ADC 药物完整解决方案

SCIEX

The Power of Precision

SCIEX 质谱 -- 超过 50 年的创新历程

SCIEX 在质谱技术领域拥有超过 50 年的创新经验。从 1981 年致力于开发突破性的技术和解决方案。

SCIEX 创立

由 Barry French 博士和 William Breukelman博士共同创立于**多伦多大学**

1970

1981

发布**第一款SCIEX的商用三重四极杆**(MS/MS)仪器

1989

发布API III，是**第一款SCIEX的商业化的液质联用系统**

2001

发布API 4000™ LC-MS/MS 系统，成为了**生物分析**的标准手段

2002

发布QTRAP® LC-MS/MS 系统，**SCIEX创新的把三重四极杆和线性离子阱技术结合起来**

2005

发布API 5000™系统，该系统是代表**当时先进水平的三重四极杆液质联用系统**

2008

发布5500系列，这是**业界知名的液相色谱三重四极杆质谱系统**

2009

成为**丹纳赫集团**的一员，并于2010年**收购Eksigent公司液相业务**

2012

SCIEX在这一年发布新产品数量创新高，这其中包括**SWATH®采集技术**

2013

整合了**Beckman Coulter®**公司毛细管电泳业务

SCIEX
The Power of Precision

成功推出第一台 SCIEX 的商业化三重四极杆质谱系统开始，一直

发布第一款SCIEX的用于台式精确质量质谱系统，**X500R QTOF 系统**
发布**SCIEX Now™**客户服务平台

五十周年纪念

2015 2018 2020

发布**Echo™ MS系统**，这是一款基于**声波激发耦合质谱(AEMS)**技术的超高检测样品通量的系统

发布专门控制三重四极杆质谱的**SCIEX OS 软件**，是SCIEX创新的核心动力

发布**BioPhase™ 8800 系统**，一款可以同时检测8份样品的多通道毛细管电泳系统

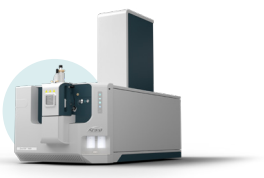
发布了**SCIEX OS软件3.0版本**，支持中文界面

2021 2022 期待未来

发布**Triple Quad™ 7500 系统**，开启灵敏度的新时代

发布**C100HT 生物制品分析系统**，**OptiFlow™ Turbo V 离子源**，和**StatusScope® 远程监控**服务

发布**ZenoTOF™ 7600系统**，这是一款高分辨精确质量质谱，是SCIEX跨跃式的质谱创新发展



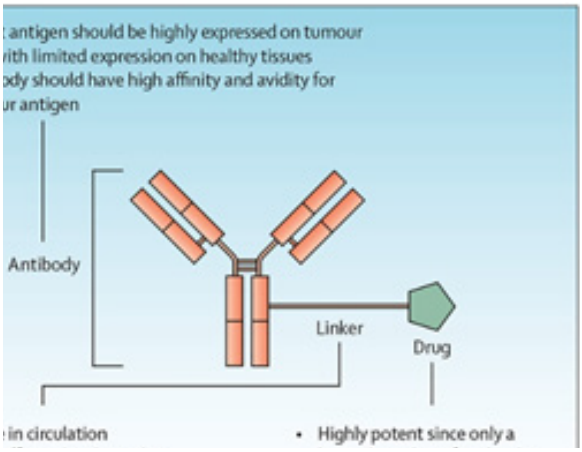
发布**ExionLC™ 2.0和ExionLC™ 2.0+ 系统**，与SCIEX质谱系统兼容，实现一体化控制

发布**Zeno™ SWATH® 数据非依赖性采集(Zeno™ SWATH DIA®)**技术，标志着生物标志物发现及转化应用中又向前迈进了重要一步

ADC 药物完整解决方案

抗体偶联药物（antibody-drug conjugate，ADC）是一类通过特定的连接子将靶向单克隆抗体与高杀伤性的细胞毒性小分子药物偶联起来的生物药，以单克隆抗体为载体将小分子细胞毒性药物高效地运输至目标肿瘤细胞中，起到治疗的目的。

近年来，随着新的连接子、小分子毒素、抗体以及靶点发现技术的不断更新，ADC 药物也发展迅速，仅 2019 年一年，FDA 就批准了三款 ADC 药物，荣昌生物的国产 ADC 维迪西妥单抗也在 2021 年 6 月获 NMPA 批准上市。目前， 全球有 100 多项 ADC 药物相关的临床试验正在进行。



ADC 药物的结构和特点
（Lancet Oncol. 2016 Jun; 17(6): e254–e262.）

小分子毒素抗体比 DAR（drug-to-antibody ratio）是 ADC 药物独特的重要质量属性，它代表抗体偶联小分子毒性药物的平均数量，较低的 DAR 会降低 ADC 的效力，但过高的 DAR 又会影响 ADC 分析的药代动力学和毒性，普遍认为 DAR 在 2-4 之间是 ADC 药物的最优选。而偶联方式是影响 DAR 的重要因素，只有选择合适的偶联技术，才能让 ADC 的毒素均一稳定地连接在抗体上。

目前主要有三种偶联方式：1) 通过暴露在抗体表面的赖氨酸 , 2) 还原链间二硫键的半胱氨酸 , 3) 定点偶联技术来进行偶联。其中赖氨酸偶联的位点较多，每个抗体上可以挂 0~8 个小分子毒素，导致每一批次的 ADC 产品异质性较大。而半胱氨酸偶联，由于二硫键的位置是固定的，所以偶联位置也相对可控，但是其链接小分子毒素的个数取决于二硫键的还原程度。当 4 条链间二硫键均被完全还原时，能携带 8 个毒素；当它被部分还原时，则可能生成 DAR 为 2、4、6、8 的产物，半胱氨酸偶联产物较赖氨酸，其均一性已经显著提高。定点偶联主要是使用生物技术人为地在单抗上引入链接位点。随着 ADC 偶联方式的进步，ADC 药物抗体比不均的问题也被逐步改善。

本文集提供 ADC 药物的解决方案，包括 ADC 药物的表征分析（分子量、DAR 值、肽图、偶连位点、翻译后修饰），大小分子的绝对定量分析，小分子代谢物鉴定及电荷异质性分析等。运用三重四极杆液质联用系统、QTOF 高分辨液质联用系统，毛细管电泳技术，提供全面的 ADC 药物深度表征方案。

内容提要

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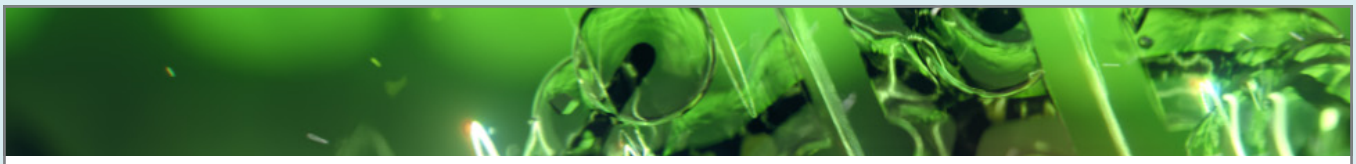

应用电子活化解离（EAD）技术进行抗体-药物偶联物（ADC）表征

采用带有EAD的SCIEX ZenoTOF™ 7600系统和Protein Metrics公司软件

Characterization of an antibody-drug-conjugate (ADC) using electron activated dissociation (EAD)

Zoe Zhang[1], Takashi Baba[2], Pavel Ryumin[2], Bill Loyd[2], Jason Causon[2], Kerstin Pohl[1]

[1] SCIEX, US; [2] SCIEX, Canada

本文对ADC样品中赖氨酸连接药物进行了表征，选择了一种自下而上的方法来确定偶联的位置。利用电子活化解离（EAD）[1,2]技术产生的离子碎片信息，可获取相关药物和连接子的详细结构信息，同时获得多肽骨架信息。

随着蛋白质工程技术的进展，抗体及其相关衍生物成为治疗药物中增长最快的一类[3]。ADCs是这些新的药物形式之一。ADCs通常由150 kDa单克隆抗体（mAb）与细胞毒性药物（有效载荷）通过特定偶联物（linker）共价偶联而成[4]。与未偶联的蛋白相比，ADC药物的结构复杂度和异质性更高，因为添加了多变的有效载荷和连接子，所以显著增加蛋白形式的数量[5]。为确保药物安全性和有效性，ADC的深度表征在其开发过程中至关重要。这不仅包括对mAb的翻译后修饰（PTMs）的鉴定和定位，还包括药物结合的验证。由于质谱技术的飞速发展，质谱（MS）已经成为ADC表征中最广泛使用的方法。完整质量分析是用于确定药物与抗体比率（DAR）的常规方法，而对结合位点的深入表征，通常依赖于自下而上的方法。现在最广泛采用的碰撞诱导解离（CID）技术能够提供氨基酸序列确认，但是这种能量比较大的碎裂技术也将有效载荷分解为更小的片段，从这种方法获得的高度复杂的谱图可能很难解析。而能量更柔和的碎裂方法可以促进此类复杂样品的解析，而且CIQ技术可能需要更长的反应时间以及灵敏度低和缺乏可重复性的困扰。

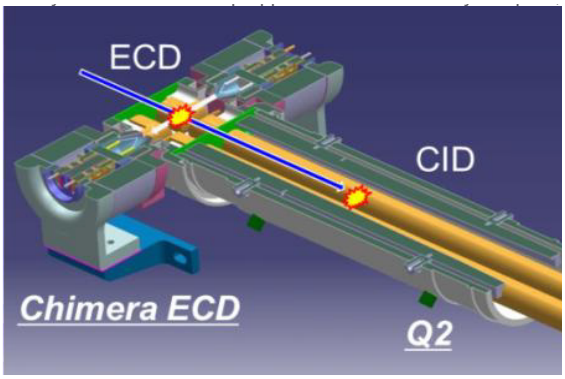

图1. EAD碰撞池的示意图。

一种基于EAD[1,2]的创新、高度可重复的碎裂方法用于分析来自商业化ADC药物的偶联肽。使用10 Hz快速非靶向的数据依赖采集（DDA）方法采集数据，并使用Protein Metrics 公司软件进行数据解析。通过此工作流程，一次进样就可以应用基于EAD的碎片进行常规和高级表征，从而获得可供每个用户使用的精简的表征方法。

