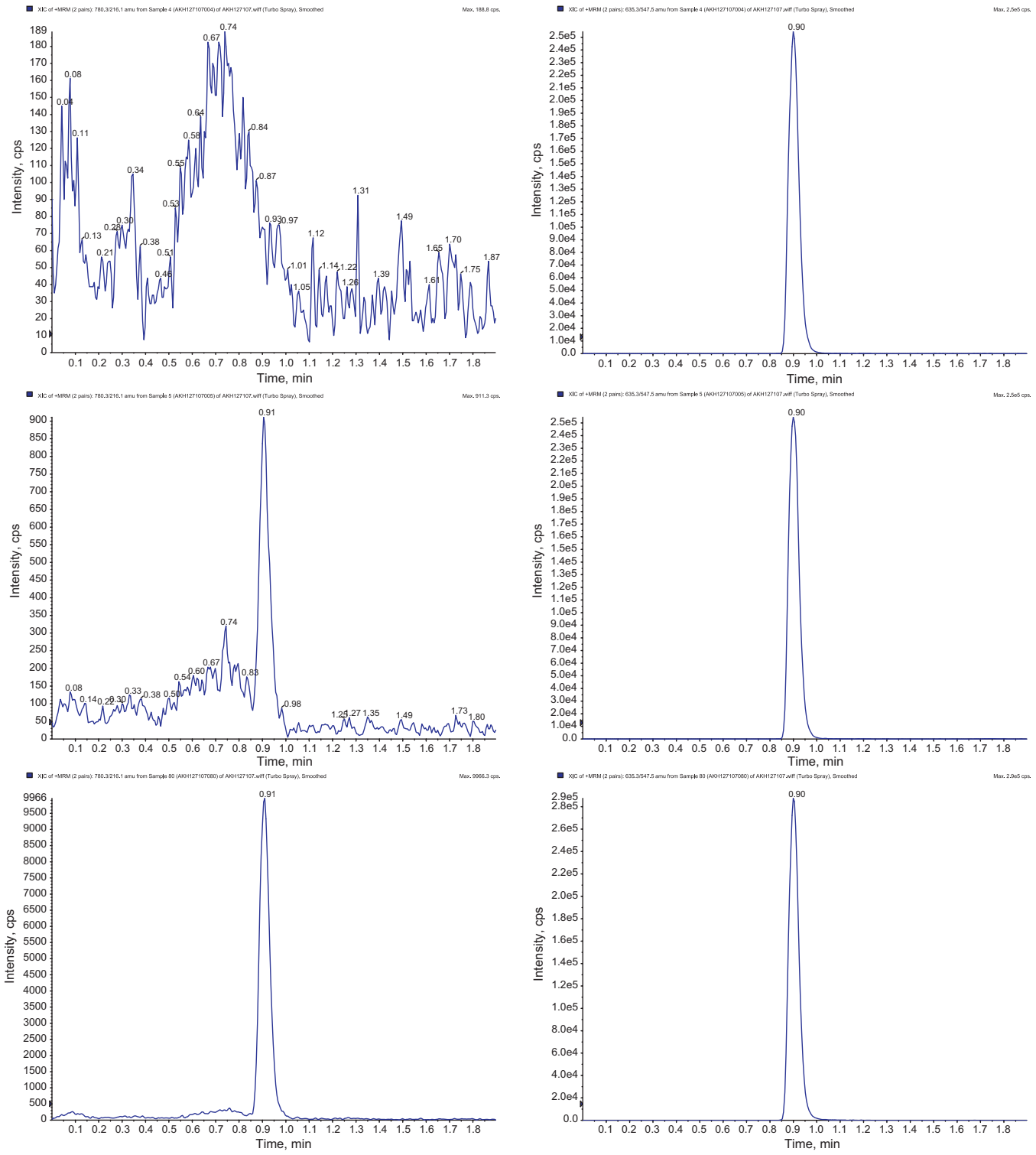

Fig. 3. Representative chromatograms in monkey plasma (left side is DM4 and right side is the IS ansamitocin) – *Top*: a blank plasma sample; *Middle*: an LLOQ sample; *Bottom*: a study sample from a monkey in the 8.34 mg/kg group.

3.3. Recovery and matrix effects

The extraction recovery of DM4 and its IS ansamitocin P-3 was determined by comparing the peak areas of extracted standards against those of nonextracted standards at three concentrations spanning the calibration curve (Table 1). In monkey plasma, the recovery ranged from 88.5 to 92.6% for DM4, and was 97.7% for ansamitocin P-3. In rat plasma, the recovery ranged from 74.2 to 95.4% for DM4, and was 87.1% for ansamitocin P-3.

