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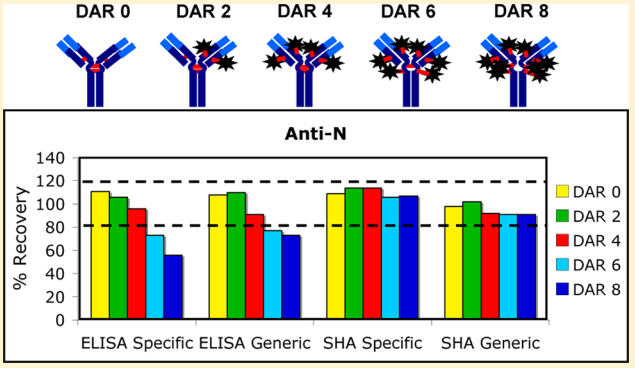
Total Antibody Quantification for MMAE-Conjugated Antibody–Drug Conjugates: Impact of Assay Format and Reagents

Katherine R. Kozak,*[,†] Siao Ping Tsai,[†] Aimee Fourie-O'Donohue,[†] Josefa dela Cruz Chuh,[†] Leslie Roth,[†] Ryan Cook,[†] Elton Chan,[†] Pamela Chan,[†] Martine Darwish,[‡] Rachana Ohri,[‡] Helga Raab,[‡] Crystal Zhang,[§] Kedan Lin,[§] and Wai Lee T. Wong[†]

[†]Departments of Biochemical and Cellular Pharmacology, [‡]Protein Chemistry, and [§]Pharmacokinetic & Pharmacodynamic Sciences, Genentech, Inc., South San Francisco, California 94080, United States

Supporting Information

ABSTRACT: Antibody–drug conjugates (ADCs) are target-specific anticancer agents consisting of cytotoxic drugs covalently linked to a monoclonal antibody. The number of ADCs in the clinic is growing, and therefore thorough characterization of the quantitative assays used to measure ADC concentrations in support of pharmacokinetic, efficacy, and safety studies is of increasing importance. Cytotoxic drugs such as the tubulin polymerization inhibiting auristatin, monomethyl auristatin E, have been conjugated to antibodies via cleavable linkers (MC-vc-PAB) through internal cysteines. This results in a heterogeneous mixture of antibody species with drug-to-antibody ratios (DAR) ranging from 0 to 8. In order to characterize the assays used to quantitate total MC-vc-PAB-MMAE ADCs (conjugated and unconjugated antibody), we used purified fractions with defined DARs from 6 therapeutic antibodies to evaluate different assay formats and reagents. Our investigations revealed that for quantitation of total antibody, including all unconjugated and conjugated antibody species, sandwich ELISA formats did not always allow for recovery of all purified DAR fractions (DAR 0–8) to within ±20% of the expected values at the reagent concentrations tested. In evaluating alternative approaches, we found that the recovery of DAR fractions with semihomogeneous assay (SHA) formats, in which sample, capture, and detection reagents are preincubated in solution, were less affected by the antibody's MMAE drug load as compared to traditional stepwise sandwich ELISAs. Thus, choosing the optimal assay format and reagents for total antibody assays is valuable for developing accurate quantitative assays.



INTRODUCTION

Antibody–drug conjugates (ADCs) are used as antibody-targeted chemotherapy to specifically deliver a cytotoxic agent to tumor cells through conjugation to a monoclonal antibody (mAb) that binds to a tumor-associated antigen.[1,2] Once the ADC reaches its target cell, the ADC internalizes into the cell where the drug can exert its cytotoxic effect. Drugs such as tubulin polymerization inhibiting auristatins including monomethyl auristatin E (MMAE) have been conjugated to antibodies and several MMAE based ADCs are under clinical development.[3−5] MMAE, an analog of dolastatin 10, is a potent antimitotic agent that exerts its cytotoxicity by binding to microtubules and inhibiting microtubule polymerization, thereby inhibiting cell division and inducing apoptosis.[6−8] MMAE has been conjugated to monoclonal antibodies via a protease-labile linker, maleimidocaproyl-valine-citrulline-*p*-aminobenzoyloxycarbonyl (MC-vc-PAB) through cysteine sulfhydryls activated by reducing interchain disulfide bonds. This method of conjugation results in a heterogeneous mixture of antibody species with drug-to-antibody ratios (DAR) varying from 0 to 8, with each species having its own distinct *in vivo* profile of pharmacokinetics (PK), efficacy, and safety.[9] The MC-vc-PAB-MMAE ADCs can be targeted to have an average DAR of 3 to 4 MMAE per antibody through controlled reduction, and the linker-drug is primarily conjugated to the solvent-accessible interchain disulfide bonds of the antibody. PK profiling of ADCs helps to characterize the impact of DAR and other ADC optimization parameters on distinct biological properties of ADCs, such as rate of drug loss (deconjugation and stability) and clearance of each DAR species. Ultimately through establishing the relationships between PK exposures to efficacy and toxicity, it helps to elucidate the mechanism of action and guide the optimization and development of ADCs.

