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Quantification for Antibody-Conjugated Drug in Trastuzumab Emtansine and Application to In Vitro Linker Stability and In Vivo Pharmacokinetic Study in Rat Using an Immuno-Affinity Capture Liquid Chromatography-Mass Spectrometric Method

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Abstract: Trastuzumab emtansine (T-DM1, brand name: Kadcyla®) is the first FDA-approved antibody-drug conjugate (ADC) for metastatic human epidermal growth factor receptor 2 positive (HER2+) breast cancer. It consists of three components: trastuzumab, an anti-HER2 monoclonal antibody, maytansinoid (DM1) as a cytotoxic drug, and maleimidomethyl cyclohexane-1-carboxylate (MCC) as a linker. In particular, the MCC linker is known as a non-cleavable linker and has a feature of being conjugated to DM1 by a covalent thioether bond. In this study, we developed an immuno-affinity capture liquid chromatography-mass spectrometric (LC-MS/MS) assay for quantifying the antibody-conjugated drug (acDrug) component of T-DM1. To quantify acDrug, desulfurated DM1 was prepared using a chemical desulfuration pretreatment and quantified as an acDrug. A quadratic regression (weighted 1/concentration), with equation $y = ax^2 + bx + c$, was used to fit the calibration curves over the concentration range of 17.09~1709.44 ng/mL for the acDrug of T-DM1. The quantification run met the in-house acceptance criteria of ±25% accuracy and precision values for the quality control (QC) samples. In conclusion, an immuno-affinity capture LC-MS/MS assay was successfully developed to quantify acDrug of T-DM1 and applied to evaluate in vitro plasma linker stability and preclinical pharmacokinetic (PK) study in rats. This assay could be helpful when applied to other ADCs with the same linker-cytotoxic drug platform.

Keywords: antibody-drug conjugate (ADC); antibody-conjugated drug (acDrug); quantification; LC-MS/MS; bioanalysis; non-cleavable linker

1. Introduction

Antibody-drug conjugate (ADC) is a promising biopharmaceutical consisting of three components: a monoclonal antibody, a linker, and cytotoxic drugs (payloads) [1–9]. ADCs undergo receptor-mediated endocytosis after reaching target cells through a specific antigen-antibody response. After endocytosis, ADCs are degraded by lysosomal enzymes, and cytotoxic drugs are released [10]. The released cytotoxic drugs cause cell death by cytotoxic effects such as mitotic arrest or the inhibition of DNA or RNA polymerization [11–13]. Because of its ability to selectively deliver these cytotoxic drugs mainly to target cells, ADCs are currently receiving a lot of interest from many pharmaceutical companies and are being developed. To date, 12 ADCs (Adcetris®, Mylotarg™, Kadcyla®, Besponsa®, Lumoxiti®, Polivy™, Padcev®, Enhertu®, Trodelvy®, Blenrep®, Zynlonta®, and Tivdak™) have been approved by U.S. Food and Drug administration (FDA) for anti-cancer therapies [14]. In

addition, it is expected that many ADC candidates in preclinical and clinical development will be approved in the future.

From the perspectives of the full pharmacokinetic (PK) properties of ADC, three main components including (1) total antibody (tAb), (2) antibody-conjugated drug (acDrug)/(or conjugated antibody), and (3) free payload should be quantitated from in vivo samples [15]. Among these three main analytes, acDrug demonstrates the concept that ADC exists in an active form as a conjugate [16]. The acDrug may also play an important role in the dose-response analysis of ADCs. However, it was very difficult to evaluate the acDrug of the first generation ADCs without cleavable linkers such as trastuzumab emtansine (T-DM1, brand name: Kadcyla®) by LC-MS, and therefore it was reported that the conjugated antibody instead of acDrug were quantitated by ELISA assay (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2013/125427Orig1s000PharmR.pdf (accessed on 1 September 2021)) for Kadcyla. Recently, more attention is paid to the importance of the in vivo acDrug levels of ADCs regarding the efficacy, in vivo drug-antibody ratio (DAR) evaluation, and toxicity. Currently, only ADCs with cleavable linkers are easily applicable to the quantitation of acDrug by the immuno-affinity capture method followed by on-bead digestion using specific enzymes such as cathepsin B or beta-glucuronidase for the LC-MS analysis of acDrug [16–18]. Due to lack of specific sites in the linker, this method was not applicable to Kadcyla, which contains non-cleavable linkers.

