

# Metabolite Identification of Payload Species of Antibody Drug Conjugates with Noncleavable Linkers using MetabolitePilot™ 2.0 Software and TripleTOF® 6600 System

*MetabolitePilot™ 2.0 Software and SCIEX TripleTOF® 6600 System*

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## Key Challenges in ADC Metabolism Studies

- Metabolism studies of ADCs must consider many different species: payload, payload plus linker, and payload plus linker conjugated an amino acid or peptide
- Potential biotransformations of all the above species must also be considered
- Missing, low-level drug metabolites in complex biological matrices
- Incomplete metabolite information leading to repeated sample analysis and decreased productivity
- Maintaining data quality for both quantitative and qualitative analysis in a high-throughput environment

## Key Features of MetabolitePilot 2.0 Software for ADCs

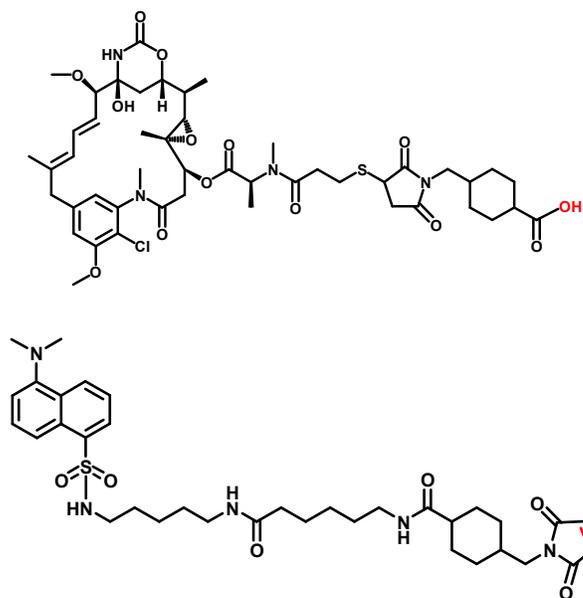
- A dedicated ADC processing workflow that performs targeted searching for all components of the ADC: payload, linker and antibody
- Multiple peak finding strategies utilizing MS and MS/MS information for targeted and untargeted searching for drug related metabolites
- Dedicated ADC structure assignment workspace and MS/MS fragment interpretation workspace where both payload and peptide are considered
- Integrated correlation function allows comparison of metabolism across multiple samples for time course studies or inter-species comparison, using both MS and analog data.

## Introduction

Antibody drug conjugates (ADC) are designed for targeted delivery of a cytotoxic molecule to a diseased or cancerous tissue. ADCs are composed of three



**Figure 1.** The SCIEX Advanced Biotransform Solution featuring the new MetabolitePilot 2.0 software, TripleTOF® 6600 and Exion AD LC system.



**Figure 2A and Figure 2B.** 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.

components: the antibody used for selective targeting, the cytotoxic drug and a linker to connect the two. The goal in design and development of ADCs is to maximize delivery to the targeted tissue and minimize delivery to untargeted tissues. There are currently two FDA approved ADC therapies on the market for the treatment of cancer and research efforts in ADC technology have grown across the pharmaceutical industry to encompass therapeutic areas other than cancer.

There are two classes of antibody drug conjugate (ADC) linkers: cleavable and noncleavable. Cleavable linkers are labile and are designed to release the unmodified cytotoxin upon internalization by the target cell due to a change in chemical environment (e.g. pH) or a specific protease inside the cell. A noncleavable linker does not contain any mechanism for payload release and relies on antibody degradation through cellular processing for release of the active species. In these ADCs the active species is the cytotoxic payload with the linker and one or more amino acids from the antibody.

In addition to studying the usual small molecule metabolism of the payload molecule separate from the antibody, a necessary step in the development of ADCs is the identification of the active payloads of both cleavable and noncleavable linkers from the ADC and any metabolism products involving active forms of likely payload products. In addition to plasma or serum incubations, *in vitro* systems of purified cellular fractions (lysosomes or S9) or purified enzymes are often used to characterize active payloads released from the ADC.

The number of possible species that can be generated when considering the metabolic/catabolic fate of an 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 plus any cleavage or degradation products. To address this challenging task SCIEX has introduced a specific ADC workflow in the new MetabolitePilot™ 2.0 software. In this tech note the ADC specific workflow features of the new MetabolitePilot 2.0 were used to identify payload products and metabolites from two noncleavable linker ADCs after incubation with human liver lysosomes and S9 fraction.