To adequately determine the PK profile and *in vivo* stability of an MMAE-conjugated antibody, it has been common to develop immunochemical assays to monitor multiple analytes, including total antibody (antibodies with and without MMAE attached). A frequent biochemical assay generally used for the

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quantification of ADC total antibody in both nonclinical and clinical samples is the enzyme-linked immunosorbent assay (ELISA).[10,11] Several total antibody ELISA formats have been utilized to quantify antibody−MMAE conjugates.[9,12−19] Antigen-binding or "specific" ELISAs are common assay designs that have been applied to several MMAE-ADCs. This type of assay uses the target antigen (generally in the form of a recombinant extracellular domain or ECD) to capture the ADC, followed by detection with antihuman IgG (whole molecule or Fc-specific).[14,16−18] An alternative type of specific assay uses an anti-idiotypic antibody as a capture reagent.[19] For nonclinical samples, "generic" assays using coat and detection antibodies against human IgG or Fc are another useful assay approach.[9,12,13,19]

Although many investigators have used ELISAs to quantify ADCs, few publications have focused on evaluating how the assay format or reagent selection can impact the quantitation of the antibody and the distinct drug antibody conjugates. Sanderson et al.[12] confirmed minimal impact of antibodies with different drug loads on their anti-idiotypic capture total antibody assay by using hydrophobic interaction chromatography (HIC) purified anti-CD30 MC-vc-PAB-MMAE DAR fractions. Stephan et al.[20] also used DAR fractions to show that different conjugation chemistries (DM1 conjugated to lysine residues of the antibody through a thioether linker, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (MCC), and monomethyl auristatin F (MMAF) coupled via a maleimidocaproyl (MC) group to the interchain cysteines) affected the antibody structure differently and thereby caused differences in assay quantification in the context of a heterogeneous ADC population. As a result, certain assay formats yielded better recovery of the DM1 and MMAF conjugates than other formats. Using standards with an average DAR of 3−4, the assays for total antibody are expected to be able to equally quantify the antibodies in the serum or plasma sample regardless of the drug load. The objective of this paper is to investigate the impact of assay format and reagent choice on MC-vc-PAB-MMAE ADC quantification in total antibody assays, including a newly developed semihomogeneous immunoassay. Evaluation of these parameters is valuable for the development of optimal immunochemical assays for quantitating MMAE-conjugated ADCs.

EXPERIMENTAL PROCEDURES

Preparation of ADCs. Antibodies against specific targets (termed anti-B, -C, -F, -M, -N, and -S) were generated at Genentech, Inc., using previously described methods.[21−23] Hybridoma supernatants were harvested and purified by affinity chromatography as previously described.[24] Purified antibodies were sterile-filtered and stored at 4 °C in PBS. The MC-vc-PAB-MMAE ADCs were prepared by partially reducing the antibodies with stoichiometric amounts of tris(2-carboxyethyl)-phosphine (TCEP) for 2 h at room temperature and then incubating the maleimido drug derivative with the partially reduced antibodies for 1 h. After quenching the reaction with excess N-acetyl-cysteine, to react with any free linker-drug, the conjugated antibody was purified. Conjugation conditions were chosen to achieve an average DAR of approximately 3.5 for all MMAE-conjugated ADCs (anti-B, DAR 3.7; anti-C, DAR 3.9; anti-F, DAR 3.6; anti-M, DAR 3.5; anti-N, DAR 3.6; and anti-S, 3.6). ADC protein concentrations were calculated from their absorbance at 280 nm and the molar extinction coefficients of the antibodies, and, on occasion, confirmed using a BCA assay (Micro BCA Protein Assay Kit, Pierce/Thermo Scientific, Rockford, IL, USA) with the corresponding unconjugated antibodies as a standard. The average DARs were calculated from the integrated areas of the DAR species resolved by hydrophobic interaction chromatography (HIC) on an analytical column (TSK butyl-NPR 4.6 mm × 10 cm, 2.5 μm, Tosoh Bioscience, King of Prussia, PA, USA). The percentages of 0, 2, 4, 6, and 8 DAR species in the ADCs are listed in Supporting Information Table S1. DAR peaks were identified by their retention time as well as by the UV absorbance ratios at 248/280 nm as described by Hamblett et al.[9] and confirmed by reversed-phase liquid chromatography mass spectrometry (LC-MS) of the reduced ADCs (as described below).

Isolation of Specific DAR Species from Antitarget-MC-vc-PAB-MMAE ADCs. The preparative scale separation of the different antitarget ADC DAR species was performed with a ProPac HIC-10 chromatographic column (PIN 069665, 10 mm × 150 mm, 5 mm, 300 Å, Dionex Corporation, Sunnyvale, CA, USA) on an Äkta Explorer system (GE Healthcare, Uppsala, Sweden). A linear gradient from 25% of mobile phase B to 80% mobile phase B over 35 min was used, at a flow rate of 4 mL/min. Mobile phase A was composed of 25 mM sodium phosphate and 1.5 M ammonium sulfate, pH 6.95, while mobile phase B contained 75% 25 mM sodium phosphate, pH 6.95, and 25% isopropyl alcohol. The sample loads were 20−50 mg of antitarget-vc-MMAE. The peaks of interest (DAR 0, 2, 4, 6, and 8) were collected. A control sample with a DAR of 0 was prepared by injecting unconjugated antitarget antibody and the peak was collected. All peak fractions were buffer exchanged into PBS by diafiltration. Identity (and >90% purity) of the isolated fractions was confirmed by reanalysis on the same analytical HIC column as described above (see Supporting Information Figure S1). Aggregation of DAR fractions was assessed as previously described[25] and values are listed in Supporting Information Table S2. Protein concentrations were calculated from the UV absorbance at 280 nm (after 320 nm reference subtraction) and the molar extinction coefficients of the antibodies at A280.

