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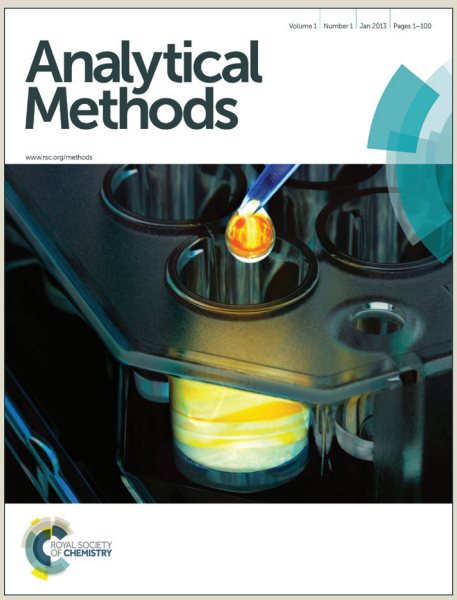

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Detection of Residual Biocides in Antibody Drug Conjugates for ImmunoPET Imaging

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Abstract

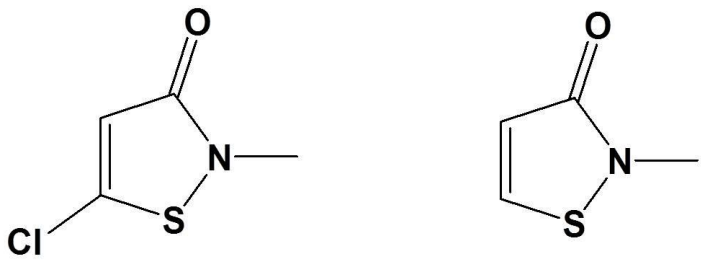
Antibody Drug Conjugates are comprised of monoclonal antibodies conjugated to a small molecule drug to combine the targeted delivery of the antibody with the mechanism of action of the small molecule component. One class of antibody drug conjugates is used in ImmunoPET and combines the selectivity of the antibody with a Positron Emission Tomography imaging agent that enables imaging tumors based on binding of the antibody. As the Positron Emission Tomography imaging reagents are limited by their radioactive half-life, they are prepared at a single patient GMP quality scale with limited advance notification of when a patient requires the dose. One aspect of this single batch manufacture is using PD-10 columns to purify the ImmunoPET agent from the excess radioactive material used in the manufacturing process. These columns are kept in a storage solution containing biocides, 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one, which prevent microbial growth in the column but could also adversely affect the patient if injected. We have developed a validatable HPLC-UV/MS method for the detection of biocides in formulated ImmunoPET drug product samples and formulation buffer. This method enables the demonstration of the clearance of biocides in the columns prior to use and quantitation of any residual biocides present in the drug product.

Introduction

Antibody Drug Conjugates (ADCs) are an emerging type of therapeutic molecule that combine the selectivity of monoclonal antibodies with the mechanism of action of a small molecule drug. As evidenced by recent approvals of Kadcyla [1] and Adcetris [2], this combined approach successfully utilizes the advantages of each class of molecule. Most commonly, ADCs consist of highly potent toxins conjugated to monoclonal antibodies which are targeted to a specific type of cancer. In these ADCs, the monoclonal antibody guides the toxin to the tumor site, binds to a specific receptor and gets internalized. Once internalized, the toxin is released and inhibits the growth of the tumor. Another class of ADC molecules, radioimmunoconjugates as used in ImmunoPET, utilizes the approach of combining the selectivity of monoclonal antibodies with Positron Emission Tomography imaging agents. By combining monoclonal antibodies and positron emitters, PET imaging can be used for tumor detection, quantitative imaging, demonstrating *in-vivo* antibody binding and localization, and even dosimetric determinations. ImmunoPET molecules have been used previously to image tumors associated with HER2 positive xenograph models [3], prostate specific membrane antigen [4], head and neck squamous cell carcinoma [5] and de2-7 epidermal growth factor receptor in Glioma [6].

The major limitation of ImmunoPET imaging is that the imaging agents are radioactive which limits their shelf life due to the radioactive half-life of the imaging agent. For example, the half-life of ^{89}Zr and ^{124}I, two common positron emitters, are 3.27 days and 4.18 days respectively [7]. While this is an effective half-life for PET imaging agents, it presents limitations for large scale GMP production as the material cannot be stored for a prolonged period of time. Typically GMP ADCs are manufactured at a sufficient scale to provide clinical trials or commercial markets with shelf lives extending two years or more. The short half-life of positron emitters, however, requires the ImmunoPET agent to be dosed within a week of manufacture. This necessitates a GMP manufacture of a single patient scale.