SCIEX ZenoTOF™ 7600系统的主要功能

- **肽图分析的新深度**：具有快速DDA的EAD技术可为下一代蛋白质治疗药物和标准单克隆抗体（mAb）的常规、深度分析提供可选择的创新碎裂方法。
- **更高级别的结构信息**：通过调节电子能量来改变碎裂模式,能会提供更高级别的结构信息。
- **更高的MS / MS灵敏度**：使用Zeno™ trap(Zeno阱)技术，可产生更多的碎片离子（5到10倍），从而提高了数据结果的置信度。
- **高重现性**：与其它碰撞碎裂技术和低重现性碎裂技术相比，使用EAD进行单电荷、双电荷和多电荷离子碎裂时，具有更高的重现性，可分析更多前体离子。
- **简单易用**：使用带有SCIEX OS软件的EAD技术，以DDA模式进行全自动数据采集，并使用Byos软件（Protein Metrics Inc.）进行自动数据解析，从而简化了分析流程、提升用户体验。

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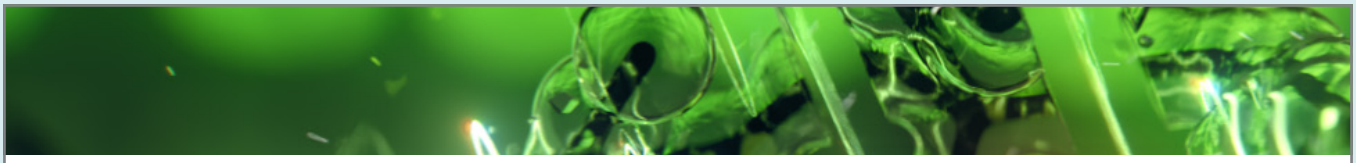
内容提要



方法

样品制备： 赖氨酸连接的ADC药物（曲妥珠单抗美坦新偶联物，T-DM1）用7.2 M盐酸胍和100 mM Tris缓冲液（pH 7.2）变性，然后加10 mM 二硫苏糖醇还原和30 mM碘乙酰胺进行烷基化。在37℃下用胰蛋白酶/ Lys-C酶消化16小时。

色谱条件： 4 µL（4 µg）的胰蛋白酶/ Lys-C消化液，采用ExionLC AD液相系统，通过CSH C18色谱柱（2.1 × 100 mm，1.7 µm，130 Å，Waters）进行分离。流动相A为含0.1％甲酸的水溶液，流动相B是含0.1％甲酸的乙腈溶液。柱温保持在50 ℃，流速为300 µL/ min，流动相梯度见表1。

表1. 肽图分析的色谱方法。

时间（min）	流动相A[%]	流动相B[%]
初始	98	2.0
5	98	2.0
6	90	10
40	55	45
44	10	90
46	10	90
47	98	2.0
50	98	2.0
51	10	90
54	10	90
55	98	2.0
60.0	98	2.0

质谱条件： 采用SCIEX ZenoTOF™ 7600系统通过信息依赖采集（IDA）方法采集数据。EAD池的电子能量设置为7 eV。详细的方法参数汇总在表2。

表2. 质谱参数。

参数	MS	MS/MS
扫描模式(Scan mode)	TOF-MS	IDA dependent
极性(Polarity)	正离子模式	
喷雾气(Gas 1)	40 psi	
加热气(Gas 2)	40 psi	
气帘气(Curtain gas)	30 psi	
离子源温度(Source temperature)	350 ℃	
离子喷雾电压(Ion spray voltage)	5200 V	
去簇电压(Declustering potential, DP)	20 V	
碰撞能量(Collision energy)	8 V	
碰撞气(CAD gas)	7	
最大候选离子(Maximum candidate ion)	5	
强度阈值(Intensity threshold)	100 cps	
离子电荷(Charge states)	2 to 10	
排除时间(Exclusion time)	6 s after 2 occurrences	
起始质荷比(Start mass)	100 m/z	150 m/z
终止质荷比(Stop mass)	1,800 m/z	2,500 m/z
电子KE(Electron KE)	NA	7 eV
电子束电流(Electron beam current)	NA	4750 nA
(ETC)	NA	100
Zeno 脉冲	NA	ON
累积时间	0.25 s	0.20 s
Time bins to sum	4	4

数据处理： 采用Byos软件（Protein Metrics Inc.）进行数据解析。

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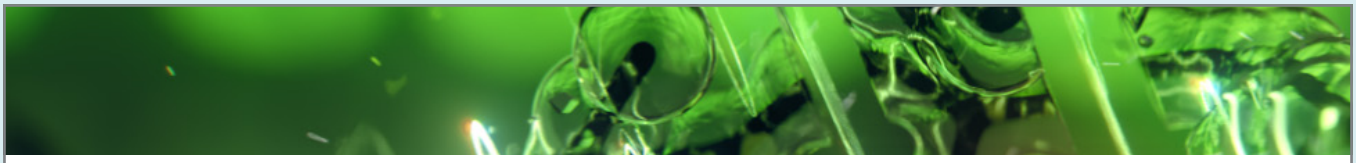

是什么，为什么，怎么做

曲妥珠单抗美坦新偶联物（T-DM1）是最早的ADC治疗药物之一，于2013年获得FDA批准用于治疗人表皮生长因子受体2（HER2）阳性转移性乳腺癌。T-DM1是由单克隆抗体曲妥珠单抗和细胞毒药物美坦新（DM1）通过不可裂解连接子共价偶联而成（图2）。将单克隆抗体（mAb）的靶标特异性与细胞毒性药物的高效率相结合，可充分利用两个方面的优势，最大限度地减少副作用[3]。T-DM1是与氨基连接，如连接在曲妥珠单抗的赖氨酸残基的侧链中。先前的完整质量研究表明，T-DM1的平均DAR约为3.5.[5,6]。但是曲妥珠单抗中有88个赖氨酸残基和4个N端基团，可能会出现450万个以上的不同分子形式[5]。有效载荷的位点和结构将直接影响药物的功效和安全性，因此将其归类为关键质量属性（CQA），并且需要在开发过程中进行全面表征和严格监控。当前，自下而上的方法是表征产品质量属性的选择方法，可同时进行鉴定和修饰位点的定位。LC-MS / MS通常采用CID技术用于验证偶联位点，因为每个DM1都会导大约957 amu的质量偏移。然而，采用CID碰撞技术除了导致肽段主链解离，还可能导致有效载荷药物产生一系列小片段，例如m/z547.221、485.224和453.199离子，这增加了谱图的复杂性[5]。虽然电子捕获解离（ECD）之类的碎裂技术有望提供有关药物偶联肽的正交信息，但是其应用尚未得到广泛探索。

SCIEX ZenoTOF™ 7600系统，引入了一种强大的选择碎裂技术，使科学家能够通过将液相分离技术结合DDA快速扫描方法获取的数据，且通过Protein Metrics 公司软件数据处理获得样品的深入图谱。这项突破性的技术实现了高重现性常规分析来解决复杂问题的梦想。

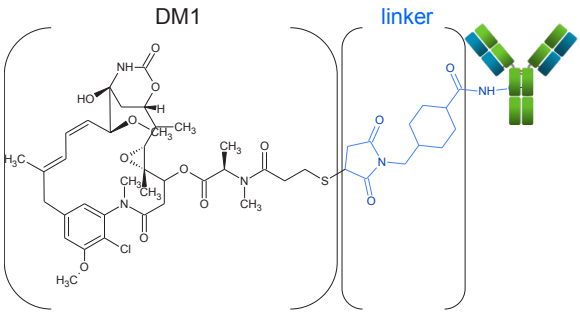

图2. 细胞毒药物有效载荷和连接子与mAb偶联的示意图。 T-DM1由DM1（黑色），靶向连接氨基残基的MCC连接子（linker，蓝色）和单克隆抗体组成。

偶联肽的分析

该研究的重点是一个商品化的ADC药物：T-DM1的表征。DM1和曲妥珠单抗之间的偶联反应具有靶向氨基的随机性。在曲妥珠单抗上的88个赖氨酸残基中，有40个是溶剂暴露的[6]，因此很容易结合。多种研究完整和亚基质量的方法已经应用于研究DAR，但是这些方法无法揭示精确的偶联位点。

本研究选择了与Zeno™ EAD相结合的DDA方法。采用这种方法，不仅可以执行常规的肽图分析，而且EAD可以在同一单针分析中进行高级表征。此外，Zeno EAD增强了碎片离子的检测能力，从而正确鉴定了低丰度物质，这种方法允许使用Protein Metrics 公司.软件直接进行数据解析。这是针对ADC样品的SCIEX EAD技术的首次探索。图3展示了在偶联肽SCDK [DM1] THTCPPCPAPELLGGPSVFLFPPKPK上观察到的碎裂模式的例子。在分析中未观察到没有连接子和药物或其部分的肽，表明其完全偶联。获得了此肽段高质量的MS / MS谱图，从而使该特定肽段的MS / MS序列覆盖率达到96.6%。一个更占优势的碎片从 m/z大于500的有效载荷产生（请见图3中的标记）。观察到的有效载荷结构的主要裂解位点是DM1的COO-C键，这种碎裂模式与先前利用CID技术产生的一系列小碎片的数据不同5。较大分子量的药物碎片可以用作特征碎片，以更具体地确认有效载荷的存在，并可以用来确认有效载荷的结构。此外，通过将Zeno EAD技术用于增强的碎片离子检测，还可以很好地检测到来自肽主链的片段信息，从而提供有关肽段的分子完整性的信息。由于酶的空间位阻，蛋白质上偶联药物的存在会导致样品制备酶解过程中的更多漏切位点。另外，赖氨酸残基和有效载荷之间的结合过程是随机反应。偶联的比率并不总是100%，这导致了多样性和低丰度物质存在。当一个肽段中存在多个潜在连接形式时，鉴定正确的连接位点可能是一个挑战。