Further analyses of the antitarget ADC, the HIC-fractionated peaks, and the unconjugated antitarget antibody were carried out by LC-MS. Samples were reduced with dithiothreitol and injected on a polymeric reversed-phase column (PIN 1912−1802, PLRPS 1000 Å, 50 × 2.1 mm, 8 mm, Varian, Inc., Lake Forest, CA, USA). The separation was achieved at 80 °C using a linear gradient from 34% B to 42% B in 10 min at a flow rate of 0.5 mL/min with detection at 280 nm. Mobile phase A was 0.05% trifluoroacetic acid in water and mobile phase B was 0.04% trifluoroacetic acid in acetonitrile. Eluted peak masses were determined by using a 9520 ESI Q-TOF Accurate Mass LC/MS instrument (Agilent Technologies, Santa Clara, CA, US). Molecular masses were derived from multiply charged ions deconvoluted using MassHunter software (Agilent Technologies). The average DAR was determined by integrating the UV A280 peak areas of the mass-identified heavy and light chain species and calculating the mole fraction of each.

Quantitative MC-vc-PAB-MMAE ADC Total Antibody ELISAs. ELISAs, demonstrating the least amount of impact to binding due to drug load, were developed to accurately measure ADC total antibody concentrations (conjugated and unconjugated antibody; Figure 1A,B). For specific ELISAs, microtiter plates (384-well with MaxiSorp surface; Nunc, Rochester, NY,

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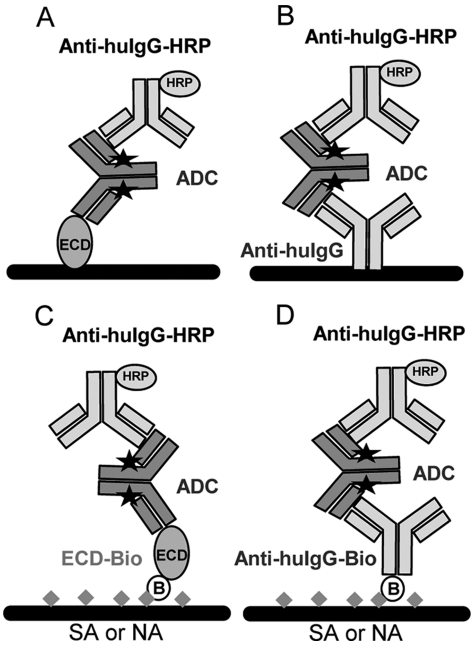

Figure 1. Illustrations of different assay formats. (A) Specific ELISA format: The ADC is captured by either an extracellular domain (ECD) of the target protein, an anti-idiotypic antibody or a peptide and then detected using a goat antihuman IgG (whole IgG or Fc) conjugated to horseradish-peroxidase (HRP). (B) Generic ELISA format: The ADC is captured by sheep antihuman IgG and detected using an antihuman IgG-HRP. (C) Specific SHA format: The ADC is complexed in solution between a biotinylated ECD, peptide, or anti-idiotypic antibody and a goat antihuman IgG-HRP and then captured on a streptavidin (SA) or NeutrAvidin (NA) plate. (D) Generic SHA format: The ADC is complexed in solution between a biotinylated sheep antihuman IgG and a goat antihuman IgG-HRP and then captured on a SA or NA plate.

USA) were coated overnight at 2−8 °C with recombinant human ECD, peptide, or anti-idiotypic antibody (Genentech, Inc.) in coat buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6). For generic ELISAs, 384-well microtiter plates were coated with sheep antihuman IgG antibody (Binding Site, San Diego, CA, USA) in coat buffer. After an overnight incubation at 4 °C, assay plates were washed with wash buffer (PBS, 0.05% polysorbate 20, pH 7.4) and treated with block buffer (PBS, 0.5% BSA, pH 7.4) for 1−2 h before a 2 h incubation with ADC standards and samples diluted in sample buffer (PBS, 0.5% BSA, 0.05% polysorbate 20, 15 ppm Proclin 300, 0.25% CHAPS, 5 mM EDTA, 0.35 M NaCl, pH 7.4). Assay plates were then washed 6 times with wash buffer and incubated for 1 h with goat or sheep antihuman IgG antibody-horseradish peroxidase (HRP) (Bethyl Laboratories, Inc., Montgomery, TX, USA; Binding Site, San Diego, CA, USA, respectively) diluted in detection buffer (PBS, 0.5% BSA, 15 ppm Proclin, 0.05% polysorbate 20, pH 7.4). After washing 6 times, the bound HRP-conjugates were detected by using tetramethyl benzidine (TMB) peroxidase substrate (Moss, Inc., Pasadena, MD, USA), and after 15 min the enzymatic reaction was stopped by the addition of 1 M phosphoric acid. Absorbance was measured at 450 nm against a reference wavelength of 620 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA, or Titertek, Huntsville, AL, USA). The concentration of DAR fractions or ADC in the samples was extrapolated from a 4-parameter fit of the unfractionated ADC standard curve. The standard curve range was specific for each ADC standard. The percent recovery of DAR fractions was calculated by taking the observed concentration of the DAR fractions as measured by ELISA and dividing by the expected concentration (as determined by absorbance). In ELISAs with the most optimal DAR fraction recoveries, rodent and monkey matrix interference was evaluated by measuring unfractionated ADC recovery in buffer with increasing percentage of serum or plasma and compared to recoveries in buffer only. The antihuman IgG antibody reagents used in the total antibody assays have been adsorbed with cynomolgus monkey serum and do not cross-react with cynomolgus IgG and minimally with rodent IgG.