One of the keys steps in the manufacture of the radiolabeled antibody is the separation of the unconjugated radioactive material from the antibody. The removal of the unconjugated radioactive material is important both from a patient safety perspective to reduce unnecessary exposure of the patient to radioactivity and from an imaging perspective to limit the background signal during PET imaging. The separation of the ADC and excess radioactive label at a single patient scale is typically performed with a PD-10 desalting column. PD-10 columns work through a size exclusion principle where the ADC is excluded from the smaller pore and elutes first whereas the smaller components such as unconjugated ^{89}Zr require longer to elute due to passing through the column matrix. The single patient approach also requires the materials to be on hand for immediate manufacture as patients requiring PET imaging delay treatment increasing the need for a fast manufacturing of the imaging agent. In order to prevent microbial growth during storage of the column, the columns are stored with a biocide. Storage without a biocide could lead to microbial growth in the column causing unacceptably high levels of endotoxin and bioburden exposure to patients. The most common biocide used for these applications is Kathon CG/ICP which is a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI) with structures illustrated in Figure 1. However, while Kathon biocides prevent microbial growth, they also have previously been shown to act as sensitizers [8] and have produced a positive response in an Ames bacterial mutation assay [9]. As a possible genotoxic impurity, the European Medicines Agency (EMEA) recommends the threshold of toxicological concern to be 1.5µg/day requiring sensitive methods for the determination of such impurities.



5-Chloro-2-methyl-4-isothiazolin-3-one (CMI) **2-Methyl-4-isothiazolin-3-one (MI)**

Figure 1: Structures for 5-Chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI).

As a potentially harmful impurity in a parenteral product, the biocide levels in the drug product need to be controlled to low levels. Previous methods to analyze and detect the biocides have been focused on cosmetics and environmental monitoring and not on pharmaceutical parental products. These have included second derivative UV analysis for Kathon standards and simulated cosmetic emulsions [10], large volume injection HPLC-MS/MS detection in water samples [11], and solid phase microextraction treated environmental samples followed by GC-MS [12] based approaches. While these approaches have been effective for cosmetics and environmental analysis, for ADCs the detection requires a validated QC method either in the presence of a formulated protein drug protein or with the proper sample preparation to remove the protein. In this work we have developed a validatable QC HPLC-UV method for the sensitive detection and quantification of the biocides in formulated ADC drug product. After development a full method assessment has been performed to demonstrate the method possesses sufficient precision, accuracy, specificity, linearity, and limit of quantification to detect the biocides in a QC environment for an ADC drug product.

Methods and Materials

Sample Information

5-chloro-2-methyl-4 isothiazolin-3-one was purchased from Santa Cruz Biotechnology, 2-methyl-4-isothiazolin-3-one was purchased from Fluka. A stock solution of 450ppm MI and 305ppm CMI was prepared in Milli-Q purified water. 1mg/mL Desferrioxamine *N*-succinate labeled monoclonal antibody was prepared at Genentech, Inc. and formulated 10mM Sodium Succinate, 240mM Sucrose, 0.02% Polysorbate 20, pH 5.5. Cold material unlabeled with ^{89}Zr was used for all experiments to minimize radioactive exposure for safety purposes.

Chromatographic Conditions

All chromatography was performed on an Agilent 1290 chromatography system with diode array detector. An XSelect CSH Phenyl-Hexyl 3.5µm 4.6 x 150mm column (Waters) was used for the separation at 50°C. Mobile phase A was composed of 0.1% Formic Acid in Milli-Q purified water with mobile phase B comprised of 0.1% formic acid in acetonitrile with the gradient listed below. UV detection was performed at 275nm with a 4nm bandwidth. Mass spectrometry was performed on the same chromatographic system with an Agilent 6150 single quadrupole mass spectrometer. Data was collected and integrated using Chemstation software with subsequent data analysis performed in Microsoft Excel.