肽段ASQDVNTAVAWYQQKPGKAPK是这种具有挑战性的另一个例子（图4）。它包含一个漏切位点和一个脯氨酸相邻的N端赖氨酸，导致偶联位点的多种选择。但是，有了从EAD技术碎裂得到丰富、高质量的MS / MS质谱图，就可以实现药物定位的自动匹配（图4A）。由于有效载荷靠近肽的C端，因此检测到的C离子比Z离子丰富（图4A），而未结合的肽显示出来自C端和N端的丰富片段（图4B）。众所周知因为电子捕获解离技术不会解离脯氨酸的N端，我们还检测到了除了C15以外的从C3到C17的全系列C片段[7]。这提供了确凿的证据表明K15未与细胞毒药物偶联。此外，z4，z5和z7表明K18（而非K21）是药物偶联的正确位点。

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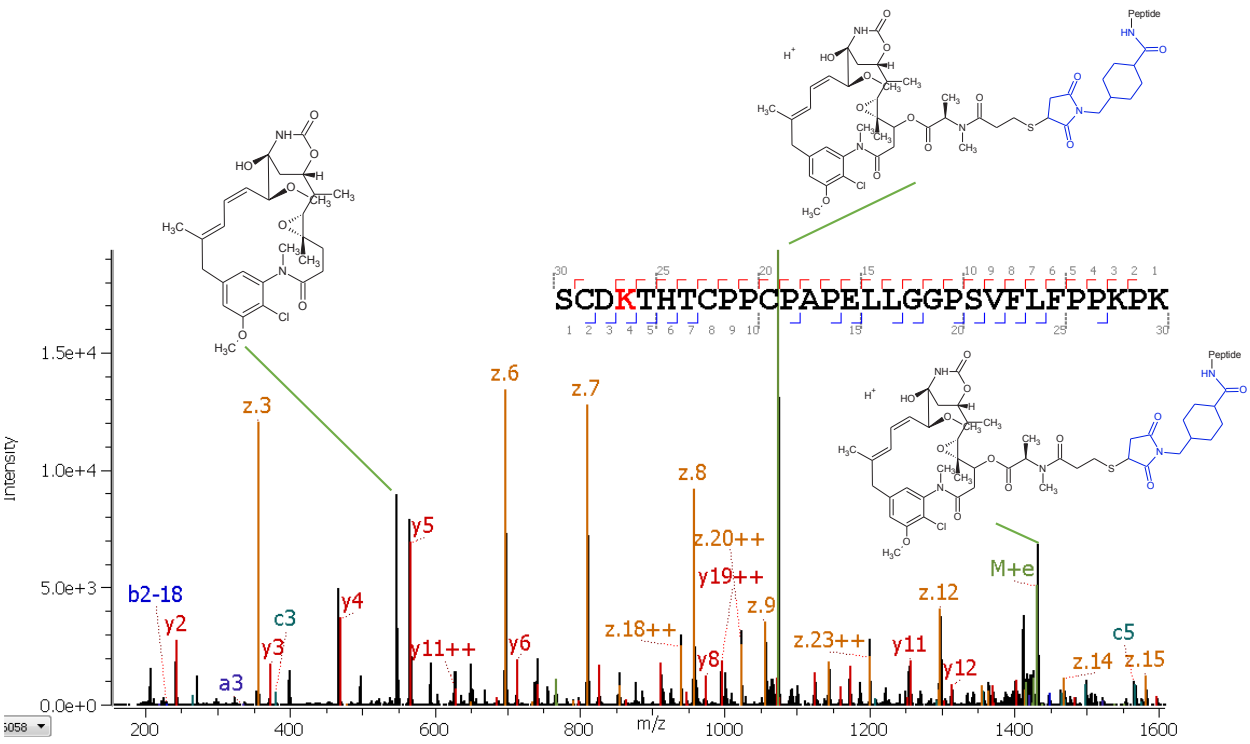

图3. 应用Zeno EAD得到的偶联肽SCDK [DM1] THTCPPCPAPELLGGPSVFLFPPKPK（z = + 4）的碎片数据。来自肽段主链指定偶联肽段离子的全扫描MS / MS数据，以及有效载荷中的碎离子信息。

这个结果进一步证明了一个事实，即偶联的赖氨酸不能被胰蛋白酶切割[5]，但K21作为连接位点除外。图4A谱图中最主要的离子（m / z 547.221）可以与有效载荷的解离联系在一起，这个同位素模式也支持这一发现，因为它适合包含卤素元素（Cl）的化合物。同时还观察到与肽连接的相关对应物（图4A）。使用SCIEX ZenoTOF 7600系统中的Zeno EAD技术可以实现在单次DDA运行中鉴定和定位偶联肽的精确表征。以前曾被认为ADC表征是LC-MS / MS的一个挑战，这是Zeno EAD如何简化ADC分析的示例。

结论

- 通过EAD的新型碎裂技术实现了具有多个潜在位点的多肽中药物偶联的准确定位
- 与传统的MS / MS分析相比，EAD技术获得更丰富的MS/MS信息。应用Zeno EAD技术，即使对于中等强度或极低强度的前体离子（例如低丰度的偶联肽），也能获得令人信服的二级碎片和出色的数据质量
- SCIEX ZenoTOF™ 7600系统强大、高重现性且易于使用的多重碎裂技术，使用户能够以简单的方式解决具有挑战性的分析问题
- 使用Protein Metrics 公司的软件进行自动数据处理，可实现复杂生物治疗药物的可重现、常规和高级表征

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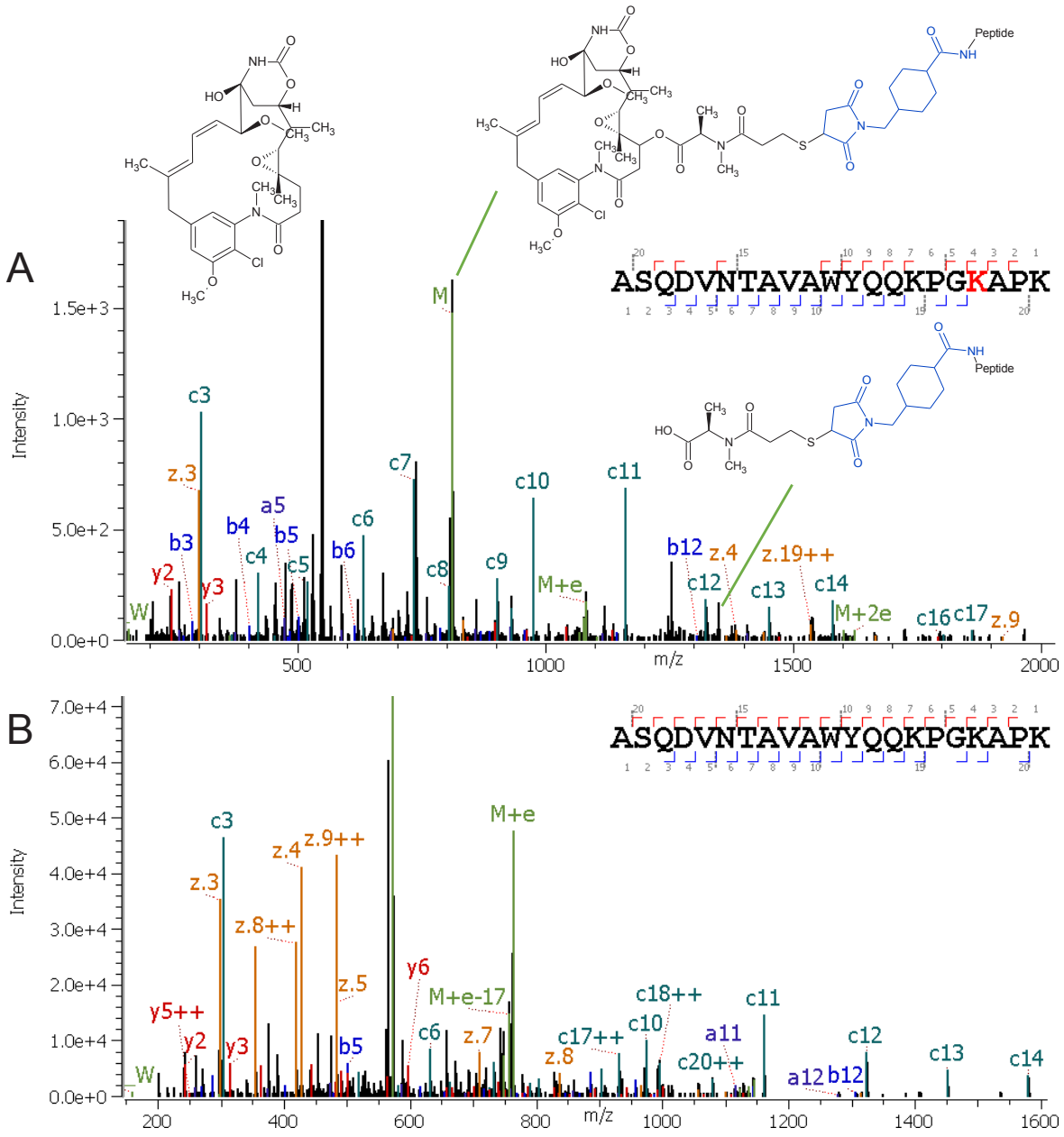

图4. 应用Zeno EAD得到的来自偶联/非偶联肽ASQDVNTAVAWYQQKPGK [DM1] APK（z = + 3）的碎片的数据。 A：来自肽段主链指定偶联肽段离子的全扫描MS / MS数据，以及有效载荷中的碎离子信息。 B：来自肽段主链指定非偶联肽的全扫描MS / MS数据。 连接子显示为蓝色，DM1药物显示为黑色