Quantitative MC-vc-PAB-MMAE ADC Total Antibody Semihomogeneous Assay. Semihomogeneous assays (SHAs), demonstrating the least amount of impact to binding due to drug load, were developed to accurately measure ADC total antibody concentrations (Figure 1C,D). Sheep anti-human IgG antibody (Binding Site, San Diego, CA, USA), target ECDs (referred to as C, F, M, and N), and anti-idiotypic antibody (S) were biotinylated. If not already in PBS, proteins were buffer exchanged into PBS using NAP-5 columns (GE Healthcare, Piscataway, NJ) or Zeba columns (Thermo Scientific, Rockford, IL, USA). Proteins were biotinylated for 1−2 h at RT on a rotary shaker using Biotin-LC-Sulfo-NHS ester (Thermo Scientific, Rockford, IL) or Biotin-X-NHS (Research Organics, Cleveland, OH) at a challenge ratio of 1:10 or 1:20 (antibody:biotin). Another buffer exchange was performed to remove unincorporated biotin. For the specific SHAs, approximately equal molar concentrations of HRP-conjugated antihuman IgG antibody and biotin-labeled ECD, peptide, or anti-idiotypic antibody were diluted in sample buffer and premixed. An equal volume of this mixture was then combined with serial dilutions of ADC-containing samples. After preincubating this solution for 2 h at room temperature with shaking, aliquots were transferred to washed 96-well streptavidin plates (preblocked, Pierce/Thermo Scientific, Rockford, IL, US) or 384-well plates coated overnight with 2 μg/mL NeutrAvidin (Pierce/Thermo Scientific) and blocked for 1 h with SuperBlock (Pierce/Thermo Scientific). The complexes were captured onto the plates during a 30 min to 2 h incubation at room temperature with shaking. After washing the plates, color was developed with TMB and read as described above. For the generic SHAs, the sheep antihuman IgG-biotin and goat antihuman IgG-HRP (Bethyl Laboratories, Inc.) were used to bridge the ADCs, and the assays were conducted as described above. Matrix interference of assays with the most optimal DAR fraction recoveries, for each ADC, was determined as mentioned above.

Pharmacokinetic Studies of MC-vc-PAB-MMAE ADC in Mice. Plasma PK studies were conducted at Genentech, Inc., in compliance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC). The studies were done in non-tumor-bearing severe combined immunodeficiency (SCID) mice. Mice were randomized into 3 groups of 15. Animals were fasted overnight before dosing with anti-N-MMAE conjugates at 0.5 or 5 mg/kg as a single intravenous dose. After dosing, mice (2 or 3 time points per mouse and 3 mice per time point over 13 time points) were anesthetized by isoflurane inhalation, and blood (100−150 μL) was collected by puncture of the retro-orbital plexus at 5 min; 1, 3, and 6 h; and 1, 2, 3, 4, 7, 10, 14, 21, and 28 days after injection. Blood was transferred to lithium heparin tubes and

immediately placed on ice until centrifugation for collection of the plasma samples. Plasma samples were diluted 1:100 in sample buffer and serially diluted 3-fold before analyzing in assays as mentioned above. The concentration of total antibody, conjugated and unconjugated, in the samples was extrapolated from a 4-parameter fit of the unfractionated ADC standard curve. The unfractionated ADC used to generate the standard curve was equivalent to the ADC used to dose the mice. The mean total antibody time−concentration data generated using the assays mentioned above were analyzed using a two-compartmental analysis (Model 8, WinNonlin-Pro, v 5.2.1; Pharsight Corporation; Mountain View, CA) to obtain the clearance (CL), maximum concentration (C_{max}), beta half-life ($t_{1/2}$), volume of distribution ($V1$), and drug exposure (AUC) for MMAE conjugated anti-N. The statistical significance of differences in PK parameters was assessed using an unpaired two-tailed t test (GraphPad Software, La Jolla, CA, USA).