In this paper, we developed an immuno-affinity capture LC-MS/MS assay using previously reported chemical desulfuration pretreatment [19] to quantify acDrug in rat preclinical PK samples even for trastuzumab emtansine (T-DM1, brand name: Kadcyla®), a representative ADC with non-cleavable linkers. T-DM1 is the first FDA-approved ADC to treat metastatic human epidermal growth factor receptor 2 (HER2)-positive breast cancer and consists of the monoclonal antibody trastuzumab, maleimidomethyl cyclohexane-1-carboxylate (MCC) as a non-cleavable linker, and maytasinoid as a payload [20]. This developed assay was applied to evaluate in vitro plasma linker stability and preclinical PK studies in rats. To our best knowledge, this is the first approach to quantitatively evaluate the acDrug of T-DM1 in vivo rat PK samples.

2. Materials and Methods

2.1. Materials

T-DM1 was purchased from Dongwon Pharmaceutical (Daejeon, Korea). Verapamil for internal standard (ISTD) was purchased from Sigma-Aldrich Korea (Seoul, Korea). Sodium borohydride ($NaBH_4$) and nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) for the chemical desulfuration pretreatment were also purchased from Sigma-Aldrich Korea (Seoul, Korea). The protein A magnetic bead was purchased from Millipore Korea (Seoul, Korea). Rat blank plasma (Sprague Dawley (SD) rat, fasted male, Na-heparin anticoagulant) was purchased from Biomedex (Seoul, Korea). Other reagents were commercially purchased for analytical purposes or reagent grading and were used without further purification.

2.2. Preparation of Stocks, Standard (STD) and Quality Control (QC) Samples

A stock solution (1 mg/mL) of T-DM1 was prepared in phosphate buffered solution (PBS) and stored at 4 °C. A stock solution was diluted with PBS and spiked into rat blank plasma to make STD and QC samples. The calibration curve was made up of eight calibrated STD samples with a final plasma concentration range of 17.09~1709.44 ng/mL. QC samples in the final plasma concentration were 42.74 (low QC), 427.36 (medium QC) and 854.72 (high QC) ng/mL.

2.3. Sample Preparation

Each 24 µL of STD and QC plasma sample was mixed with 350 µL of 0.1% tween 20 in PBS and 30 µL of protein A magnetic bead. In addition, 20 µL of all study samples were also mixed with 354 µL of 0.1% tween 20 in PBS and 30 µL protein A magnetic bead (https://www.merckmillipore.com/KR/ko/product/PureProteome-Protein-

A-Magnetic-Bead-System,MM_NF-LSKMAGA10#anchor_BRO (accessed on 1 September 2021)) [8]. After vortexing, the mixture samples were incubated gently for about 2 h at room temperature and then the samples were fixed on a magnetic rack and washed with 200 µL of 0.1% tween 20 treated PBS and 200 µL of PBS.

For the chemical desulfuration pretreatment, the washed samples were treated with 90 µL of 0.2 M glycine hydrochloric acid (Gly-HCl) and incubated for 20 min at 60 °C. After incubation, samples were chilled for 5 min at 4 °C. Then, 100 µL of methanol, 40 µL of $NiCl_2 \cdot 6H_2O$ (100 mg/mL) and 30 µL of $NaBH_4$ (40 mg/mL) were added to the chilled samples [19]. Figure 1 shows the chemical desulfuration pretreatment mechanism of the thioether bond between the MCC linker and the DM1 of T-DM1.