Matrix effects were evaluated by preparing QC low samples with six different lots of monkey or rat plasma (Table 2). The accuracy of samples from each lot was within the acceptance criteria (±15%).

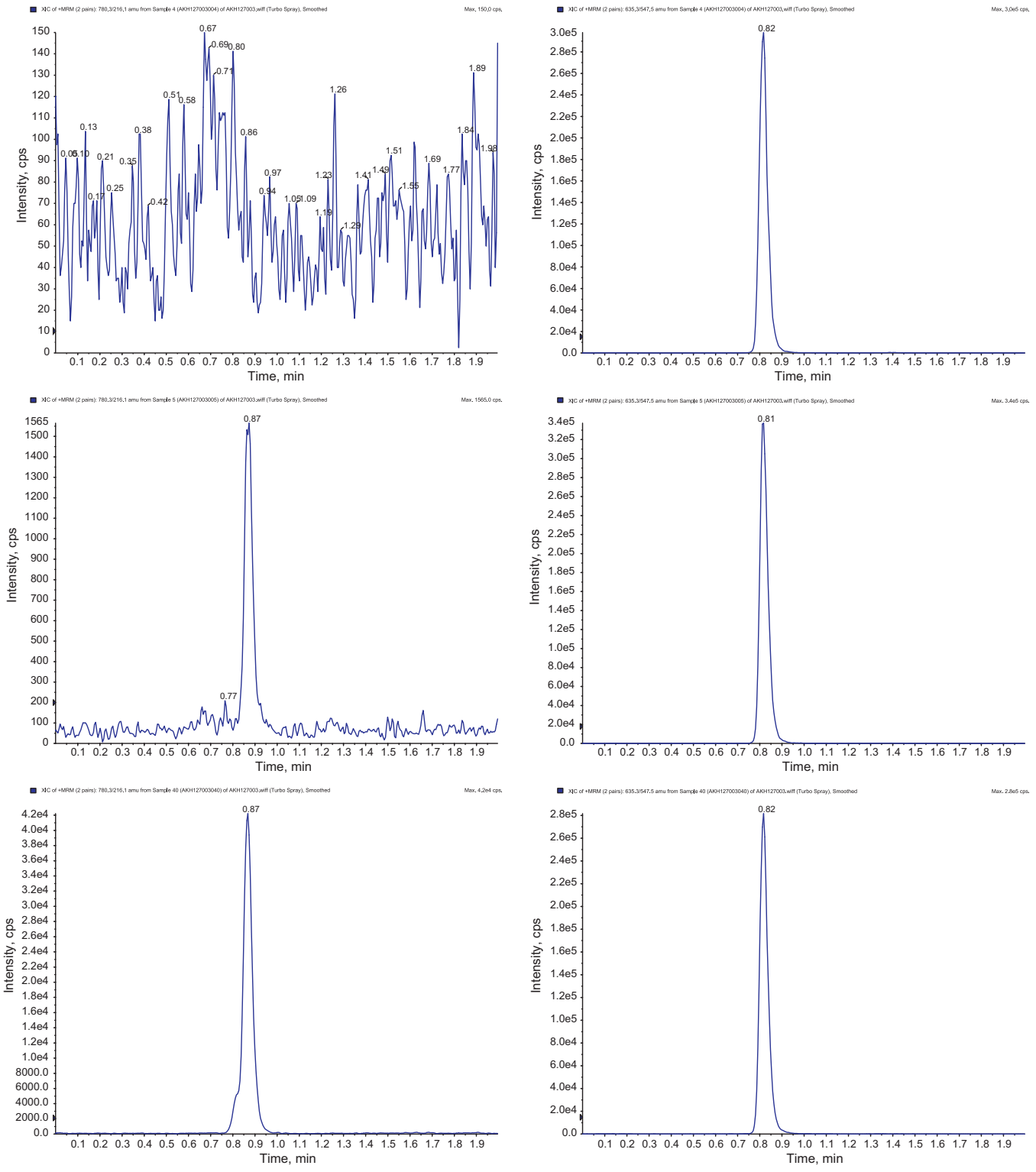

Fig. 4. Representative chromatograms in rat plasma (left side is DM4 and right side is the IS ansamitocin) – *Top*: a blank plasma sample; *Middle*: an LLOQ sample; *Bottom*: a study sample from a rat in the 20 mg/kg group.

