

Determination of Released Payload Species of Antibody Drug Conjugates with Noncleavable Linkers by High Resolution Mass Spectrometry and MetabolitePilot™ 2.0 Software



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ABSTRACT

In this study the Advanced Biotransform Solution and Antibody Drug Conjugate (ADC) specific workflow features of the new MetabolitePilot™ 2.0 software were used to identify released payload products and metabolites from two noncleavable linker ADCs after incubation with human liver lysosomes (HLL) and S9 fraction. The commercial ADC trastuzumab emtansine and the ADC mimic from Sigma were used as example compounds.

INTRODUCTION

The number of possible species that can be generated when considering the metabolic/catabolic fate of an Antibody Drug Conjugate (ADC) is vast. One must consider payload-linker species with one or more amino acids attached plus potential biotransformations of the linker and/or payload in addition to any cleavage or degradation products. Not all ADCs are completely stable in circulation and nonspecific release of the drug or transfer of the drug to other serum components has been reported. Understanding and identifying the major released drug-containing species and metabolites is an essential part of ADC drug development. Until recently no specific ADC metabolism workflow existed in a commercial metabolite ID software package making consideration of all these species a difficult process.

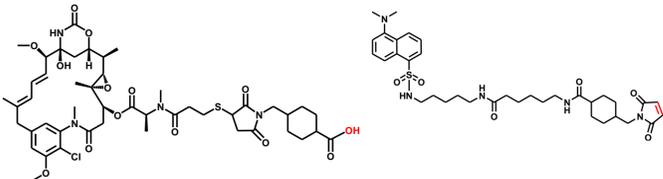


Figure 1. The structures of the linker plus payload of trastuzumab emtansine (T-E) and SigmaMab Antibody Drug Conjugate mimic (MSQC8), sites of conjugation to the mAb are highlighted in red.

METHODS

Sample Preparation:

- 50 µg/mL ADC incubated with 0.25 mg/mL HLL or 1 mg/mL S9, 37° C.
- Total volume = 200 µL (70 µL H₂O+20 µL catabolism buffer pH 5+100 µL analyte stock).
- Remove 50 µL aliquot at 1, 2, 4 and 26 hours.
- Add 250 µL of cold ACN, then centrifuge at 14 x g for 15 minutes.
- Remove supernatant to a clean tube and dry under vacuum at 37° C.
- Reconstitute in 50 µL 5% mobile phase B for injection.

LC-MS Data Collection:

- SCIEX TripleTOF® 6600 System using SWATH® acquisition (Advanced Biotransform Solution)
- 25 variable SWATH windows focused around 1103.47 for T-E and 789.37 for MSQC8
- Total scan time for the SWATH method was 850 ms
- SCIEX Exion AD system
- Phenomenex Aeris C18 Peptide column (2.0 x 150 mm), 1.7 µm
- Elution was performed using a linear gradient from 5% to 80% B over 26 mins. A – H₂O, B – ACN 0.1% CH₂O₂



Data Processing with MetabolitePilot 2.0:

Peak Finding Algorithms:

- T-E – Predicted and generic TOF MS peak finding plus TOFMSMS of at least 1 characteristic product ion
- MSQC8 – Predicted and generic TOF MS peak finding
- Charge state 1 to 3

Biotransformations

- Loss of water, demethylation, hydrogenation and dihydrogenation

Payload Cleavages

- Break 2 bonds, filtered by site of attachment

Antibody Details

- Non-specific cleavage of 2 bonds, break disulfide bonds
- Max aa count = 5
- T-E – trastuzumab, lysine conjugation with loss of water
- MSQC8 – SigmaMab, cysteine conjugation with addition

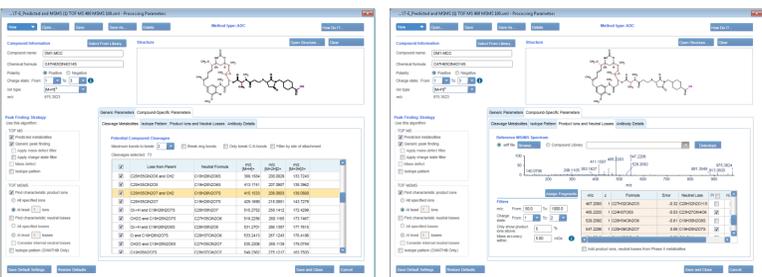


Figure 2. The payload cleavage metabolites and reference MS/MS spectrum from the processing method used for T-E.