The above mixture samples were gently shaken at room temperature overnight and centrifuged at 12,000 rpm for 10 min. The supernatant samples were transferred into another Eppendorf tube and evaporated to dryness under vacuum in a centrifugal evaporator (CVE-3110, Eyela, Tokyo, Japan) connected to a cold trap (UT-1000, Eyela, Tokyo, Japan). The dried residue samples were reconstituted using 30% acetonitrile (ACN) solution dissolved with 100 ng/mL of verapamil as an ISTD solution, vortexed, and then centrifuged at 12,000 rpm for 10 min. The supernatant samples were transferred into LC vials for LC-MS/MS analysis.

2.4. Method Qualification

Method qualification was carried out with a fit-for-purpose approach [8]. The qualification run contained duplicate STDs at eight concentrations and QCs at three different level concentrations. The in-house acceptance criteria for STDs and QCs in the qualification run were within ±25% of the precision and accuracy values. A quadratic regression (weighted 1/concentration), with an equation $y = ax^2 + bx + c$, was used to fit the calibration curves. In addition, two blank plasma samples were run. The intra- and inter-day run for the accuracy and precision assays were calculated at each QC concentration.

Preliminary stability tests were conducted in rat plasma samples under different conditions, such as short-term, long-term, and freeze-thaw stability at three different levels of QC. The short-term stability was determined at room temperature for 6 h. The long-term stability was determined by analyzing QC samples kept frozen at −80 °C for 3 months. For the freeze-thaw stability, the samples were subjected to three freeze-and-thaw cycles at −80 °C. The in-house acceptance criteria for all preliminary stability tests were also within ±25% of the precision and accuracy values.

2.5. Application for In Vitro Linker Stability Test and Preclinical Pharmacokinetic (PK) Study in Rats

For the in vitro linker stability test, 10 µL of 500 µg/mL T-DM1 in PBS was spiked into 50 µL of rat blank plasma to make an incubation sample. After incubation at 37 °C for 0, 1, 3, 5, and 7 days, the incubated samples were stored at −80 °C until analysis.

The preclinical PK studies were conducted in SD rats. T-DM1 was administered to rats via single intravenous bolus injection (3 mg/kg). Blood samples were collected at 3 min; 1, 4 and 7 h; 1, 2, 4, 7, 14, and 21 days post-dose in heparinized tubes. The blood samples were centrifuged, and the supernatant plasma were stored at −80 °C until analysis. Animal experiments followed the animal care protocol (No. 202003A-CNU-024) approved by the Chungnam National University. All procedures related to animal experiments were also performed in accordance with the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). PK parameters were calculated by non-compartmental analysis (NCA) using WinNonlin® version 8.0.0 (Pharsight Corporation, Mountain View, CA, USA).