Gradient

Time (min)	%MPA	%MPB
0.0	98.0	2.0
5.0	98.0	2.0
20.0	50.0	50.0
20.1	0.0	100.0
30.0	0.0	100.0
30.1	98.0	2.0
40.0	98.0	2.0

Results and Discussion

As a drug product used for PET imaging, ADCs are administered as an IV injection directly into the bloodstream of the patient. This requires the biocides be cleared from the PD-10 column prior to use and as well as assurance that the biocide levels are below ppm levels in the final drug product. In

order to demonstrate clearance of the residual Kathon biocides in the protein drug product, we focused on developing an HPLC assay with both UV and MS detection capability. The chromatographic method was developed through column screening along with column temperature and gradient optimization on MI and CMI standards. The sensitivity between mass spectrometric and UV detection at 275nm was also assessed using biocide standards. The final method is described in the experimental section with a representative chromatogram shown in Figure 2. In this sample MI and CMI are spiked into formulated ADC.

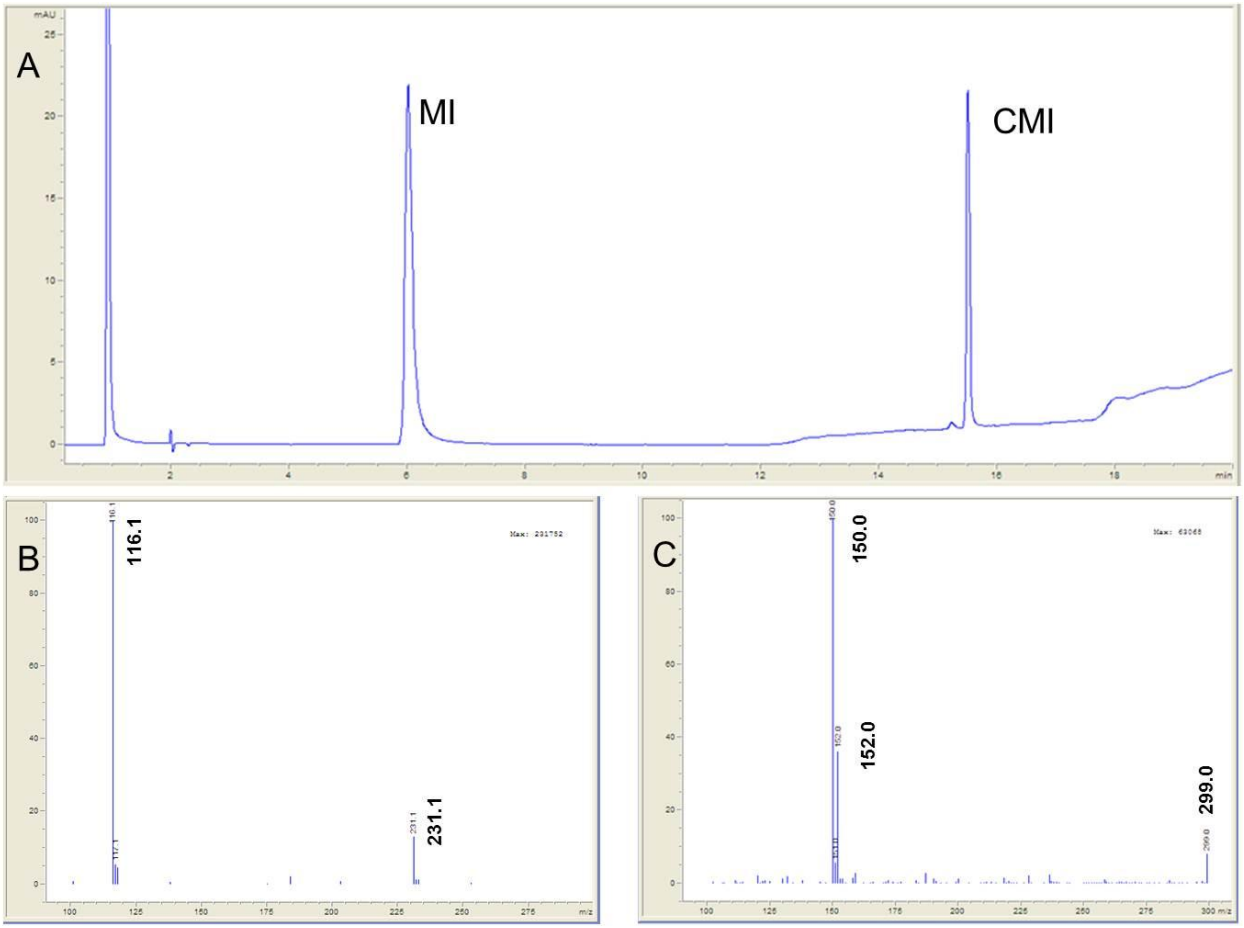


Figure 2: A) Representative Chromatogram of the biocides MI and CMI using detection at 275nm. B) Mass Spectrum of the peak labeled MI. C) Mass Spectrum of the peak labeled CMI.