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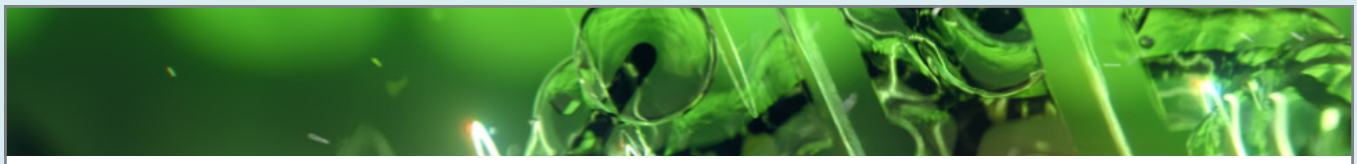

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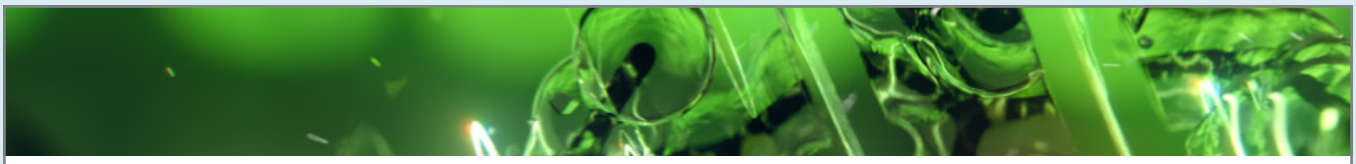

Simple and fast-tracking of DAR distribution using intact multiple attribute methodology (Intact MAM)

DAR determination for cystine-linked ADC (Vorsetuzumab) featuring SCIEX OS software

Kazuko Inoue[1], *Ryo Yokoyama[2],* Chie Inagaki[2], Greg Roman[3], and Zoe Zhang[3]

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This technical note highlights the utility of SCIEX's flexible solution for intact MAM within SCIEX OS software. This example shows the drug antibody ratio (DAR) monitoring for antibody-drug conjugates (ADCs). The streamlined MAM data flow from data acquisition to data processing in compliance-ready SCIEX OS software will also be demonstrated.

ADCs are biotherapeutics composed of small molecule drugs conjugated to an antibody scaffold. An ADC molecule consists of three components: monoclonal antibody, linker, and cytotoxic drug. As a result, ADCs have a very complex molecular structure with macromolecules and small molecule characteristics. The information about the average DAR is essential for assessing ADC qualities. For instance, a low DAR could reduce the efficacy of a drug. Therefore, DAR values are one of the critical quality attributes (CQAs) for the therapeutic index of ADCs.

In this technical note, we demonstrate SCIEX's intact MAM workflow in DAR determination of a cystine-linked ADC from monkey and mouse plasma. A time course study was performed to showcase the streamlined LC-MS based workflow, combining SCIEX high-resolution QTOF mass spectrometry with user-friendly data analysis software.

Key features of SCIEX's intact MAM solution for DAR monitoring

- **High throughput:** DAR monitoring for a large sample set
- **Intuitive software:** Powerful product attribute definition, tracking, and quantification with flexible custom calculations for CQAs based on specific user needs.
- **Streamlined solution:** Complete software solution for acquisition and quantitative tracking of DAR changes.
- **Compliance-ready**: DAR quantification and monitoring are performed in compliance-ready SCIEX OS software.

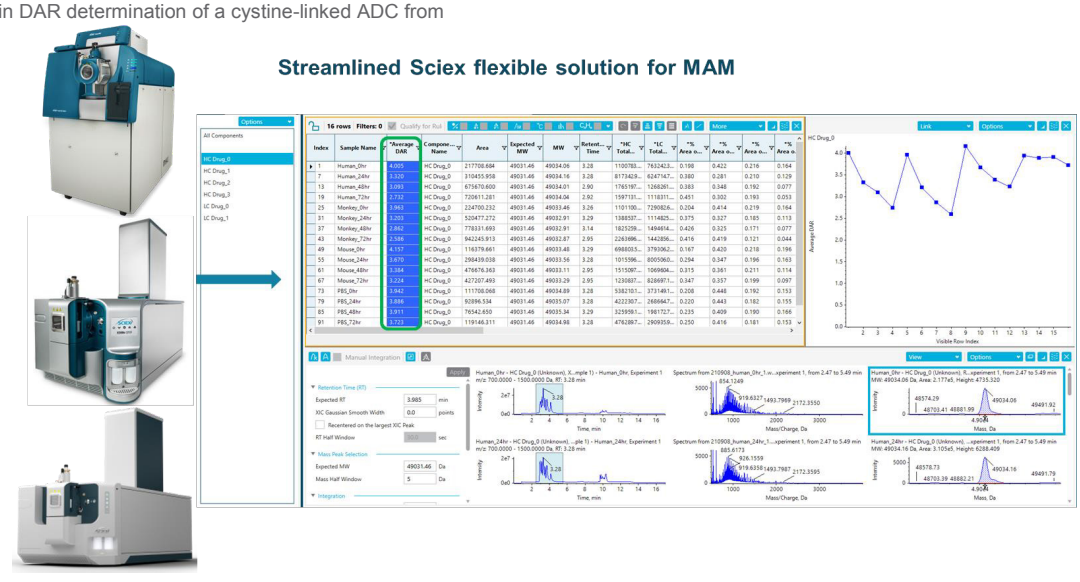

Figure 1. A streamlined workflow for DAR monitoring using SCIEX intact MAM

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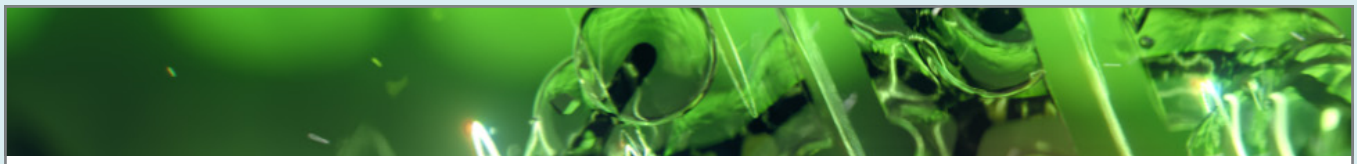

Methods

Sample preparation:

Vorsetuzumab Mc-VC-PAB-MMAE ADC was purchased from Creative Biolab Inc., (Shirley, NY). Human, monkey, mouse plasma, and PBS samples were incubated with vorsetuzumab MMAE ADC (30 µL, 0.1 mg/mL) for time intervals of 0, 24, 48, and 72 hrs. These samples were diluted with HBS-EP buffer (260 µL), followed by the addition of biotinylated human CD27 ligand as antigen of vorsetuzumab (10µL of 0.2 mg/mL aqueous solution). The mixture was incubated at room temperature for 15 minutes to generate conjugates with biotinylated human CD27 and vorsetuzumab MMAE ADC. Subsequently, streptavidin immobilized magnetic beads (Dynabeads M-280 streptavidin) were suspended into the resulting mixture and incubated at room temperature for 15 minutes with continuous mixing. After incubation, the resulting magnetic beads collected from the mixture were washed with HBS-EP buffer twice.

Table 2. Chromatography for intact and subunit analysis.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
Initial	*80*	*20*
8	*50*	*50*
9	*10*	*90*
12	*10*	*90*
12.1	*80*	*20*
17	*80*	*20*

The biotinylated vorsetuzumab MMAE ADC was released from the streptavidin magnetic beads using a pre-mixed PNGaseF reaction solution (1uL of rapid PNGaseF diluted with 37°C prewarmed MilliQ water). Then it was incubated at 37°C for 60 minutes to conduct on-bead deglycosylation. After collecting the magnetic beads from the rapid PNGaseF reaction mixture, the beads were washed with HBS-EP buffer. The vorsetuzumab MMAE ADC captured on the beads was eluted with 50 uL of acetonitrile/MilliQ water/formic acid (10/90/0.01 V/V/V). The eluates were treated with 10 mM TCEP at a final concentration for 30 minutes at 37°C to reduce the intra-chain disulfide bond of the ADC. The reduced ADC sample was injected into a TripleTOF 6600+ system with a UPLC system.

Chromatography:

Subunits were separated using an ACQUITY BEH protein C4 1.7 um, 2.1 x 50 mm analytical column (Waters), which was kept at 80°C in the column oven of a Nexera 30A (Shimadzu) UHPLC system. Table 1 shows the LC gradient used for subunit separation at a flow rate of 0.3 mL/min with mobile phases A and B consisting of 0.1% formic acid in water and 0.1% FA in acetonitrile, respectively.

Mass spectrometry:

LC-MS data were acquired using the TripleTOF 6600+ system. The key TOF parameters are listed in Table 2.

Table 2. MS parameters for subunit and intact mass analysis.

Parameter	Setting
Scan mode	*TOF-MS*
Polarity	*Positive*
Intact protein mode	*ON*
Gas 1	*60 psi*
Gas 2	*60 psi*
Curtain gas	*30 psi*
Temperature	*450 °C*
Ion spray voltage	*5500 V*
CAD gas	*6*
Time bins to sum	*80*
Accumulation time	*0.5 s*
Start mass	*500 m/z*
Stop mass	*4,000 m/z*
Declustering potential	*120 V*
Collision energy	*10 V*

Data processing:

Reconstruction and average DAR calculation of cystine-linked ADCs were performed in compliance-ready SCIEX OS software, version 2.2.