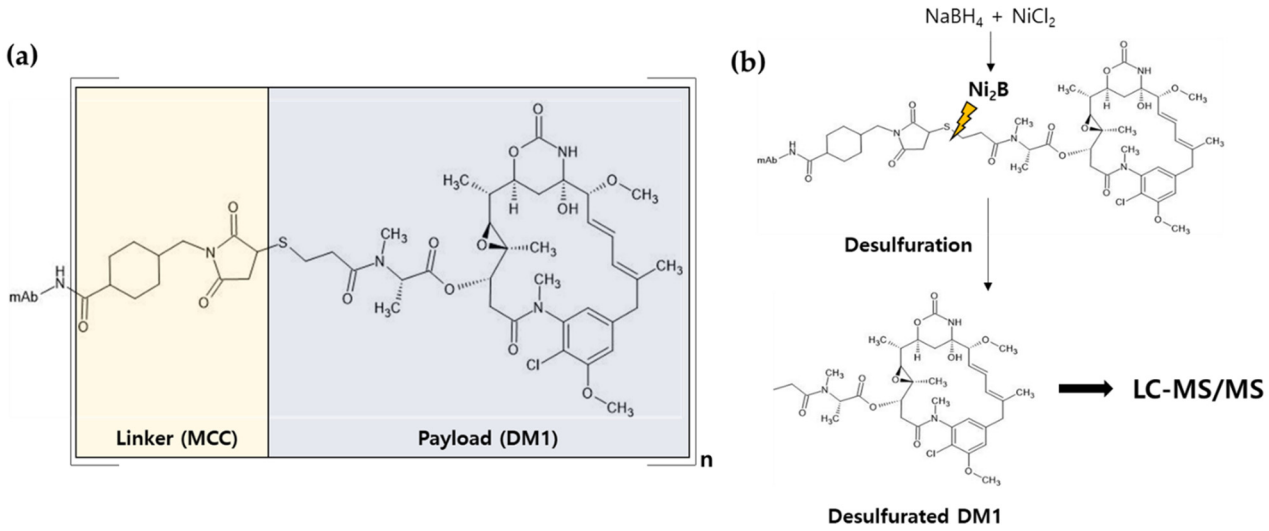

Figure 1. (**a**) Chemical structure of T-DM1 and (**b**) desulfuration reaction in the thioether bond between MCC linker and DM1 of T-DM1 by sodium borohydride ($NaBH_4$) and nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) chemical reagents.

2.6. LC-MS/MS Conditions

Two Shimadzu LC-20AD pumps, a Shimadzu CBM-20A HPLC pump controller (Shimadzu Corporation, Columbia, MD, USA), a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC, USA) and a quadrupole time-of-flight TripleTOF™ 5600 mass spectrometer (Sciex, Foster City, CA, USA) equipped with a Duospray™ ion source (Sciex, Foster City, CA, USA) were used for LC-MS/MS analysis.

The pumping mode of two Shimadzu LC-20AD pumps was binary flow and the composition of mobile phases were as follows: mobile phase A was distilled and deionized water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The mobile phase B percent of gradient elution increased from 10% mobile phase B to 95% mobile phase B during run time. The LC gradient for this analysis is summarized in Table 1. The injection volume was 10 µL and LC flow rate was 0.4 mL/min. The desulfurated DM1 was separated through a Phenomenex Kinetex XB-C18 column (2.1 × 50 mm, 2.6 µm).

Table 1. The LC gradient elution for the separation of desulfurated DM1.

Time (min)	Mobile Phase B (%)
0.0	10
0.5	10
1.1	95
1.5	95
1.6	10
3.0	10

The TOF-MS scan mass spectra and the product ion scan mass spectra were recorded in the positive ion mode. The scan range was at m/z 100~900 in the TOF-MS scan and at m/z 100~750 in the product ion scan. For the quantification, $[M+H]^+$ ions of desulfurated DM1 and verapamil were selected at m/z 706.3 and m/z 455.3, respectively and their product ions at m/z 547.2 and m/z 165.1 were used for quantitative analysis, respectively. The temperature of the ion source was 500 °C and the ion spray voltage was at 5500 V. For the desulfurated DM1 and verapamil of ISTD, the declustering potential was 10 V and 125 V, respectively, and the collision energy was 29 V and 30 V, respectively. The analytical run was performed after sufficient system equilibrium was performed. In addition, before the corresponding analytical run, calibration was performed on the TOF-MS and a positive

mode scan using a calibration solution standard for TripleTOF™ 5600, and the error ppm was less than 1 ppm.

3. Results and Discussion

3.1. Method Development and Qualification

An immuno-affinity capture LC-MS/MS assay was used for the quantification of desulfurated DM1 as acDrug of T-DM1. Desulfurated DM1 is the cleaved form of the sulfur element from DM1, and the MS/MS spectrum is shown in Figure 2. The parent $[M+H]^+$ ion was detected at m/z 706.3, and the most abundant product ion was at m/z 547.2.