3.4. Method performance

Quantitation was performed using a weighted linear least squares regression with the equation $y = a + bx$, where "a" is the y-intercept and "b" is the slope of the calibration curve. The weighting of $1/X^2$ was found to give excellent fit and therefore was chosen for the regression. The mean values of the slope, intercept, and R^2 are listed in Table 3. The R^2 was in a range of 0.996 –0.999.

The precision and accuracy results are listed in Table 4. Intra-run and inter-run results for samples prepared at QC low, QC medium and QC high demonstrated results that were within the acceptance criteria (+15%). The dilution integrity was demonstrated by the precision and accuracy of dilution QC sample.

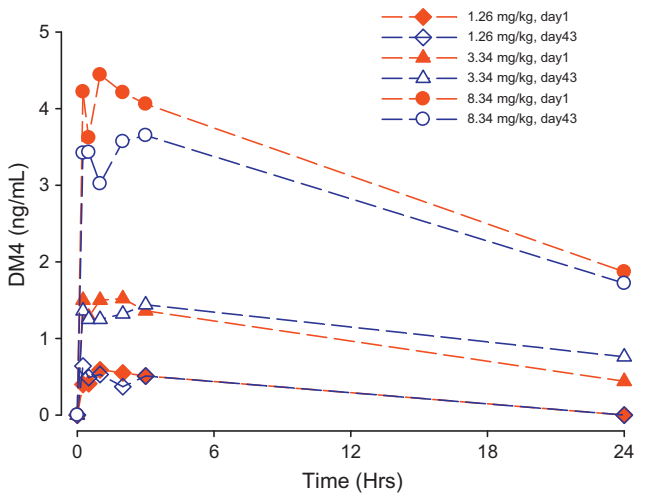

Fig. 5. Time-concentration profiles of free DM4 after IV administration of B3F6-DM4 to monkeys (intravenous injection, 0, 1.26, 3.34 and 8.34 mg/kg every 3 weeks).

The sensitivity of the method at 0.500 ng/mL (LLOQ) was tested by analyzing six samples prepared at the LLOQ level in each of three independent batches over the course of 3 days. For monkey samples, the intra-run CV ranged from 3.7% to 7.2% and the intra-run percent bias ranged from −16.8% to −1.0%. The inter-run cumulative CV of all replicates was 9.1% with a total mean bias of −7.2%. For rat samples, the intra-run CV ranged from 6.6% to 16.4% and the intra-run percent bias ranged from −12.2% to −8.4%. The inter-run cumulative CV of all replicates was 13.1% with a total mean bias of −0.8%.

In testing the method for selectivity, six different lots of blank plasma were analyzed for interferences. No interference was detected at the retention times of DM4 and IS in each lot. Representative chromatograms of extracted blank rat and monkey samples are presented in Figs. 3 and 4.

3.5. Stability

Stability testing was conducted and the results showed DM4 was sufficiently stable under method handling conditions (Table 5). A negative bias was observed, particularly with exposure of samples to higher temperatures in the thawed state. Plasma samples were found to have acceptable stability when they were kept in an ice water bath for no more than 6 h for monkey plasma, and no more than 12 h for rat plasma. Freeze/thaw, storage stability at −70 °C, and processed sample stability were also demonstrated.

3.6. Method application

The validated LC–MS/MS method was successfully applied to nonclinical studies in monkeys and rats. Examples of LC–MS/MS chromatograms from these studies are presented in Fig. 3 (monkey studies) and Fig. 4 (rat studies). DM4 concentrations were found to be <20.0 ng/mL in monkey samples and <40.0 ng/mL in rat samples. The ULOQ of the method was found to be sufficient, so sample dilution was not necessary. The LLOQ was sensitive enough to quantitate samples from all dosing groups and all time points except at 24 h after dosing (1.26 mg/kg in monkey). Examples of time-DM4 concentration profiles are presented in Fig. 5 from a GLP monkey study (Section 2.5). Well defined time-concentration profiles were obtained from LC–MS/MS data, allowing reliable pharmacokinetic analysis of the free DM4 component in circulating blood.

4. Conclusion

A sensitive liquid–liquid extraction LC–MS/MS method was developed and validated for the quantitative determination of free maytansinoid DM4 in monkey and rat plasma. The method demonstrated good linearity in the range of 0.500–100 ng/mL in both monkey and rat plasma. The within- and between-run precision and accuracy of the calibration standards and QC samples met FDA acceptance criteria for bioanalytical method validation. The LC–MS/MS method was proven to be sensitive, accurate, and reproducible. The method was used to support GLP nonclinical studies of the novel antitumor agent B3F6-DM4.

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