## Experimental

### Reagents

Trastuzumab emtansine (T-E) was purchased commercially. SigmaMAb Antibody Drug Conjugate mimic (MSQC8) was provided by Sigma-Aldrich. Human liver lysosomes (HLL), S9 fraction and catabolism buffer were kindly provided by Xenotech.

### Sample Preparation Incubations

ADCs were incubated with either HLL (0.25 mg/mL) or S9 fraction (1 mg/mL). ADC concentration was 50 µg/mL in each incubation. Control samples included ADC without HLL or S9 and HLL or S9 without ADC. Total reaction volume was 200 µL, consisting of 70 µL water, 20 µL catabolism buffer (10x), 100 µL HLL or S9 and 10 µL analyte stock solutions.

At different time points (1, 2, 4 and 26 hours) a 50 µL aliquot was removed and processed. Sample processing involved: adding 250 µL of cold ACN, then centrifuging at 14 x g for 15 minutes. The supernatant was removed to a clean tube and dried under vacuum at 37 °C. Dried samples were then reconstituted in 50 µL 5% mobile phase B for injection.

### Mass Spectrometry Data Collection

Data was collected on a SCIEX TripleTOF® 6600 System using SWATH® acquisition. TOF MS acquisition covered m/z 300 to 2000. MS/MS data were collected using 25 variable SWATH windows focused around the m/z of the linker payload plus the amino acid of conjugation. Five 13 Da windows centered around the parent mass (1103 for T-E and 789 for MSQC8) and expanding to windows of 50, 100 and 200 to cover a mass range of 50 to 2000 Da. A collision energy of 40 with collision energy spread of 15 was used. Total scan time for the SWATH method was 850 ms.

### Chromatography

Samples were chromatographed on a SCIEX Exion AD system using a 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. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid.

### Data Processing

An ADC specific processing method was created in MetabolitePilot 2.0 software for both T-E and MSQC8. Briefly, the first step is to provide the chemical structure of the payload and linker, designate the amino acid of attachment, the type of conjugation chemistry involved and then enter the antibody sequence. Next the size and identity of protein/peptide sequence fragments from the antibody linked to the payload were chosen. Non-specific cleavage of two peptide bonds plus disulfide bond cleavage was used and up to five amino acids were considered. Then a biotransformation list and set of cleavage metabolites of the payload was chosen. Since the incubation in HLL and S9 occurs at ~pH 5 in a reducing environment the biotransformation list contained

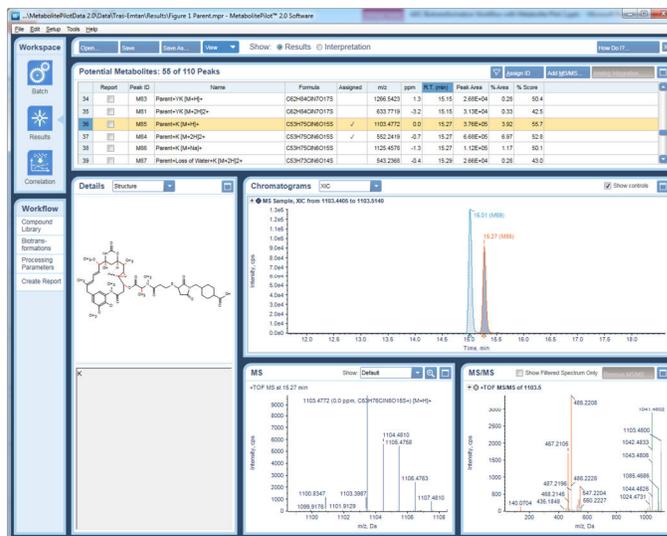
only: loss of water, demethylation, internal hydrolysis and the parent (payload plus linker). Phase I and II metabolism of the parent was not considered for this *in vitro* system. A reference MS/MS spectrum was available for DM1+MCC and used to designate characteristic product ions and neutral losses. A reference spectrum for the MSQC8 payload was not available.

Peak finding for both ADCs included TOF MS with both predicted and generic peak finding algorithms. The TOF MSMS peak finding algorithm was used for T-E since a reference MS/MS spectrum was available, at least 2 characteristic product ions (485.22, 547.22) and 1 neutral loss were selected and the advanced MS/MS filter was set to 1 unit below confident. A retention time window of 3 to 29 minutes was used to search for metabolites products. A sample to control comparison ratio of >3 was used and metabolites up to a charge state of 3 were considered.