RESULTS

Purified ADC DAR Fractions with High MMAE Drug Load Were Under-Recovered in Total Antibody ELISAs Using Various Formats and Reagent Conditions. MMAE-conjugated ADCs are heterogeneous by nature. We therefore investigated the effect of total antibody assay format (Figure 1A−D) and reagent conditions on the detection of purified MC-vc-PAB-MMAE ADC fractions with uniform DARs. We isolated different DAR species from various MMAE conjugated ADCs (anti-B, -C, -F, -M, -N, and -S) by HIC (DARs of 0, 2, 4, 6, and 8 MMAE per antibody) post antibody purification and drug conjugation. A representative HIC profile of different DAR species (DARs of 0, 2, 4, 6, and 8 MMAE per antibody) is shown in Figure 2A and Supporting Information Figure S1. With the exception of anti-F DAR 6 (38%) and DAR 8 (70%) fractions, purified DAR fractions contained less than 10% aggregate (see Supporting Information Table S2). These purified fractions of MMAE-conjugated ADCs with varying DARs were assayed in both the specific and generic total antibody ELISA formats (Figure 1A,B) using multiple capture (peptide, ECD, antibody) and detection antibody concentrations. Assay performance was assessed by quantifying the percent recovery of purified DAR antibody based on the unfractionated ADC standard antibody concentration in buffer. DAR fractions were evaluated in buffer after ELISA assays were confirmed to tolerate matrix interference. The standards used to quantitate the DAR fractions were unfractionated ADCs with an average DAR between 3 and 4 (see Supporting Information Table S1). DAR fraction recoveries for a representative ADC molecule (anti-N) using specific ELISAs with different reagent concentrations are shown in Figure 2B. The percent recovery of DAR fractions was calculated as mentioned in the methods. For MMAE-conjugated anti-N (Figure 2B), the DAR 6 and 8 recoveries were less than 80% using all conditions tested. Analysis of 6 different MMAE-conjugated ADCs with ELISAs showed that the mean recovery (±SD, n = 6) of the lowest drug loaded species (DAR 0 or unconjugated anti-S antibody) was 101% ± 10% and 101% ± 8.9% in the specific and generic ELISAs, respectively, while for the highest drug loaded species (DAR 8, excluding anti-B), the recoveries were 58% ± 12% and 71% ± 15% (mean ± SD, n = 5) in the same specific and generic ELISAs (Figure 3). Thus, in most of the ELISA formats evaluated, antibodies with high drug loads resulted in concentration recoveries that were on average 20% lower than expected.

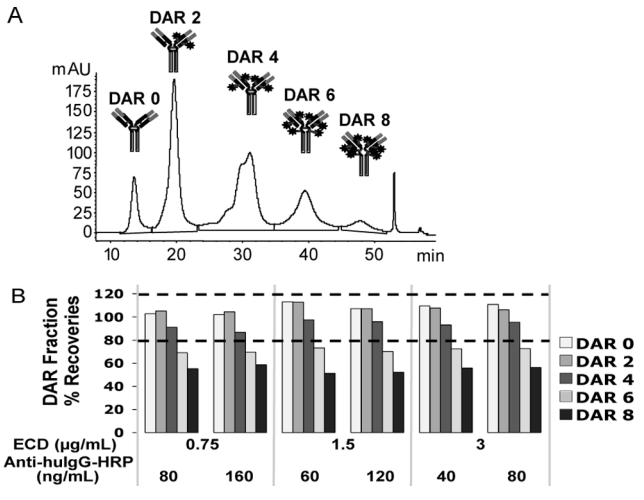

Figure 2. (A) Representative hydrophobic interaction chromatography (HIC) profile of different DAR species (a DAR of 0, 2, 4, 6, and 8 MMAE per antibody). DAR fractions were isolated from various MMAE-conjugated ADCs (anti-B, -C, -F, -M, -N, and -S). Different capture and detection reagent concentrations were evaluated in the specific total antibody ELISA and ADC fraction recoveries were analyzed for anti-N (B), a representative MMAE-conjugated antibody. DAR: Drug to Antibody Ratio. DAR 0: no drug on antibody, DAR 8: 8 drugs on antibody.

Quantification of Purified ADC DAR Fractions in Total Antibody Semihomogeneous Assays Resulted in Improved Recovery. The various DAR fractions were used to help develop and evaluate additional assay formats for optimal and equal quantification of DAR fractions, irrespective of the drug load. We proceeded to develop a total antibody ELISA where preformation of protein complexes in solution occur prior to capture onto ELISA plates (semihomogeneous assay or SHA). This ELISA format, with a homogeneous solution phase preincubation step and a reduced number of washing steps, was evaluated to determine whether this assay format would improve quantitation of the higher drug-loaded antibodies. We optimized specific and generic SHAs (Figure 1C,D) for all 6 ADCs using multiple capture and detection conditions. After confirming that assays tolerated rodent and monkey serum and/or plasma, we performed DAR fraction analysis in the absence of added matrix. SHA formats demonstrated equivalent matrix interference as compared to stepwise ELISA formats for ADCs tested (data not shown). The DAR fraction recovery results showed that almost all the ADC DAR fractions, particularly those with higher drug loads, had improved recovery in the SHA format as compared to the stepwise sandwich ELISA formats, using unfractionated ADCs with average DARs of ∼3.5 as standards (Figure 3). Thus, the purified fractions of MMAE-conjugated ADCs with DAR of 0, 2, 4, 6, and 8 MMAE per antibody recovered more similarly to each other as compared to the stepwise ELISAs. The average recoveries of the DAR 0 (unconjugated antibody for anti-S) fractions were 97% ± 20% and 96% ± 18% in the specific and generic SHAs, respectively (mean ± SD for the 6 ADCs). The average recoveries of DAR 8 (excluding anti-B) in the same SHA assays were 81% ± 18% and 86% ± 11%, respectively (mean ± SD for the 5 ADCs). Most of the DAR fractions analyzed using SHAs resulted in recovered concentrations