Based on the standards of MI and CMI, the single quadrupole MS detection had slightly better sensitivity however using the UV signal at 275nm gave an LOQ of 50ppb for the biocides which was sufficient sensitive for this application. UV detection also has the advantages of being simpler from a QC and instrumental perspective and was therefore selected as the detection method for this application. Looking at the mass spectrum associated with each peak, the first peak labeled MI has an $[M+H]^+$ of 116.1 m/z which is consistent with MI while the peak labelled CMI has an $[M+H]^+$ of 150.0 m/z which is consistent with the mass of CMI. The mass spectra also indicate that neither biocide has any coeluting excipients that may interfere with obtaining accurate results.

As this method needs to quantify the amount of each biocide in an ADC, a spiked recovery experiment was also performed as part of the sample preparation method development. MI (2.25ppm) and CMI (1.53 ppm) were spiked into both formulation buffer and formulated ADC to assess the impact of the proteins themselves on the analysis. Additionally, since removal of the protein prior to analysis is desirable when trying to detect small molecules in an ADC, two common methods of protein removal techniques (protein precipitation and filtration) were evaluated along with direct injection (no protein precipitation). The precipitation was performed by adding 2 equivalents of acetonitrile followed by incubation at -20°C for 30 minutes. This was followed by centrifuging the sample at 4000rpm for 10 minutes and transferring the supernatant to an HPLC vial. Filtration was performed using an Amicon Ultra Ultracel 10K 10,000 molecular weight cutoff centrifuge filter and centrifuging at 4000rpm for 45 minutes. The flow-through from the filter was then transferred to an HPLC vial and analyzed. All preparations were performed in triplicate with the average integrated peak areas for each preparation technique shown in Figure 3.

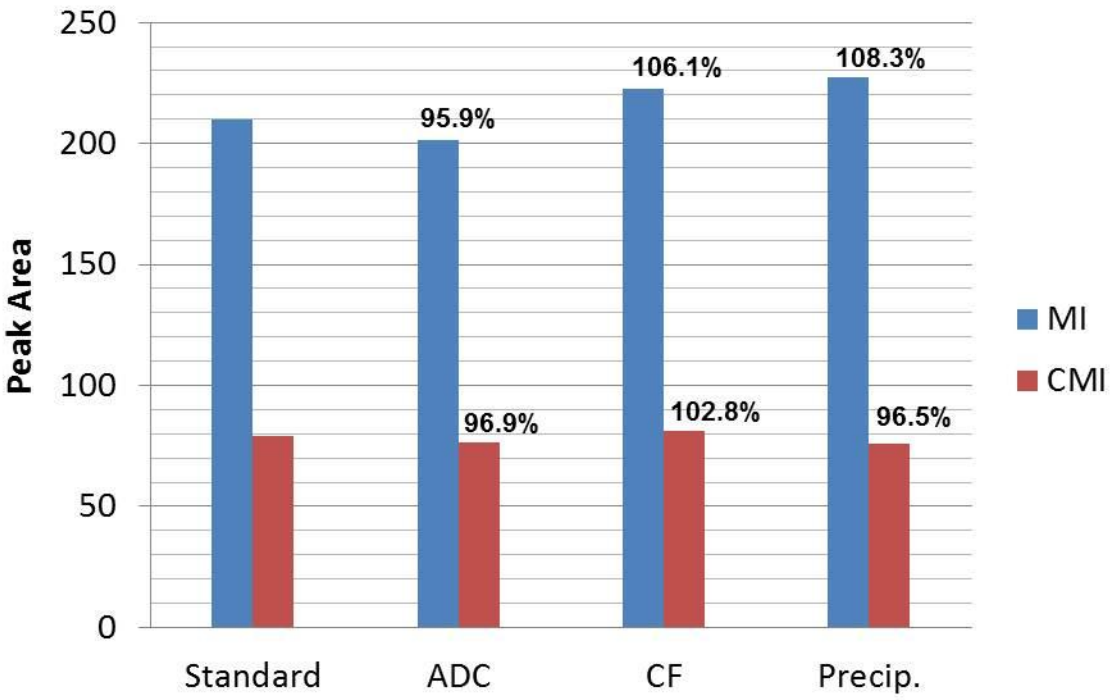


Figure 3: Average integrated peak areas and spike recoveries for each preparation method including the MI and CMI in formulation buffer (Standard), in formulated ADC (ADC), samples prepared via centrifuge filtration (CF), and samples prepared through precipitation in acetonitrile corrected for the dilution factor (Precip.).