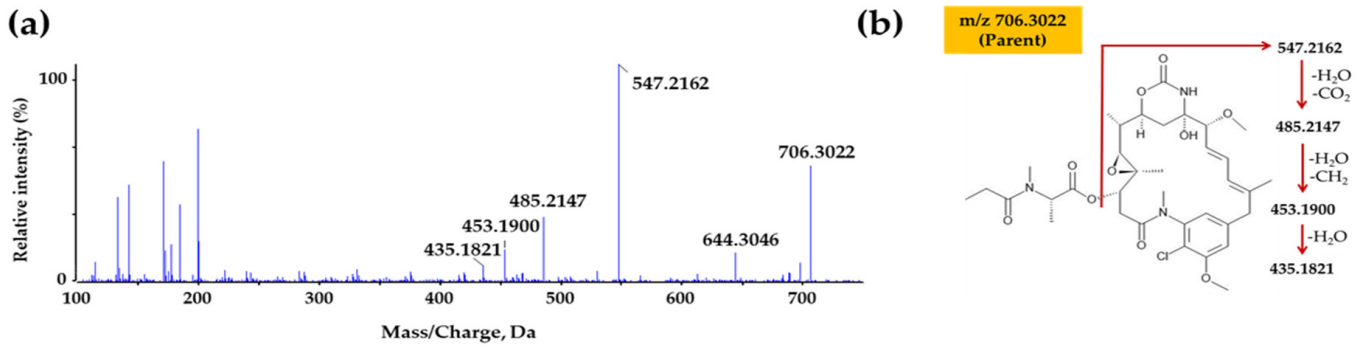

Figure 2. (**a**) MS/MS spectrum of desulfurated DM1 and (**b**) expected fragment ions of parent ion (m/z 706.3022).

The calibration curves with eight points in the range of 17.09~1709.44 ng/mL in duplicate were freshly prepared for all data sets. The quadratic regression using the ratios of peaks versus concentrations was weighted by 1/concentration. The acceptance of the curve was ≥0.99 for the desulfurated DM1. Figure 3 shows the calibration curve of desulfurated DM1.

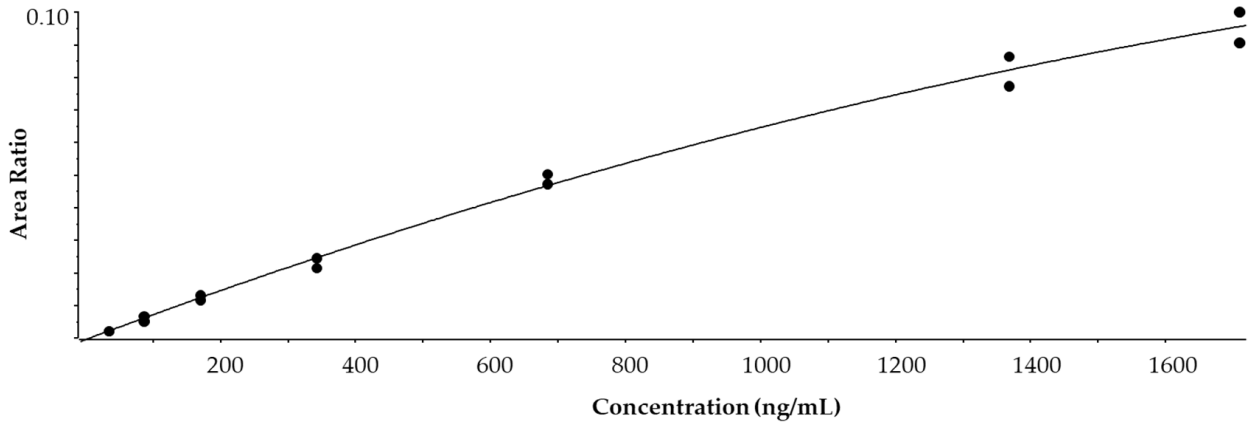

Figure 3. Calibration curve for quantification of desulfurated DM1 (r = 0.99746).