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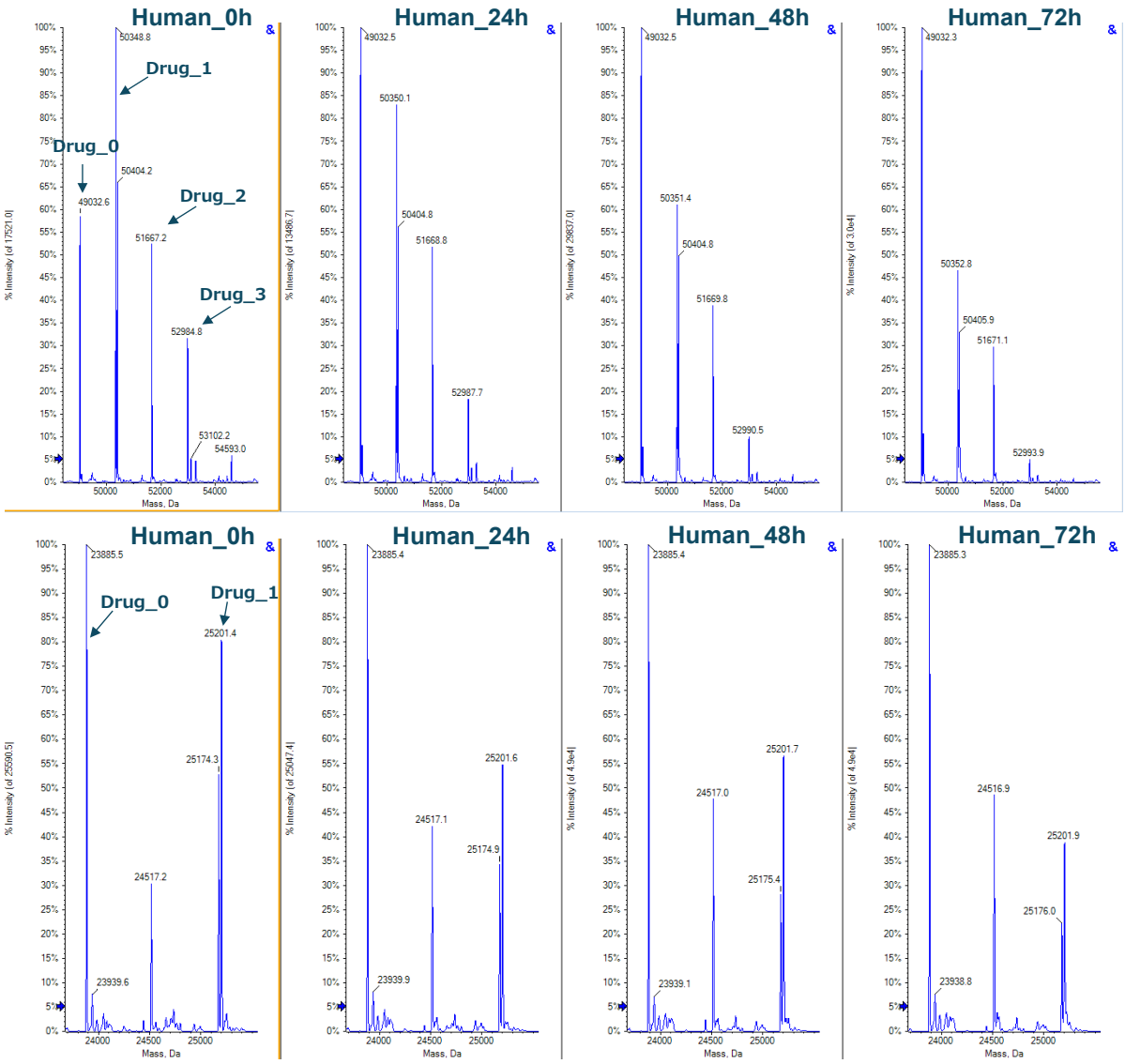

Figure 2. Reconstructed spectrum from Cys-linked ADC incubated in human plasma. Top: Heavy chain. Bottom: light chain. Peaks corresponding to the molecule carrying a higher number of drug payloads decreased throughout incubation time.

Data collection for DAR calculations

Compared to traditional antibodies, ADCs go through more potential biotransformations with a payload *in vivo* and *in vitro*, which can change DAR distribution significantly. [1] The change of *in vivo* DAR distribution of trastuzumab emtansine was recently reported in literature .[2,3] DAR is one of the CQAs that must be closely monitored during ADC manufacture and storage as it can affect drug efficacy and safety. In this work, a time course stability study was designed to investigate the changes of DAR distribution from incubation of vorsetuzumab in different plasma. Data were collected, evaluated, and reconstructed using SCIEX

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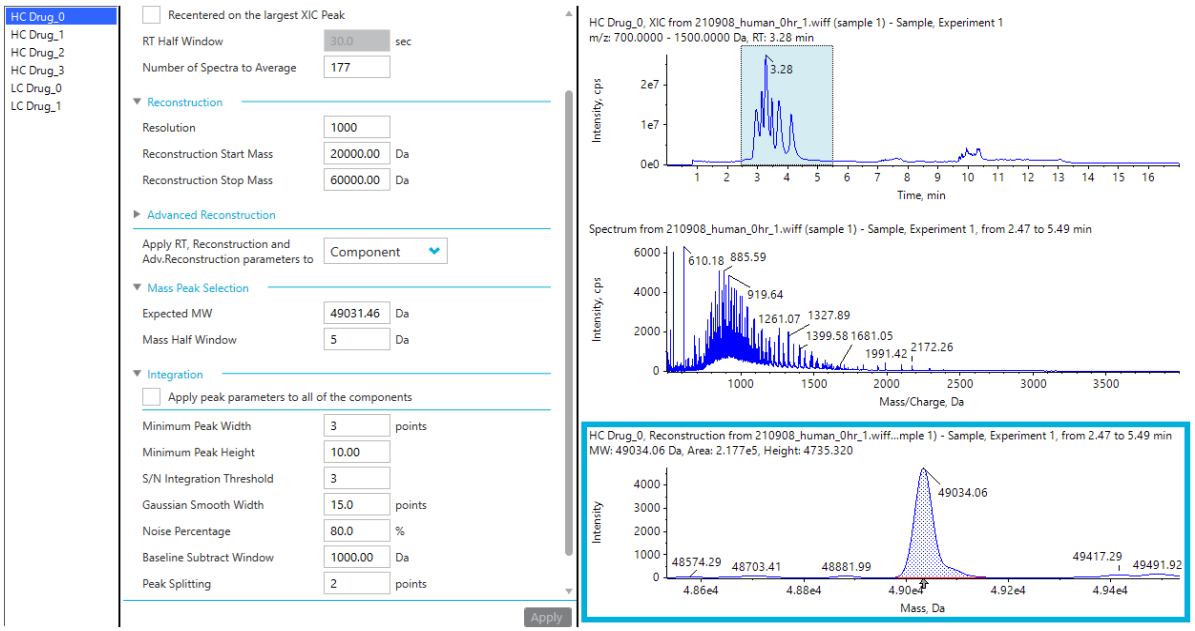

Figure 3. Parameter definition for each subunit of vorsetuzumab MMAE ADC in SCIEX OS software. SCIEX OS software allows for convenient deconvolution of the respective subunits related to the ADC.

OS software over the time course study for vorsetuzumab MMAE ADC in human plasma, as shown in Figure 2. The ADC peaks with a different number of drug payloads were labeled in the reconstructed spectra. The dominant species observed for the heavy chain carried 0-3 drug molecules, while the light chain was mainly conjugated with 0 or 1 drug payload. The DAR distributions of both chains were shifted toward the lower payloads throughout incubation.

Method generation for DAR calculation

SCIEX OS software offers a simple and streamlined intact MAM solution[4]. Attributes can be easily defined, and integration parameters for individual components can be adjusted to achieve accurate quantitation in SCIEX OS software, as shown in the previous technical note. [4] Figure 3 displays reconstruction and peak integration parameters for developing intact MAM. Using the advanced reconstruction parameters, the user can define the number of spectra to average, S/N threshold, resolution, and mass range for the assay. The attribute level can be calculated within each attribute group using flexible custom calculations. In this technical note, a method was built for automated DAR calculation, as shown in Figure 4. The percentage of peak area for each payload from each subunit was calculated using the formula displayed in Figure 4A. As vorsetuzumab MMAE ADC has two heavy chains and two light chains, the total ratio of each payload needs to be multiplied by 2, as shown in Figure 4B. Finally, the average DAR was calculated by adding all the ratios from each payload (Figure 4C).

Intact MAM for automated DAR monitoring

Vorsetuzumab MMAE ADC samples incubated with plasma were purified through immunocapture. On-bead reduction and deglycosylation were performed to reduce sample complexity, and a TripleTOF 6600+ system was employed to measure the ADC's heavy chain and light chain. Molecular mass and quantitative information of each subunit were obtained using SCIEX's intact MAM solution. The average DAR of the entire ADC can be calculated by building a custom formula within SCIEX OS software, as illustrated in Figure 4. Detailed result tables can be obtained and customized for accelerating data review (Figure 5). The table includes all the quantification results from intact MAM workflow, such as the ADC's absolute and

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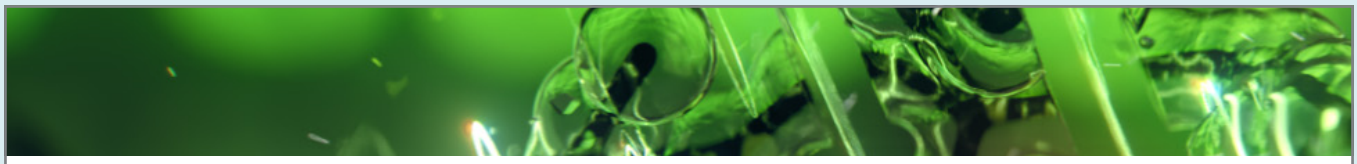

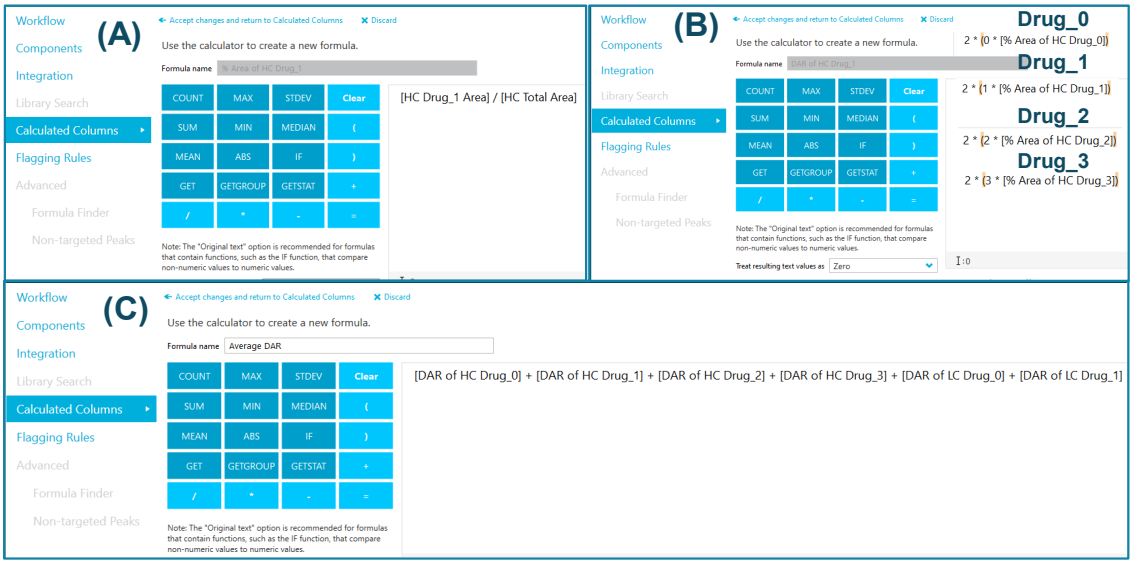

Figure 4. Custom formula for DAR calculation. (A) The calculation for the % area of each payload. Drug_1 is shown as an example. (B) The calculation for the DAR of each payload. Drug_0, Drug_1, Drug_2, and Drug_3 on the heavy chain are shown as an example. (C) The calculation for the average DAR of the entire ADC.