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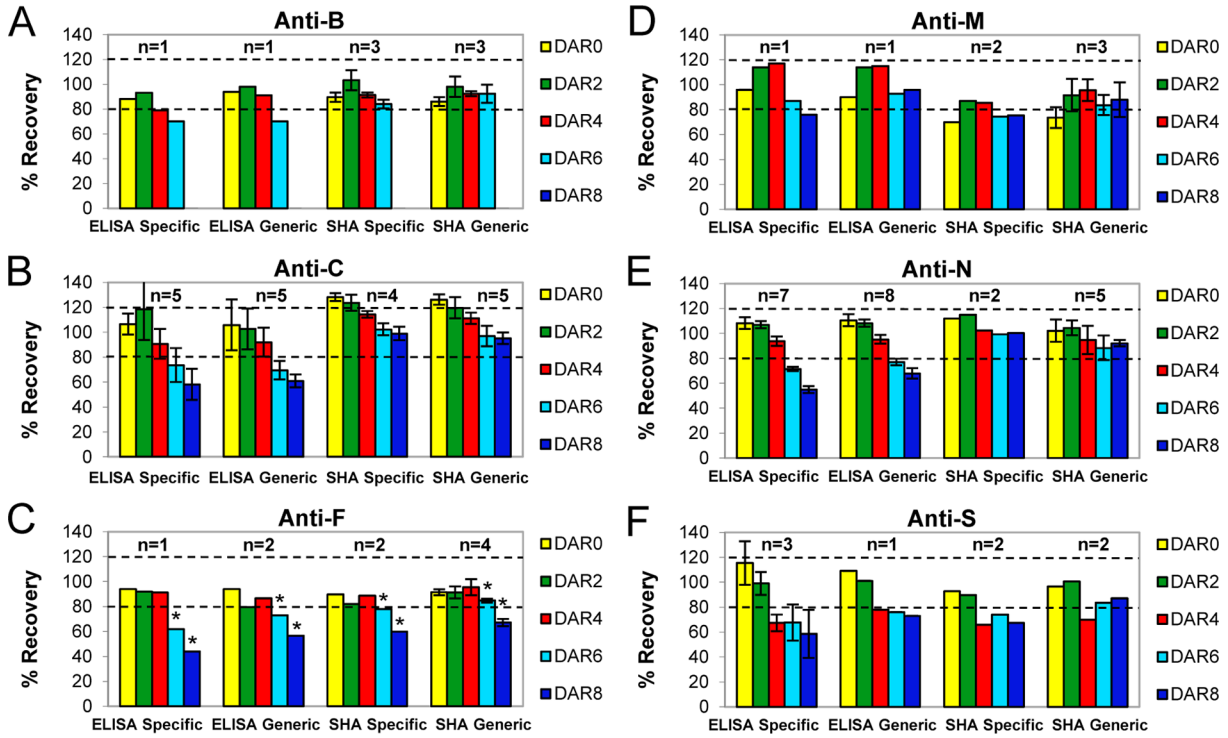

Figure 3. Evaluated fraction recovery of purified MMAE-conjugated ADC DARs in optimized specific and generic ELISAs and SHAs. DAR fraction recoveries for six different MMAE-conjugated antitarget antibodies were compared. An unconjugated antibody was used as DAR 0 for anti-S in place of a mock conjugated DAR 0, as the latter sample was not available. The highest DAR fraction analyzed for the MMAE-conjugated anti-B ADC was a DAR 6. The anti-F ADC DAR fractions 6 and 8, designated by an *, were both highly aggregated (37% and 70%, respectively). For those fractions and assays where at least three independent runs were performed, data is shown as mean ± SD, while duplicate runs (n = 2) are presented as the mean.

within the expected 100% ± 20%; an exception was the highly aggregated fractions (for anti-F, DAR 6 and DAR 8 were 38% and 70% aggregated, respectively; Figure 3C). Of the 6 ADCs analyzed, 5 yielded better recovery of the DAR fractions in the SHA than in the stepwise ELISA, while the recoveries for anti-M were fairly similar in all assay formats (Figure 3D).

Different Assay Formats Resulted in Distinct PK Profiles. We further evaluated the impact of assay choice on plasma PK profiles of anti-N-ADC (shown to have improved DAR fraction recovery in the SHA format; Figure 3E) in SCID mice. Samples were analyzed using the two total antibody assay formats that resulted in the greatest difference in average DAR recoveries based on all ADCs evaluated: the specific ELISA (average DAR 8 recovery = 58% ± 12%) and the generic SHA (average DAR recovery = 86% ± 11%). As expected for a nontarget binding antibody and its conjugates, the PK profiles of the 0.5 and 5 mg/kg dose levels were similar and dose proportional. The total antibody concentrations measured by the generic SHA were consistently higher than those measured by the specific ELISA (Figure 4A,B; Table 1). Consequently, total exposures measured as AUC were statistically different at both dose levels when measured by the two different assay formats (Table 1). The calculated total antibody clearance rate was 10.9 and 10.1 mL/day/kg at 0.5 and 5 mg/kg, respectively, when assayed using the specific ELISA (the assay with DAR 6 and 8 recoveries <80% of expected) versus 7.89 and 8.07 mL/day/kg at 0.5 and 5 mg/kg, respectively, when assayed using the generic SHA (the assay with DAR recoveries >80% of expected). At both dose levels, the clearance rates derived from the two analytical formats were statistically different (p < 0.01).