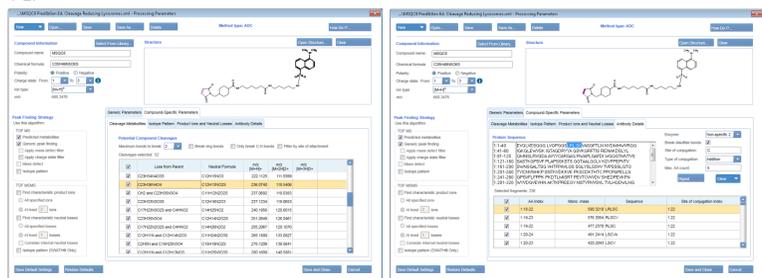


Figure 3. The payload cleavage metabolites and antibody catabolite details from the processing method used for MSQC8.

RESULTS

A total of 59 potential metabolites of T-E were found in the 26 hour time point of the incubation with HLL within the retention time window of 3 to 29 minutes. As T-E contains a non-cleavable linker the released payload species consists of DM1-MCC plus a lysine residue. Using the results workspace of MetabolitePilot 2.0 Software this released product was found at retention times 15.01 and 15.27 minutes as the released payload is a pair of diastereomers (Figure 4). The released payload was found in the TOF MS spectra as singly charged and doubly charged protonated species and as the singly charged sodium adduct all within ±2.0 ppm (m/z 1103.4763, 552.2145 and 1125.4573).

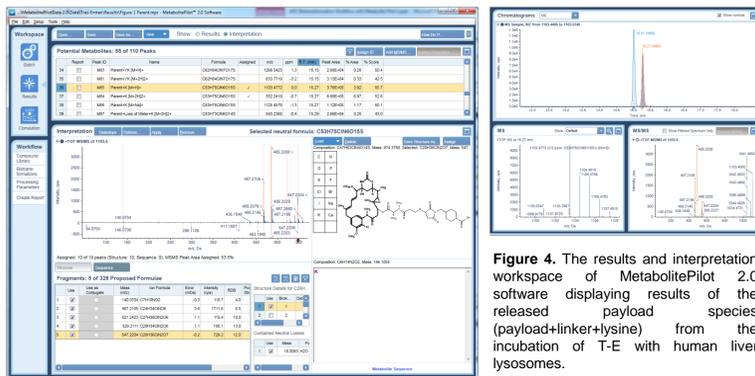


Figure 4. The results and interpretation workspace of MetabolitePilot 2.0 software displaying results of the released payload species (payload+linker+lysine) from the incubation of T-E with human liver lysosomes.

After identification of the released payload species in the results workspace further confirmation was performed using MS/MS in the interpretation workspace (Figure 4). The assign fragments feature is used to calculate potential structures for the fragments and neutral losses in the MS/MS spectrum. For the singly charged released payload species 4 common product ions (indicated in orange) were found in the MS/MS at 140.0701, 467.2086, 485.2209 and 547.2208 within ±4.0 mDa.

Four other catabolites containing the unmodified DM1-MCC conjugated to a dipeptide sequence were found that had at least two product ions in common and appeared as a pairs of diastereomeric peaks. Table 1 lists their identity and sequence. No catabolites conjugated to anything larger than a dipeptide were confirmed in the sample, this likely due to a combination of the length of the incubation and the sample work-up which involved an organic precipitation.

Table 1. List of released payload plus linker species from the incubation of T-E with HLL and S9.

Catabolite	RT 1	RT 2
Parent	17.6	
Parent+YK	15.17	15.40
Parent+K	15.01	15.27
Parent+KV	15.1	15.4
Parent+KS	15.0	15.3
Parent+KA	14.8	15.1

MSQC8 is a cysteine linked ADC that is not a therapeutic molecule but an ADC mimic. A reference MS/MS spectrum was not available for the dansyl fluorophore plus linker so peak finding was performed using predicted metabolites and generic peak finding. A total of 119 potential metabolites and catabolites were found within the retention time window of 3 to 25 minutes and ±5.0 ppm. The released payload species (dansyl+linker+cysteine) was found in both single and doubly charged forms at 14.92 minutes as a single peak.