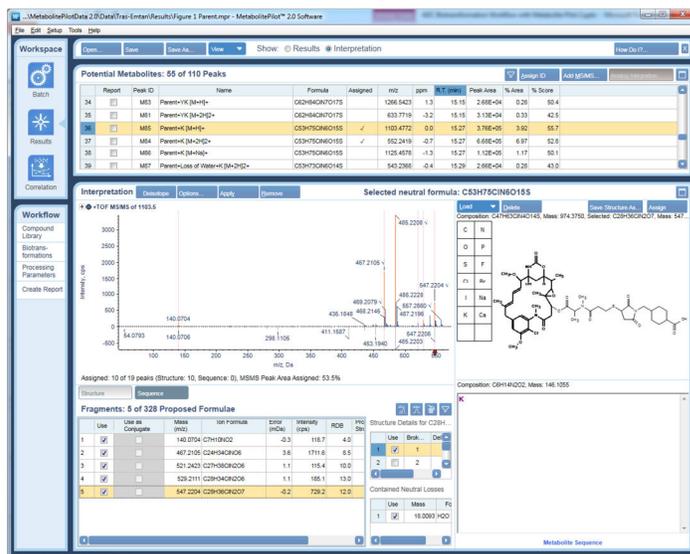
### Results

The MetabolitePilot™ 2.0 batch workspace was used to setup and process the SWATH data collected from incubations of both ADCs with HLL and S9 incubations at all time points using the data processing parameters described above. Both control samples were used during processing of each time point. The batch processing table of MetabolitePilot 2.0 has been expanded from 30 to 200 rows to increase throughput and the results from each sample may be viewed individually in the results workspace as they are completed before the whole batch is finished.

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 3). 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 3.** The results workspace of MetabolitePilot 2.0 software displaying results from the incubation of T-E with human liver lysosomes. The released payload species (payload+linker+lysine) was found at retention times 15.01 and 15.27 minutes as a pair of diastereomer peaks. The structure of the payload and linker species is displayed on the left with the amino acid of conjugation below. Also displayed is the MS and MS/MS spectra of the released payload species.



**Figure 4.** The interpretation results 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 product ions in the MS/MS spectra are assigned to the identified metabolite. There are separate assignments for both the small molecule payload and sequences of the mAb.

After identification of the released payload species in the results workspace further confirmation was performed using MS/MS in the interpretation workspace (Figure 4). Once a putative metabolite is identified the structure of the

payload and linker is loaded into the fragment interpretation workspace and the putative peptide sequence is loaded into the sequence workspace and the residue of conjugation is assigned. Next 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  $\pm 4.0$  mDa. Three of the four common product ions had structures proposed as indicated in bold on the chemical structure.

A similar workflow was followed to identify and confirm other released payload species containing larger portions of the antibody sequence in the 26 hour sample. 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

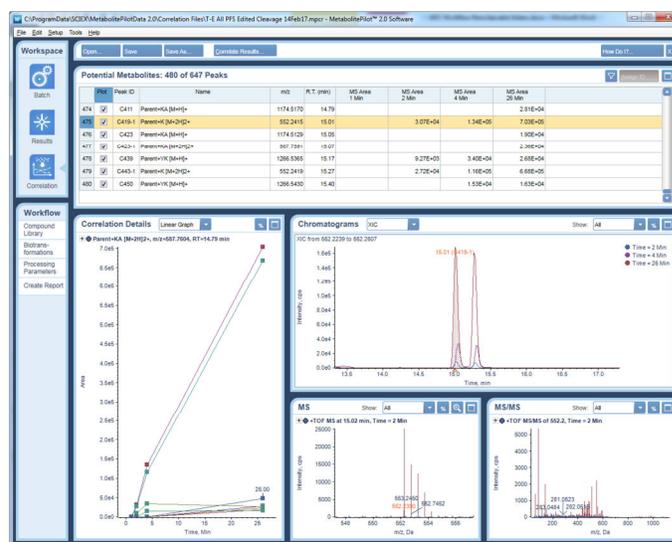
**Table 1.** List of released payload plus linker species from the incubation of T-E with HLL and S9.

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

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.