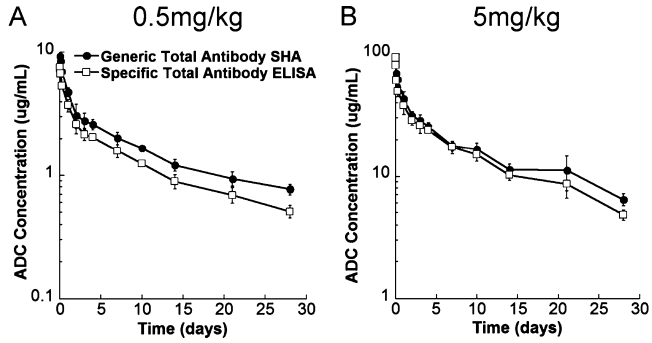

Figure 4. Plasma PK profiles of ADCs in mice with different assay formats and reagents. Mice were injected with a single IV dose of MMAE-conjugated anti-N at 0.5 mg/kg (A) and 5 mg/kg (B) and plasma samples were collected over time up to 28 days. Sample analysis was conducted using two different total assay formats: Specific ELISA and generic SHA. Data points represent mean ± SD, n = 3.

DISCUSSION

Although ELISAs are commonly used to quantify MMAE-conjugated ADCs, few publications have reported on assay sensitivity to ADC-specific parameters, such as DARs, assay performance optimization through assay format and reagent selections, and subsequent PK interpretations. While both Sanderson et al.[12] and Boswell et al.[19] mention that their total antibody assays are minimally impacted by antibody drug load, Stephan et al.[20] demonstrated that the different effects of the MCC-DM1 and MC-MMAF conjugation chemistries on the antibody structure affected their quantification in ELISAs. The DM1 drug load on the antibody not only reduced the

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Table 1. Pharmacokinetic Parameters Based on the Total Antibody Concentrations as Measured Using Two Assay Formats with the Greatest Difference in DAR Fraction Recovery[a]

		total antibody assays	
PK parameters	dose (mg/mL)	generic SHA	specific ELISA
CL* (mL/kg/day)	0.5	7.89 ± 0.289	10.9 ± 0.331
	5.0	8.07 ± 0.366	10.1 ± 0.334
*T*1/2(days)	0.5	14.0 ± 1.04	12.4 ± 0.789
	5.0	13.9 ± 1.29	10.6 ± 0.590
*V*1 (mL/kg)	0.5	53.9 ± 2.23	67.5 ± 2.70
	5.0	60.6 ± 3.09	59.2 ± 3.53
AUC* (day x ug/mL)	0.5	63.4 ± 2.32	45.7 ± 1.38
	5.0	619 ± 28.0	496 ± 16.4
C_{max}	0.5	9.28 ± 0.384	7.41 ± 0.297
	5.0	82.5 ± 4.21	84.4 ± 5.03

[a]Error represented by standard deviations (n = 3). *Indicates the differences between assay formats are statistically significant ($p < 0.01$ by two-tailed unpaired t-test).

antibody's ability to bind to the capture antigen, but also decreased the ability of the goat antihuman IgG (Fab'2)-HRP detection antibody to bind to the ADC. The anti-CD22 DM1 PK profiles resulted in differences when serum samples were analyzed in these two assays, although they were not significant. Most other reports quantitating ADCs focus mainly on describing the PK[9,13−18] without including assay characterization information.

We therefore evaluated the impact of assay format and reagent selection on quantitation of protease-cleavable MMAE ADCs in total antibody ELISAs typically used to generate PK data. Fractions of MMAE-conjugated antitarget antibodies with 0, 2, 4, 6, and 8 MMAE per antibody were purified using hydrophobic interaction chromatography and characterized by LC-MS. We then evaluated the quantification of the DAR fractions in different total antibody ELISA formats performed using various concentrations of the assay reagents. Once we determined that the DAR fractions for some ADCs could not be recovered to within 100% ± 20% of the expected concentrations under any of the conditions tested for both the specific and generic total antibody ELISAs, we evaluated an alternative immunoassay format involving a homogeneous solution phase incubation prior to capture of antibody complexes onto the ELISA plate. In this semihomogeneous assay (SHA) for total antibody, the ADC is preincubated together with the biotinylated and HRP-conjugated assay reagents followed by capture onto a NeutrAvidin- or streptavidin-coated plate and addition of substrate. Theoretically, the SHA format could improve quantification of the higher MMAE-loaded antibody species due to the reduced number of wash steps and the fact that the capture reagent is presented to the ADC without the denaturation effects that could potentially occur upon direct coating onto plastic plates. Using the specific and generic SHA formats (Figure 1C and D), we observed improved DAR fraction recovery (Figure 3), including the highly drug-loaded species and highly aggregated DAR fractions (for anti-F, DAR 6 and DAR 8 were 38% and 70% aggregated, respectively).