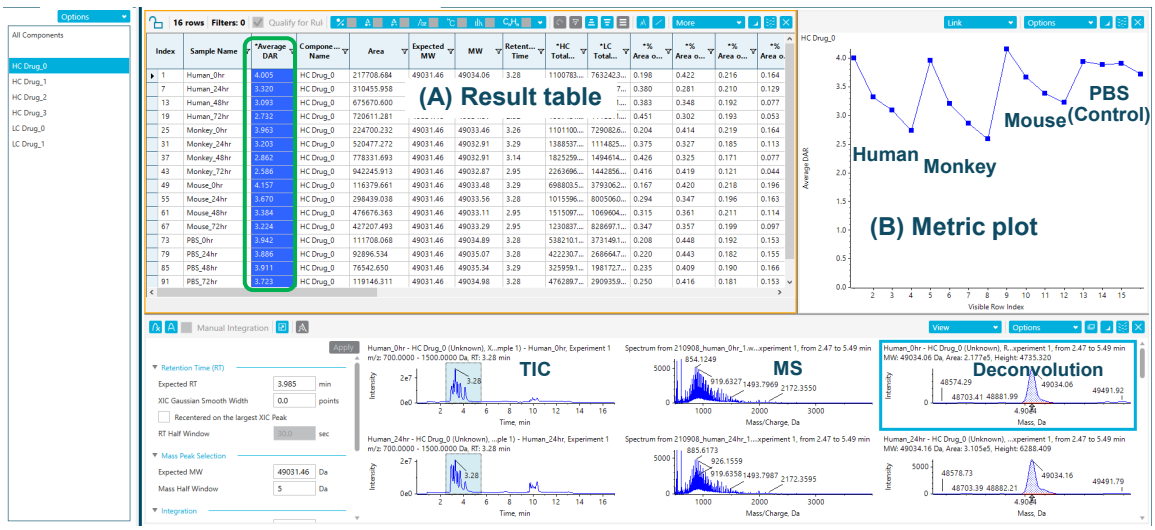

Figure 5. Result table and metric plot of DAR in SCIEX OS software. (A) Result table showing reconstructed peak area and DAR calculated customized formula. TIC, MS, and reconstructed spectrum were displayed on the same page. (B) Metric plots allow for easy visualization of DAR distribution changes.

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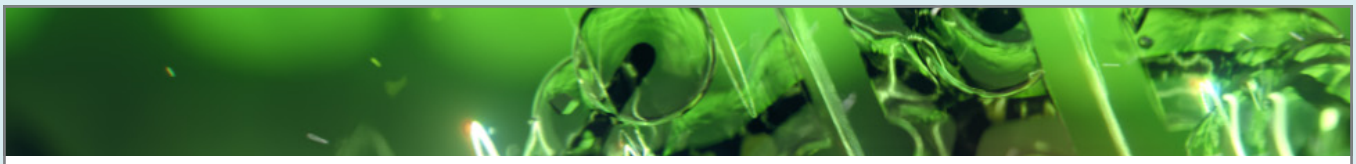

percentage peak areas with a different number of drugs and average DAR (Figure 5A). The results can be sorted by sample, targeted attribute, or modification event.

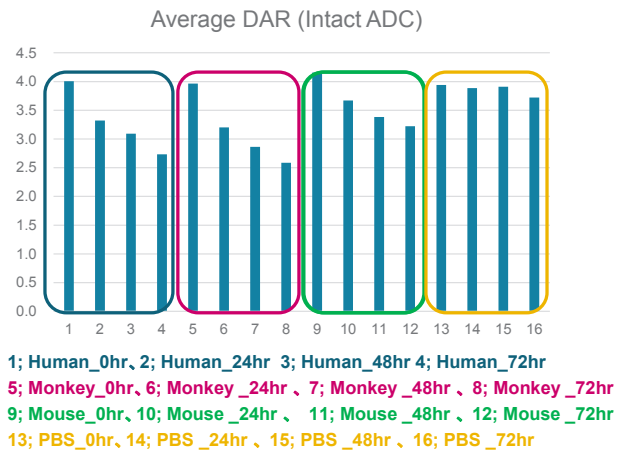

Figure 6. Average DAR of Intact ADC and DAR of each payload results. A; Average DAR monitoring from each sample. B; DAR monitoring of each payload result.

A metric plot can be created to visualize the change of each attribute (Figure 5B), offering a quick way of detecting changes in the molecule. The metric plot in Figure 5B clearly shows that the DAR distribution decreased throughout incubation of vorsetuzumab MMAE ADC in different plasma (human, monkey, and mouse). By comparison, the control samples incubated with PBS did not show this trend, as the DAR remained consistent over 3 days of incubation. The change in DAR distribution can also be visualized using a bar graph, as displayed in Figure 6. These results suggested that vorsetuzumab MMAE ADC underwent a potential biotransformation process of the payload in plasma, leading to a decrease in the average DAR value.

Conclusions

- SCIEX's flexible solution for intact MAM within SCIEX OS software offers a powerful workflow for ADC analysis by providing a streamlined and compliant software package, from data acquisition through data analysis
- The combination of SCIEX high resolution mass spectrometry and streamlined software presents a cutting-edge solution for attribute monitoring in process development, enabling faster decision making
- The TripleTOF 6600+ system and SCIEX OS software provide an excellent DAR monitoring tool.

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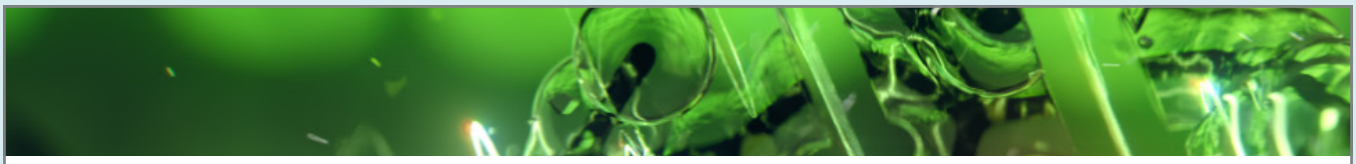

Analysis of non-deglycosylated antibody-drug-conjugates by TripleTOF® high resolution quadrupole-time-of-flight instrument and effective reconstruction software

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Introduction

Antibody drug conjugates (ADC's) are an important class of biotherapeutic compounds delivering a targeted, usually cytotoxic drug selectively to the target cell. Lysine –linked ADC's can be very complex with multiple payloads conjugated to the same biotherapeutic protein. The drug-antibody-ratio (DAR) needs to be characterized and monitored from batch-to-batch, to ensure safety and efficacy of the biotherapeutic. Analysis time bottleneck in a conventional set-up would be the deglycosylation step, which is usually required to eliminate complexity in the sample. Here we present data acquired from a non-treated ADC with multiple payloads, and N-glycosylation intact. Despite the complexity, reproducible analysis and processing was achieved. Differential ion mobility was used to further reduce the complexity and the background interferences.

Intact protein analysis for antibodies and ADCs is a rapid method for global observations of changes to product from lot-to-lot. Intact protein analysis is used to control mass of complete protein product, (i.e. to discover possible clipping or truncation), glycosylation heterogeneity, and to assess the DAR of ADCs. These aspects should be vigorously controlled, as biological therapeutics are produced in cells, and glycosylation patterns may vary due to changes in production. The drug conjugation efficiency should also be assessed to ensure safety and efficacy.

Reproducible, reliable and trustworthy data and processing are the most important factors to assess the information from intact protein analysis.

Lysine linked ADC's are a common class of ADCs, with a high number of possible conjugation sites in the structure (IgG amino acids sequence typically has more than 70 Lys –residues). Monitoring and characterization of the DAR calls for strong data quality and confidence in data processing, with automated calculations to allow fast decision making on the quality of the product. The tools allowing for good quality spectra and automated batch processing in the biopharmaceutical industry allow not only for the sample preparation time to be reduced, but also to allow the response time to the synthetic chemist department to be reduced. The speed of the analysis would also allow for the opportunity to look at more synthesis options in the same timeframe, and therefore faster synthesis method optimization.

Total analysis time depends on the sample preparation. The simpler this can be made, the more samples can be analyzed in a reasonable time frame. To reduce the sample preparation time high data quality, and orthogonal separation methods can be used to evaluate product complexity. Differential ion mobility has been shown to effectively reduce the data complexity and to separate features of different sizes and mobility. Here, the interferences in the spectra were separated from the ADC of interest.

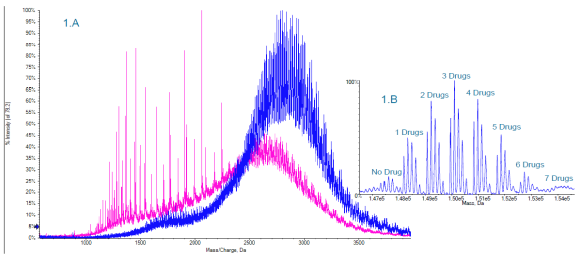

Figure 1 (A) comparison of raw spectra from the chromatographic system 1: reverse phase separation. In Blue: spectra with SelexION® technology cell optimized for the transmission of ADC ions. In Pink: no differential mobility separation applied. (B) Reconstructed spectrum of the SelexION technology-acquired data.