The direct injection and centrifuged filtered samples gave good recoveries and had %RSDs for the three preparations below 3% for both MI and CMI. The precipitation preparation corrected for the dilution factor had slightly reduced recoveries and a much larger %RSD for the three replicates at 10.6% for MI and 10.1% for CMI. The addition of acetonitrile for protein precipitation also impacts the sensitivity due to the overall dilution of the sample. Chromatographically, the analyte peaks in the 2:1 Acetonitrile:Formulation buffer exhibited peak splitting with the entire split peak integrated for the recovery calculation. Further dilution with Milli-Q purified water at a 1:1 ratio did not significantly improve the peak shape. Both the direct analysis of the ADC sample and the centrifuge filter preparation gave acceptable results for %RSD and recovery. Based on the acceptable performance and simplicity of the sample preparation, direct analysis was used in the actual method assessment experiments.

Method Assessment

With the sample preparation established in the previous experiment, the method was assessed based on several common validation parameters, including the method and system precision, linearity, and the limit of quantitation. Demonstration of the adequacy of the other additional validation parameters was not deemed necessary as spiked recovery was assessed in the prior experiment while solution stability was not needed due to nature of the same day GMP manufacture and QC release of the ImmunoPET ADCs. Demonstration of method precision involved six replicate preparations of the sample while the system precision used six replicate injections from the same sample. The samples for these measurements used 1mg/mL ADC in the formulation buffer spiked with 2.25ppm of MI and 1.53 ppm of CMI. The results for the six replicate injections and sample preparations are shown in Table 1.

Table 1: System and Method Precision Results for the detection of Biocides in ADC samples.

	System Precision				**Method Precision**			
	MI		**CMI**		**MI**		**CMI**	
Sample	**RT**	**Peak Area**	**RT**	**Peak Area**	**RT**	**Peak Area**	**RT**	**Peak Area**
1	6.03	114.13	15.50	45.08	6.02	114.25	15.46	44.62
2	6.01	114.57	15.40	44.36	6.03	113.77	15.65	42.94
3	6.03	115.76	15.56	45.10	6.03	114.15	15.51	42.21
4	6.03	115.29	15.54	44.15	6.05	112.32	15.73	41.96
5	6.03	113.65	15.53	44.32	6.04	113.63	15.71	42.89
6	6.02	114.45	15.38	43.78	6.05	113.07	15.69	42.60
Avg	**6.03**	**114.64**	**15.48**	**44.46**	**6.04**	**113.53**	**15.63**	**42.87**
St.Dv.	0.01	0.77	0.08	0.53	0.01	0.73	0.11	0.94
%RSD	**0.14**	**0.67**	**0.50**	**1.18**	**0.23**	**0.64**	**0.71**	**2.19**

The method demonstrated suitable system and method precision with respect to the peak areas of MI with %RSDs of 0.67% and 0.64% for the system and method precision respectively. The %RSDs for CMI in both the system and method precision were higher at 1.18% and 2.19% respectively although the results are still acceptable and indicate the method is suitable for its intended purpose.

The linearity of the method is critical for quantitation as the assay response must be linear and proportional to the actual amount in the sample. The linearity of MI and CMI were assessed from the low ppm levels to 10's of ppbs (the relevant concentration range for the biocide assay) in 1mg/mL ADC in formulation buffer. Figure 4 shows the response curves for both MI and CMI at various concentrations.