In our hands, besides improved DAR fraction recovery, an additional advantage of the SHA format was that it seemed to exhibit greater robustness than the stepwise ELISA, since changes in reagent concentrations had less impact on DAR recovery in the SHA (data not shown) as compared to the ELISAs (Figure 2B). Another advantage of the SHA is that the incubation time of the capture step could be shortened to 30 min for faster throughput. SHA formats also had acceptable plasma and serum matrix interference tolerance similar to the ELISAs, allowing all assays used to measure DAR fraction recoveries and PK analysis (ELISA and SHA formats), to be performed in the absence of added matrix. Unlike the ELISA, however, the SHA displayed a hook or Prozone effect at ADC concentrations above the standard curve range (see Supporting Information Figure S2). This was overcome by analyzing the samples at multiple serial dilutions. Any dilutions that were nonlinear and fell within the Prozone/hook effect were excluded. The final sample concentrations were based on at least 3 linear consecutive dilutions outside the area of the Prozone/hook effect.

Our results suggested that the generic assays, whether a stepwise sandwich ELISA or a SHA (Figure 1B and D), appear to have slight to moderate improvement in recovery of the higher drug-loaded ADC DAR species as compared to the specific assays (DAR 8 recovery: ELISA, specific 58% ± 12% and generic 71% ± 15%; SHA, specific 81% ± 18% and generic 86% ± 11%; mean ± SD, n = 5). These results may imply that the MMAE conjugation could interfere with the antigen binding capacity in various ELISA formats. This effect was observed for purified DARs as low as 4 MMAE per antibody. The binding of generic assay reagents to the antibody, such as the polyclonal antihuman IgG antibody, may be less impacted by conjugation of drug to the antibody because these reagents are not solely dependent on binding to the variable region. It appeared that the specific assay, which relies on antigen binding, could potentially produce less accurate results than total antibody quantification using other assay designs when standard composition is different from sample. Based on our DAR recovery data for multiple ADCs, the generic SHA yielded recoveries closer to the expected concentrations of the DAR fractions as compared to the other three total antibody assays (generic SHA > specific SHA > generic ELISA > specific ELISA), and therefore we utilized this assay format to evaluate MMAE-conjugated ADC PK profiles for preclinical animal studies. Moreover, the generic SHA displayed minimal matrix interference and was suitable for analysis of PK samples from multiple species.

For MMAE-conjugated anti-N ADC quantitation in the total antibody assays, the improvements in the DAR fraction recovery seen with the SHA formats translated into statistically significant changes in terms of key PK parameters, such as clearance and AUC. In this study, we observed that the differences in PK profiles reflected the DAR recovery difference in assay formats. For example, data generated from the specific SHA format situated between data generated from the generic SHA and specific ELISA formats (Supporting InformationTable S3) as expected from the respective recovery data (Figure 3E). The divergence of PK profiles was more pronounced at later time points, which could be driven by multiple factors. First, the unfractionated ADC standard was used to quantitate the mouse PK samples dosed with the same unfractionated ADC. At early PK time points, the amount/ratio of high DAR species are similar to the standard, and the impact from recovery differences may not be observable. At later time points, as drug is being released *in vivo* over time, the drug load on the sample ADC would be less likely to match the standard, which makes recovery issues more noticeable. Second, the high

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DAR species may have higher clearance[9] and their diminishing presence over time could also contribute to the difference between assays. Although it is not clear whether the purified DAR preparations are structurally identical to the DAR species found *in vivo*, or if improved DAR fraction recovery translates into changes in PK profiles, our study showed that utilizing DAR fractions can be helpful in evaluating and optimizing total antibody assays for quantitating antibodies with varying drug loads.

In conclusion, we evaluated how assay formats and reagent choice affected quantitation of protease-cleavable MMAE conjugated ADCs. Using MMAE-conjugated ADC DAR fractions with 0, 2, 4, 6, and 8 MMAE per antibody, we found that the total antibody ELISAs were not all able to recover different DAR fractions similarly when utilizing an unfractionated ADC representative of the dosing material for the assay standard curve. Of the molecules tested, both specific and generic SHA formats seemed to show improved recovery of ADC fractions, with the generic SHA format resulting in recoveries slightly closer to expected. The improvement in DAR fraction recovery as seen with the SHA format translated into statistically significant changes in key PK parameters for the anti-N conjugate in a mouse PK study, such as clearance and AUC. Of the six different MMAE-conjugated ADCs tested, the SHA format was shown to improve DAR fraction recovery for five out of the six molecules as compared to the sandwich ELISA total antibody assay formats. Thus, the SHA format is our preferred method for quantitating MMAE-conjugated ADCs in preclinical animal study samples. However, appropriate assay format and reagents should nevertheless be evaluated carefully for each molecule. Given that the drug loss and the clearance rate for the varying DAR species of MMAE conjugate can differ, resulting in a change of the DAR distribution profile during the PK sampling time course, it is important to develop and choose a total antibody assay format that does not discriminate drug load.

■ ASSOCIATED CONTENT

Supporting Information

Percent of DAR species, Table S1; Percent aggregation of DAR fractions, Table S2; PK parameters of SHA format, Table S3; HIC profile of anti-B purity, Figure S1; Prozone/hook effect of anti-N in generic SHA, Figure S2. This information is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: kkozak@gene.com; phone: (650) 225-1831.

Notes

The authors declare the following competing financial interest(s): Authors are employees of Genentech, a member of the Roche group, and hold financial interest in Roche.

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■ ABBREVIATIONS

ADC, antibody−drug conjugate; DAR, drug-to-antibody ratio; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; HIC, hydrophobic interaction chromatography; mAb, monoclonal antibody; SHA, semihomogeneous assay; PK, pharmacokinetics

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