Experimental

Lysine-linked ADC was kindly provided by Sanofi-Vitry (France). The amino acid sequence and drug moiety structure are proprietary information.

The ADC sample was analyzed using TripleTOF® 5600 coupled to Shimadzu Nexera UHPLC, by two different chromatographic set-ups:

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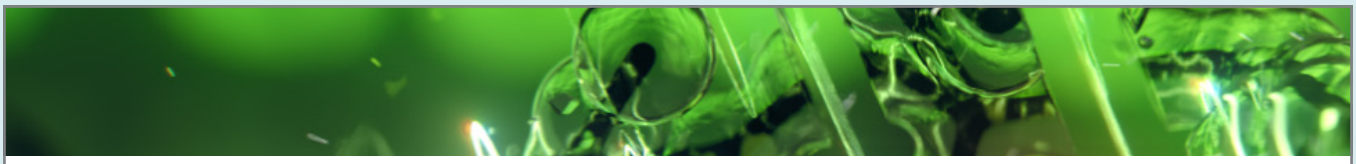

1. Agilent SB C-8 (5µm 1mmx75mm). Gradient with 20 min run time, 200 µL/min flow rate. Solvents A: 0.1% formic acid (FA) water; B: 0.1% FA Acetonitrile.
2. Waters Acquity UPLC BEH 200 SEC (1,7µm 4,6mmx300mm). Gradient with 35 min run time, 300-400 µL/min flow rate. Solvents A: Ammonium formate 25mM, 1% FA; B: Acetonitrile. Column temperature 60 ºC in both cases. 5-10µg of ADC loaded on column per run.

SelexION DMS technology was used in chromatographic system 1. Data was acquired with a TOF-MS scan over the mass range (m/z = 400-4500).Peak reconstruction was performed using BioPharmaView™ software.

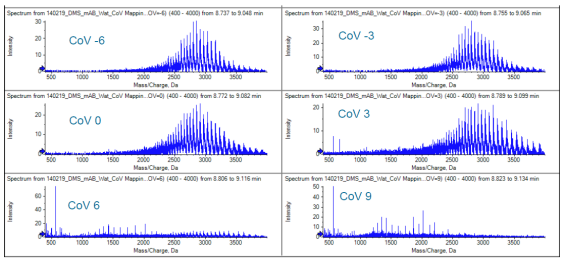

Figure 2 Optimization of the SelexION technology compensation voltages (CoV) on a monoclonal antibody (mAb). Panes have been marked with the applied CoV (here shown from -6 to 9). The separation voltage was optimized to 3500V. For large proteins such as mAbs, the transmission optimum was found with negative CoVs; for the smaller proteins (Ab light chain) and interferences, the transmission optimum was found with positive CoVs.

Results

In the initial (higher through-put) RP (C-8) set-up with SelexION differential mobility separation technology was used to reduce background interference to allow for cleaner spectra and better processing (Figure 1). The signal from the light chain (MW around 25kDa) and the formulation background interference was separated from the signal of the ADC (MW around 150 kDa) by separation voltage (SV) of 3500 V and compensation voltage (CoV) of 3. The SelexION parameters were optimized by multiple injections to ramp through compensation voltages from -12V to +12V, with the lower molecular weight features having transmission optimum at the higher end (6-12V) and the higher molecular weight features having their transmission optimum at lower CoV values (-3 - 0) (Figure 2).

The second chromatographic set-up, with size exclusion type separation, allowed for reconstruction quality spectra to be acquired in normal mode with the TripleTOF system (Figure 3).

Data processing was achieved in BioPharmaView™ software which employs maximum entropy reconstruction, here 100 iterations employed (Figure 4). Batch submission in BioPharmaView software demonstrated high level of reproducibility in both, analysis and reconstruction processing.

Non-deglycosylated ADC was identified to carry up to 7 payloads per antibody (Figures 4 and 5). The glycosylation pattern showed three glycan structures to be the most significant ones (combinations of G0F-G0F, GOF-G1F, G1F-G1F, G1F-G2F, G2F-G2F; Figure 4.). The raw data was complex, carrying heterogeneity from both, the drug conjugation and the glycosylation. The charge state envelope showed highest intensity signal for charge states from about +40 to +65 in chromatographic system 1, and +35 to +60 in the chromatographic system 2.

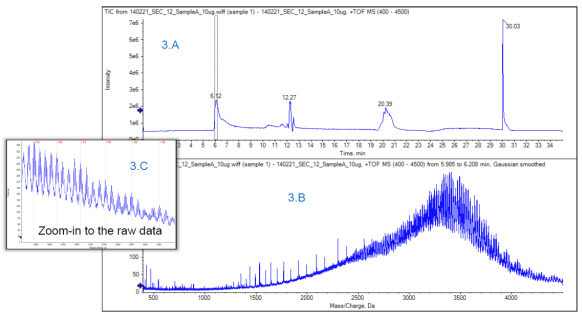

Figure 3 Chromatographic system 2 (SEC separation) Total ion Chromatogram (3A) and the obtained ADC raw spectrum (3B) and a zoom-in of the spectrum (3C) with the charge states highlighted

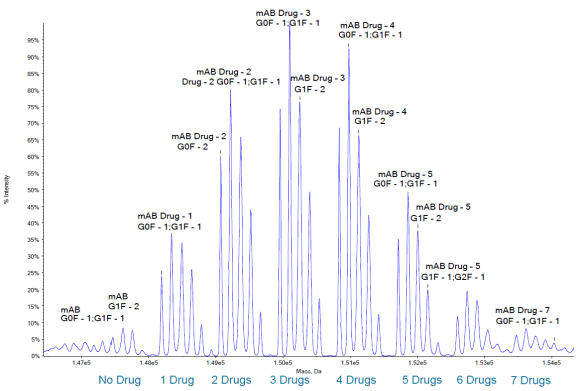

Figure 4 BioPharmaView software reconstruction of the data presented in Figure 3 Drug load was up to 7 per antibody moiety, with all the different drug conjugations demonstrating the same glycosylation pattern of combinations of G0Fs to G2Fs.

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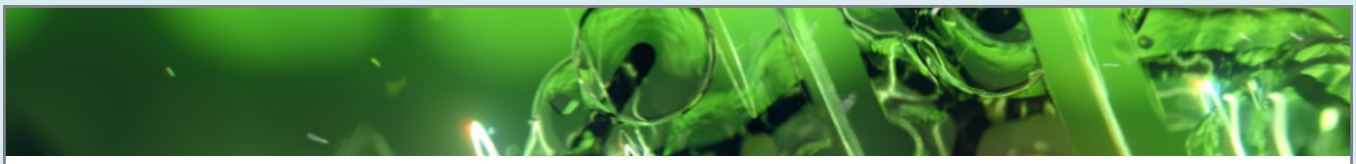

In addition to reconstruction, BioPharmaView software performs automated DAR calculations. The calculations for the samples shown in Figure 4 are shown in Figure 5. The batch processing of six injections of the same sample are shown in Figure 6.

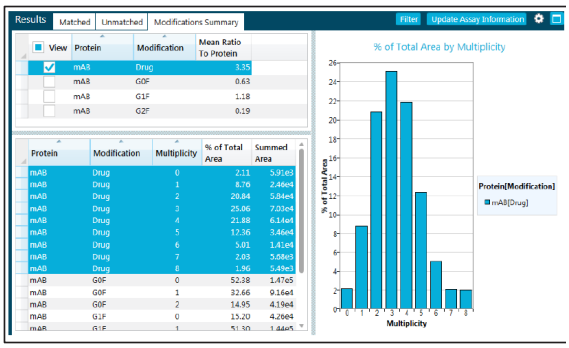

Figure 5 Automated DAR calculations for the non-deglycosylated ADC. The reconstruction area is given as a value, and also as a percentage of the total area per each multiplicity of conjugation. The multiplicity is represented as columns and the final DAR ratio is calculated.

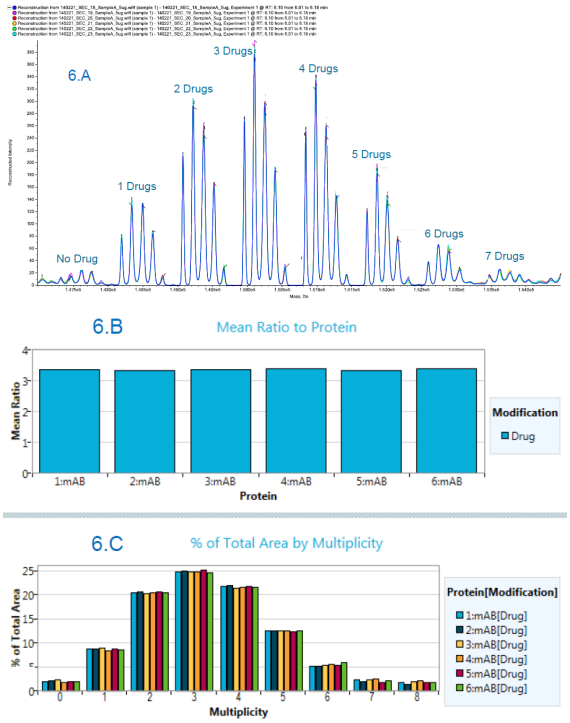

Figure 6 Reconstruction of analysis of six injections of the non-deglycosylated ADC: (6A) the overlay of six reconstructed spectra, (6B) the DAR ratios and (6C) the multiplicity represented as columns for the six injections.