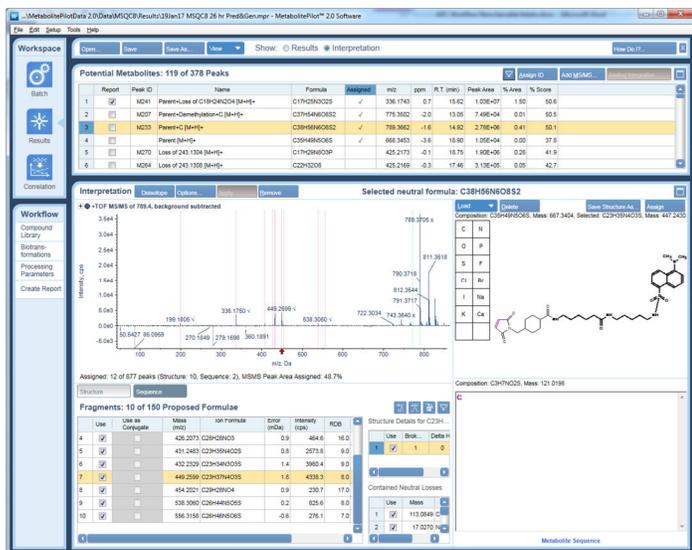
The correlation workspace of MetabolitePilot™ 2.0 software was next used to confirm the presence of the identified metabolites across the assay time points and between liver fraction experiments. The correlation workspace has an interactive graph window where results can be displayed as an x, y plot, bar graph or table. In addition to the correlation workspace overlaid XIC's, TOF MS and TOF MS/MS spectra are also displayed for the chosen metabolites/catabolites. Figure 5 displays the correlation plot of the released payload plus lysine from the HLL incubation. It was also observed in the 2 and 4 hour samples but not in the 1 hour time point. The peak area of the compound (15.01 and 15.27 peaks) increased by 4-fold between the 2 and 4 hour time points and by 5

fold between the 4 and 26 hour time points. A similar fold increase was seen for the S9 incubations. The dipeptide payload species were seen predominantly in the 4 hour and 26 hour samples and did not increase in peak area as rapidly as the released payload plus lysine. The rate of appearance of released payload species is a complex proposition due to the heterogeneous nature of the T-E molecule with DAR species from 0 to 8 and different lysine residues involved in conjugation within each DAR species.



**Figure 5.** 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.

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  $\pm 5.0$  ppm. The linker in MSQC8 is also a non-cleavable linker and the released payload species for this ADC mimic would be the dansyl fluorophore plus linker and cysteine. This molecule is found in both single and doubly charged forms at 14.92 minutes as a single peak. The interpretation workspace (Figure 6) 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  $\pm 2.0$  mDa and three of the four ions contained the dansyl ring.



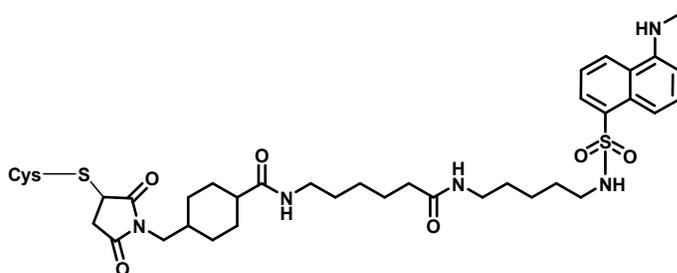
**Figure 5.** The interpretation results workspace of MetabolitePilot™ 2.0 software displaying results of the released payload species (payload+linker+cysteine) from the incubation of MSQC8 with human liver lysosomes. There are 3 product ions with proposed structures assigned to the payload species that include the dansyl fluorophore: 449.2599, 432.2329 and 336.1750.

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 (Figure 6).

The correlation workspace was used to visualize the appearance of both of these metabolites across all time points and sample sets. The payload+linker+cysteine was found in both HLL and S9 fraction and at all time points and increased over time. The demethylated payload+linker+cysteine were observed in only the 4 and 26 hour time points in both the HLL and S9 incubations.

**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.

Catabolite	Released Payload	Demethylated Released Payload
TOF MS	789.3662	775.3502
TOF MS/MS 1	449.2599	435.2390
TOF MS/MS 2	432.2329	418.2311
TOF MS/MS 3	336.1750	322.1596



**Figure 6.** Structural assignment of the demethylated MSQC8 released payload species.

## 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. This comprehensive knowledge base combined with untargeted searching for drug related metabolites and peak finding strategies utilizing MS and MS/MS information ensures success for scientists studying ADC metabolism.

## Acknowledgments

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