Representative chromatograms of the limit of quantification (LLOQ) and high QC are also shown in Figure 4. The peak retention time was the same in all samples.

The intra- and inter-day runs for the accuracy and precision assays were performed for method qualification and the results are shown in Table 2. The qualification run met the acceptance criteria of ±25% accuracy and precision for QC samples.

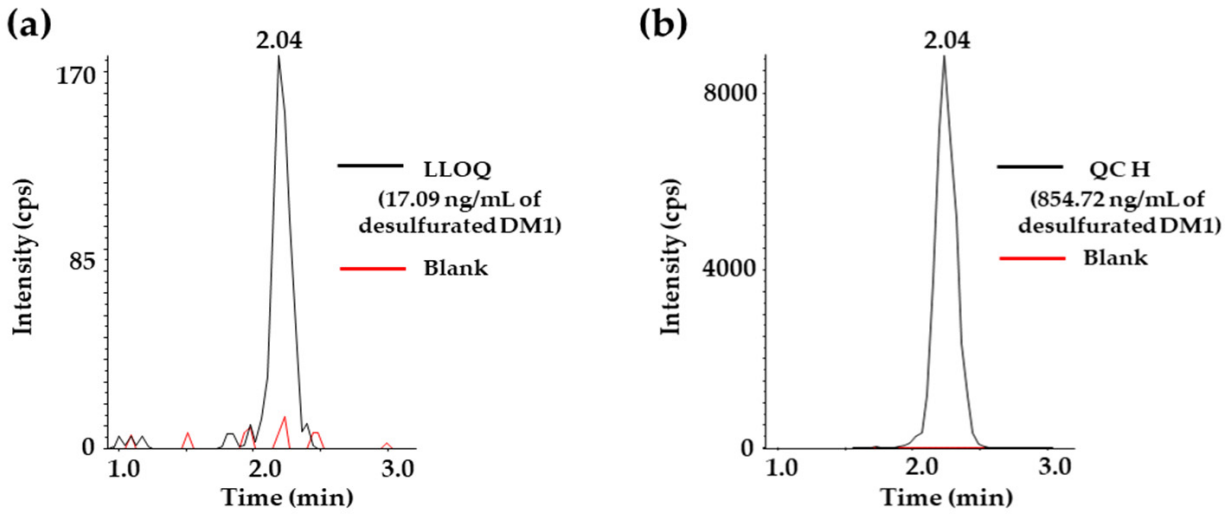

Figure 4. Representative chromatograms of (**a**) limit of quantification (LLOQ, 17.09 ng/mL) and (**b**) high level quality control (QC H, 854.72 ng/mL) of desulfurated DM1.

Table 2. The intra/inter-run assays for QC samples of desulfurated DM1.

Intra-Run Assay				
Run No.	**Statistics**	**Low QC (42.74 ng/mL)**	**Medium QC (427.36 ng/mL)**	**High QC (854.72 ng/mL)**
Day 1	Mean concentration (ng/mL)	43.76	419.10	870.05
	Accuracy (%)	102.40	98.07	101.79
	Precision (%, CV)	10.15	8.53	6.06
	n *	3	3	3
Day 2	Mean concentration (ng/mL)	47.98	395.68	877.40
	Accuracy (%)	112.27	92.59	102.65
	Precision (%, CV)	2.15	6.54	10.30
	n	3	3	3
Day 3	Mean concentration (ng/mL)	44.45	373.00	745.26
	Accuracy (%)	104.00	87.28	87.19
	Precision (%, CV)	8.03	13.75	12.04
	n	3	3	3
Inter-Run Assay (Day 1~3)				
Statistics		**Low QC (42.74 ng/mL)**	**Medium QC (427.36 ng/mL)**	**High QC (854.72 ng/mL)**
Mean concentration (ng/mL)		41.06	425.54	850.22
Accuracy (%)		96.09	99.57	99.47
Precision (%, CV)		13.98	5.74	10.12
n		9	9	9

* Number of samples.