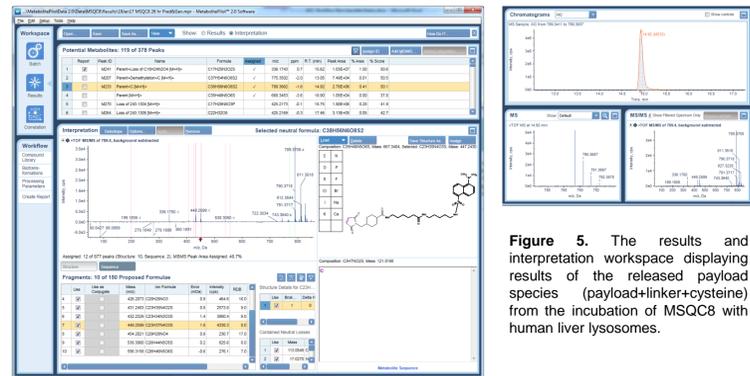


Figure 5. The results and interpretation workspace displaying results of the released payload species (payload+linker+cysteine) from the incubation of MSQC8 with human liver lysosomes.

The interpretation workspace (Figure 5) was then used to assign peaks in the MS/MS, this was a critical step as no reference spectra of the payload and linker was available. The structural interpretation tool assigned structures to four of the ions in the MS/MS all within ±2.0 mDa and three of the four ions contained the dansyl ring, Table 2.

Among the list of potential catabolites/metabolites a putative demethylated metabolite of the payload+linker+cysteine was identified in the TOF MS at 13.05 minutes. Again the interpretation workspace was used to assign ions in the MS/MS to the metabolite structure for confident ID. Three ions in the MS/MS spectra were found with a mass difference of 14 Da when compared with the ions assigned in the released payload species. Based on the assigned fragment structures from MetabolitePilot™ 2.0 software and their mass accuracies; the site of demethylation was assigned to the dimethylamino group of the naphthalene ring of the payload (Table 2).

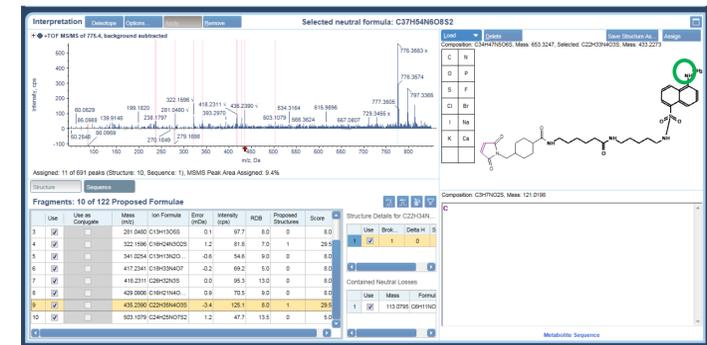


Figure 6. The interpretation workspace displaying the MS/MS interpretation results for the demethylated metabolite of the MSQC8 payload.

Table 2. List of ions with m/z shifts of 14 Da found in the MS and MS/MS spectra of MSQC8 after incubation with HLL and S9.

Mass (Parent)	Mass (M-1)	Structure	Mass (Parent)	Mass (M-1)	Structure
789.3662	775.3502		336.1750	322.1596	
449.2599	435.2390		432.2329	418.2311	

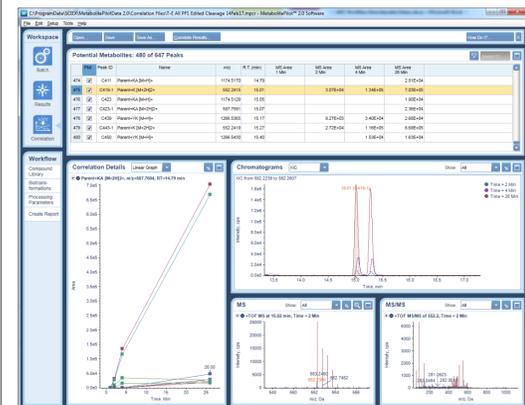


Figure 6. The correlation workspace of MetabolitePilot 2.0 software displaying results of the released payload species (payload+linker+lysine) from the incubation of T-E with human liver lysosomes. In this workspace overlaid XICs from the LC chromatogram, MS and MS/MS spectra are overlaid. The most abundant species found was payload+linker+lysine, payload+linker plus dipeptide fragments containing lysine were also observed.

CONCLUSIONS

The SCIEX Advanced Biotrans solution with the TripleTOF® 6600 system and new MetabolitePilot 2.0 software effectively addresses the complex task of performing metabolite and catabolite ID on ADCs from comprehensive peak detection with SWATH® acquisition to metabolite finding. MetabolitePilot 2.0 software features a dedicated ADC processing workflow that performs targeted searching for all components of the ADC: payload, linker and antibody plus cleavage metabolites and biotransformations.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

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