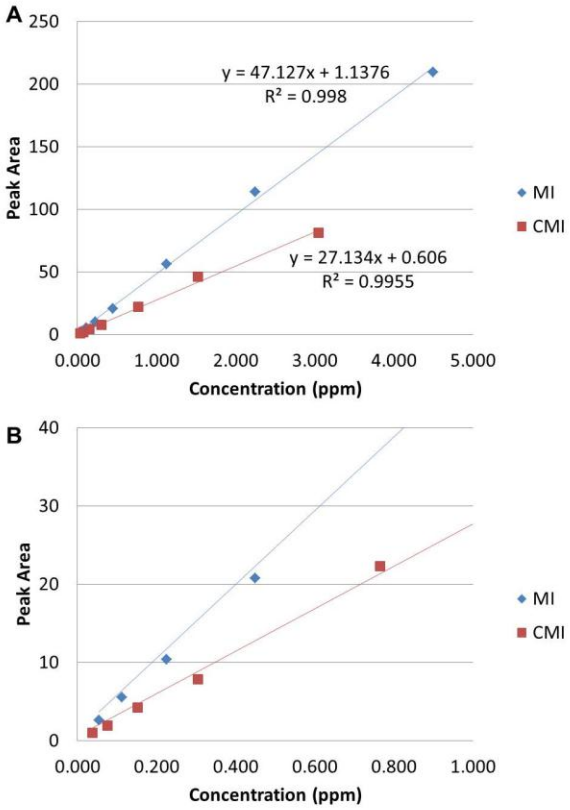


Figure 4: A) Response curves for MI (4.50ppm, 2.25ppm, 1.13ppm, 0.45ppm, 0.23ppm, 0.11ppm, and 0.055ppm) and CMI (3.05ppm, 1.53ppm, 0.77ppm, 0.31ppm, 0.15ppm, 0.077ppm, and 0.037ppm). B) Response curve in Figure 3A enlarged to show lower concentrations.

The response curves for both MI and CMI demonstrated good linearity with R^2 values greater than 0.995 for both analytes. The response curves also show good linearity for the lower concentrations tested that were below 1ppm indicating the method is suitable for quantitating residual levels of the biocides even in a formulated ADC sample.

In order to determine the lowest amount that the assay could quantitate, the Limit of Quantitation was experimentally determined. Replicate injections of 55ppb MI and 37ppb CMI spiked into a formulated ADC sample were analyzed and the %RSD and the signal to noise ratio of the MI and CMI peaks were assessed. Acceptable results for a limit of quantitation were defined as an average signal to noise ratio of greater than 10 with a corresponding %RSD of below 10.0%. The results of the limit of quantitation experiment are shown in Table 2.

Table 2: Results of the LOQ determination for MI and CMI.

	MI			CMI		
Injection	**RT**	**Peak Area**	**S/N**	**RT**	**Peak Area**	**S/N**
1	6.031	2.620	19.393	15.570	0.977	17.328
2	6.029	2.466	18.926	15.533	0.984	18.688
3	6.016	2.311	18.660	15.272	0.981	16.301
Avg	**6.025**	**2.466**	**18.993**	**15.458**	**0.980**	**17.439**
St.Dv.	0.008	0.154	0.371	0.162	0.004	1.197
%RSD	**0.135**	**6.264**	**1.953**	**1.051**	**0.363**	**6.865**

The results show the assay was capable of quantitating biocides down to 55ppb for MI and 37ppb for MI based on both the %RSD numbers and the signal to noise ratios. The MI signal to noise ratio averaged 19 at the 55ppb concentration with a %RSD of the peak area of 6.3%. CMI had a much better %RSD of the peak area at 0.36% with an average signal to noise ratio of 17. These results demonstrate the ability of the assay to quantitate the residual biocide levels to extremely low levels for a formulated ADC drug product.

Discussion

There are many unique challenges inherent to the development and manufacturing of ADCs. In particular detecting residual compounds is exceedingly challenging in the presence of the formulated protein where not only the protein itself can impact the analysis but also the buffer and excipient

components can increase the background or affect the chromatography. Extending these challenges to a single patient level GMP manufacture of radioactive species also presents its unique obstacles, including the purification of the bound and unbound radioactive molecules from the ADC at an individual dose scale. In order to protect patient safety, residual levels of many compounds must be determined in the formulated drug product, including residual solvents, residual free drug, and even residual biocides from the purification storage media.

We have developed a sensitive validatable assay for residual Kathon biocides. The method uses an HPLC separation with UV-Vis detection and exhibited excellent performance in formulated drug product. The method demonstrated suitable system and method precision, linearity, spiked recovery, and limit of quantitation. With a limit of quantitation of 55 ppb for MI and 37 ppb for CMI, the method can accurately detect and quantify the Kathon biocides at a limit that is low enough to support the release of the drug product for parenteral usage. We also demonstrated the method was compatible with some standard protein preparation procedures to overcome cases in which the protein or other formulation components interfere with the assay. In conclusion, this sensitive and robust method can be utilized to obtain accurate results and ensure safety of the patient from potentially toxic residual biocides.

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