Preliminary stability tests were conducted in rat plasma under various conditions, such as short-term, long-term, and freeze-thaw, and the results are shown in Table 3. The accuracy (%) and precision (%CV) of stability samples met the acceptance criteria of ±25%. As a result, the desulfurated DM1 in rat plasma was stable under the different experimental conditions.

Table 3. Stability tests of T-DM1 in rat plasma under various conditions.

Assessment	Statistics	Low QC (42.74 ng/mL)	Medium QC (427.36 ng/mL)	High QC (854.72 ng/mL)
Short-term stability (Room temperature, 6 h)	Mean concentration	46.15	436.99	874.26
	Accuracy (%)	108.00	102.25	102.29
	Precision (%, CV)	4.90	2.14	9.75
	n	3	3	3
Long-term Stability (−80 °C, 3 months)	Mean concentration	40.57	398.93	866.68
	Accuracy (%)	94.93	93.35	101.40
	Precision (%, CV)	22.55	5.08	4.12
	n	3	3	3
Freeze-thaw Stability (−80 °C, 3 cycles)	Mean concentration	40.63	429.47	910.96
	Accuracy (%)	95.07	100.49	106.58
	Precision (%, CV)	8.00	0.75	6.70
	n	3	3	3

3.2. Application to In Vitro Plasma Stability Test and Preclinical PK Studies in Rats

This qualified LC-MS/MS assay was applied to the in vitro plasma stability test and a preclinical PK study in rats. The results of the in vitro linker stability test are shown in Figure 5. The remaining (%) of acDrug in T-DM1 was calculated by relative ratio to the concentration of desulfurated DM1 at day 0, which was the initial incubation time. The results show that desulfurated DM1 as acDrug decreased dramatically to 36.97% at day 3, and then slowly decreased until day 7 in rat plasma.

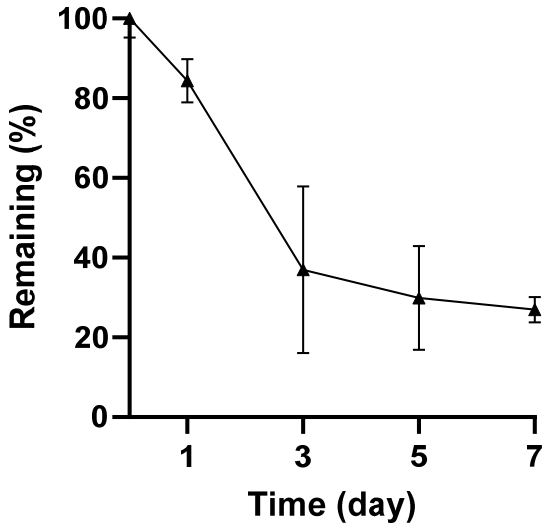

Figure 5. In vitro linker stability of T-DM1 and remaining (%) of acDrug in T-DM1, calculated from desulfurated DM1.

Rat plasma samples obtained from preclinical PK study after single intravenous administration of 3 mg/kg for T-DM1 were also analyzed using this qualified LC-MS/MS assay. The PK profiles and parameters for desulfurated DM1 are shown in Figure 6 and Table 4, respectively.

As a result, the half-life ($T_{1/2}$) was 4.56 ± 1.11 days, the maximum plasma concentration (C_{max}) was 1396.50 ± 267.92 ng/mL, and the clearance (CL) was 22.55 mL/day/kg. From the PK perspective, the acDrug of T-DM1 as a desulfurated form appeared to show relatively higher CL than the total antibodies of conventional therapeutic monoclonal antibodies. As shown from the in vitro plasma stability results, it is assumed that the linker instability of T-DM1 in rat plasma might play a role to some extent regarding the higher CL in vivo.

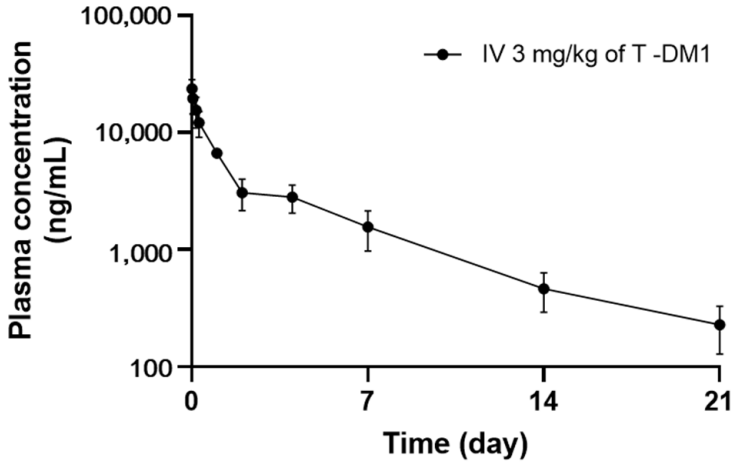

Figure 6. Pharmacokinetic (PK) profile of desulfurated DM1 after intravenous dose of 3 mg/kg for T-DM1 in rats.

Table 4. PK parameters of desulfurated DM1 after intravenous dose of 3 mg/kg for T-DM1 in rats.

	PK Parameters						
	$T_{1/2}$ (Day)	C_{max} (ng/mL)	AUC_{last} (Day*ng/mL)	AUC_{INF} (Day*ng/mL)	$AUC_{\%Extrap}$ (%)	CL (mL/Day/kg)	V_{ss} (mL/kg)
Mean	4.56	1396.50	2212.16	2318.94	4.56	22.55	107.98
SD	1.11	267.92	357.96	383.13	0.50	4.00	14.65

$T_{1/2}$, half-life; C_{max}, maximum plasma concentration; AUC_{last}, area under the curve up to last measurable concentration; AUC_{INF}, area under the curve from time zero to infinity; $AUC_{\%Extrap}$, the relative ratio of AUC_{last} to AUC_{INF}; CL, clearance; V_{ss}, volume of distribution.

4. Conclusions

In this paper, an immuno-affinity capture LC-MS/MS method for the acDrug of T-DM1 in rat plasma was successfully developed and applied to in vitro linker stability and preclinical PK assessment in rats. The chemical desulfuration pretreatment used in this study was able to specifically cleave thioether bonds between the MCC linker and DM1 of T-DM1. The method was qualified in a dynamic range of 17.09~1709.44 ng/mL using quadratic regression with 1/concentration weighting, which was sufficient to cover all time courses for this rat PK study with a dose level of 3 mg/kg for the acDrug of T-DM1. This immuno-affinity capture LC-MS/MS method was accurate and reproducible for the determination of acDrug concentration. This method would be also helpful for the acDrug analysis of innovative ADCs with similar non-cleavable linker platforms.

Author Contributions: Conceptualization, S.-j.P., B.i.L. and M.-H.P.; methodology, S.-j.P. and B.i.L. and M.-H.P.; software, S.-j.P.; validation, B.i.L., J.C., Y.P., M.-j.P. and J.-h.L.; formal analysis, S.-j.P. and B.i.L.; investigation, S.-j.P. and B.i.L.; resources, Y.G.S.; data curation, S.-j.P., B.i.L. and M.-H.P.; writing—original draft preparation, S.-j.P.; writing—review and editing, B.i.L., J.C., Y.P., M.-j.P., J.-h.L., J.L. (Jiyu Lee), S.H., J.L. (Jeongmin Lee) and Y.G.S.; visualization, S.-j.P.; supervision, Y.G.S.; project administration, Y.G.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Chungnam National University (protocol code 202003A-CNU-024, approval date: 1 April 2020). All procedures related to animal experiments were also performed in accordance with the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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