Conclusions

Complex ADC's can be reproducibly analyzed without time consuming sample preparation to deglycosylate the protein. The reconstruction with BioPharmaView software enabled both the DAR to be calculated and the glycosylation pattern to be monitored within a single sample and single analysis for this complex biotherapeutic. The sample analysis time was reduced to obtain higher through-put by excluding sample pretreatment time, combining shorter analysis time with SelexION technology separation, and by enabling fast data-analysis batch-processing by BioPharmaView software

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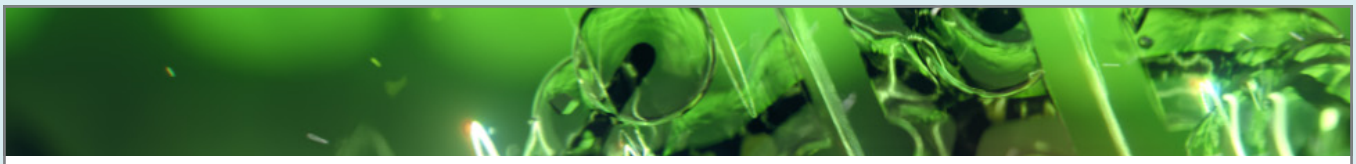

Native LC-MS Analysis of an Intact Cysteine-Linked Antibody-Drug-Conjugate

Featuring SCIEX TripleTOF® 6600 system with IonDrive™ Turbo V source and BioPharmaView™ Software 3.0.1

Jennifer Schanz[1], Jason Tonillo[1], Nicolas Rasche[1], Kerstin Pohl[2], Denise Scherbl[3], Roland Kellner[1], Sean McCarthy[2]
[1]Merck KGaA, Darmstadt, Germany; [2]SCIEX Framingham, MA, USA; [3]SCIEX, Villebon sur Yvette, France

The concept of antibody-drug-conjugates (ADC) delivering a cytotoxic compound specifically to malignant target cells with the help of a (at that time called) “heptophore” was described by Paul Ehrlich in 1913. This very reasonable concept increases selectivity for delivery and minimizes off-target toxicity. More than 100 years later, much development focusing amongst others on the protein itself, cytotoxic drugs, linkers, and conjugation chemistry have been developed. Usually, conjugation of payloads are obtained via lysine-linkage or cysteine-linkage using native or engineered residues. Linkers binding to the protein through thiols from cysteines offer an advantage of less heterogeneity compared to lysine-linked ADCs and the potential to be reduced in cellular compartments releasing the drugs inside the cells while keeping the conjugates intact during circulation.

With heterogeneity being a major challenge in the development of ADCs, a variety of analytical tools are applied throughout characterization. While conjugated light and heavy chains can be analyzed separately after reduction of remaining inter chain disulfide bonds using reversed-phase LC-MS detection. To monitor the intact ADC, native LC-MS analysis provides a non-denaturing approach to analyze intact ADCs. For native analysis, size exclusion chromatography (SEC) with volatile, aqueous buffers enables scientists to determine the drug-antibody-ratio (DAR) on intact level for ADCs. The DAR is a critical quality attribute (CQA) of ADCs which needs to be assessed during the characterization of biotherapeutics. Furthermore, proteins take up fewer charges during native analysis compared to reversed phase conditions, resulting in less distribution of the signal and an increased spacing of different features of the sample on the m/z-axis. Finally, it is less unlikely to induce artefacts during the analysis.

Here, the advantages applying native conditions for the characterization of ADCs using a TripleTOF 6600 system are described. A cysteine-linked ADC sample obtained from Merck KGaA, Darmstadt, Germany was used as a model molecule for which MS parameters were optimized and the DAR was assessed on intact level. In addition, the heterogeneity of the sample was investigated and reconstructed masses were assigned to different protein features.

Key Feature of TripleTOF 6600 System with IonDrive Turbo V source and BioPharmaView Software

- High resolution and high sensitivity for native MS analysis of proteins and protein complexes
- Efficient desolvation of native eluents without disruption of protein complexes
- Automatic matching or protein forms and DAR calculation of ADCs

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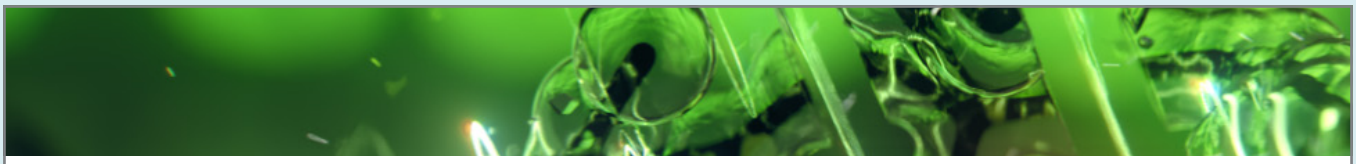

Materials and Methods

Sample

A cysteine-linked ADC sample (see Figure 1) based on a mouse monoclonal antibody (mAb) against hen egg lysozyme (HEL)

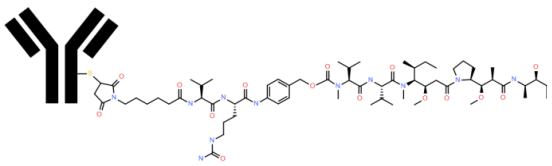

Figure 1. Conjugation example via cysteines for MC vc-PAB-MMAE.

coupled with maleimidio caproyl (MC) valine-citrulline (vc) p-aminobenzyl oxycarbonyl (PAB) monomethyl auristatin E (MMAE) (anti-HELxMC-vc-PAB-MMAE) obtained from Merck KGaA, Darmstadt, Germany (c = 4.09 mg/mL).

Chromatography and Mass Spectrometry

An ExionLC™ system coupled to a SCIEX accurate mass spectrometer TripleTOF 6600 system equipped with an IonDrive Turbo V source were used for data acquisition. An isocratic chromatography was used with conditions as shown in Table 1. The MS parameters used are shown in Table 2.

Table 1. Chromatographic Conditions.

Parameter	Value
Column	Phenomenex bioZen SEC-3 150×4.6 mm, 1.8 µm
Mobile Phase A	50 mM ammonium acetate, pH 7.2
Mobile Phase B	50 mM ammonium acetate, pH 7.2
Flow Rate	150 µL/min
Column Temperature	26 °C
Injection Amount	25 µl / 100 µg

Data processing:

Data was processed using BioPharmaView Software 3.0.1.

Table 2. MS Parameters.

Parameter	Setting
Scan Mode	TOF-MS positive
GS1	60 psi
GS2	60 psi
Curtain Gas	35 psi
Source Temperature	250 °C
Ion Spray Voltage	5000 V
Time Bins to Sum	80
Accumulation Time	1 sec
TOF Start	1,000 *m/z*
TOF Stop	7,000 *m/z*
Declustering Potential	100 V
Collision Energy	7 eV

Results

Analyzing the ADC sample under native conditions using the TripleTOF 6600 system fitted with the IonDrive Turbo V source resulted in a charge state envelope at higher *m/z* values compared to denaturing analysis as expected (see Figure 2). Using a source temperature of 250°C, reduced ion spray voltage of 5000 V, and a low declustering potential, the non-covalent sample was efficiently ionized, desolvated, and detected in its intact state. Under native-like conditions the protein maintains a 3-D structure and therefore does not take up as many charges as when being analyzed under denaturating conditions (envelope between 5000-6700 *m/z*, Figure 2). Even though soft source conditions are needed for keeping the molecule intact, the desolvation of the molecules with the IonDrive Turbo V source could be performed successfully. The source ensures high efficiency in transferring heat to the molecule via two efficient heaters, so that even when using low temperatures desolvation, nicely separated protein features on an *m/z* scale (Figure 2, upper right-hand corner), are obtained. The high quality raw spectrum was reconstructed to a “zero charge state” using BioPharmaView software allowing to assign the major proteoforms (Figure 3) with theoretically calculated masses based on the sequence and known modifications such as glycosylations and linker/drug information. The protein was detected without any drug load (naked mAb), with two drugs and with four drugs attached. For

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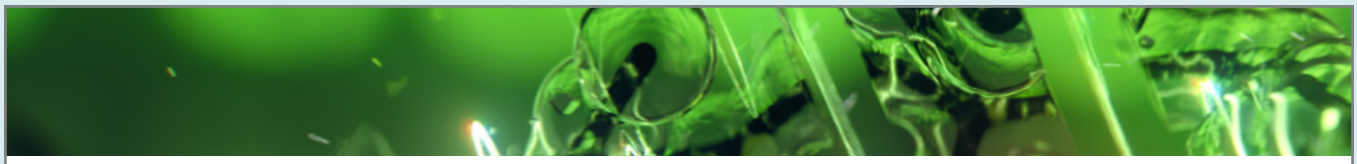

each drug load, different glycoforms were observed as expected (see Figure 3).

By processing with BioPharmaView software the main proteoforms were automatically assigned and used to calculate a DAR value of 2.78 based on the reconstructed peak areas (Figure 4). Furthermore, the drug load was visualized within the software as a bar graph for easy and quick comparison of different samples or batches (Figure 4, upper right hand corner).

Additional peaks were found in the reconstructed data which were confirmed by the high quality spectral raw data using the help of the so called hypermass tool: Any given peak in the reconstructed data can be calculated back to the potential charge states in the raw data helping the user to discriminate between real peaks being supported by raw data and potential artefacts (see figure 5). These peaks could be linked back to partial loss of the MC-vc-PAB-MMAE load (delta mass of 760 Da) and to the loss of glycosylation with a delta mass of 1445 Da (see green and orange labelling in Figure 3). It is not clear if the losses are induced during sample preparation, storage, or from the analysis. Interestingly, loss of glycosylation has not been observed in native studies of non-ADC molecules.[1]

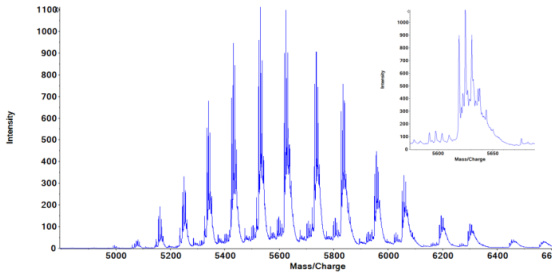

Figure 2. TOF-MS raw data for intact ADC sample. Upper right corner shows zoomed view of charge state z-28.

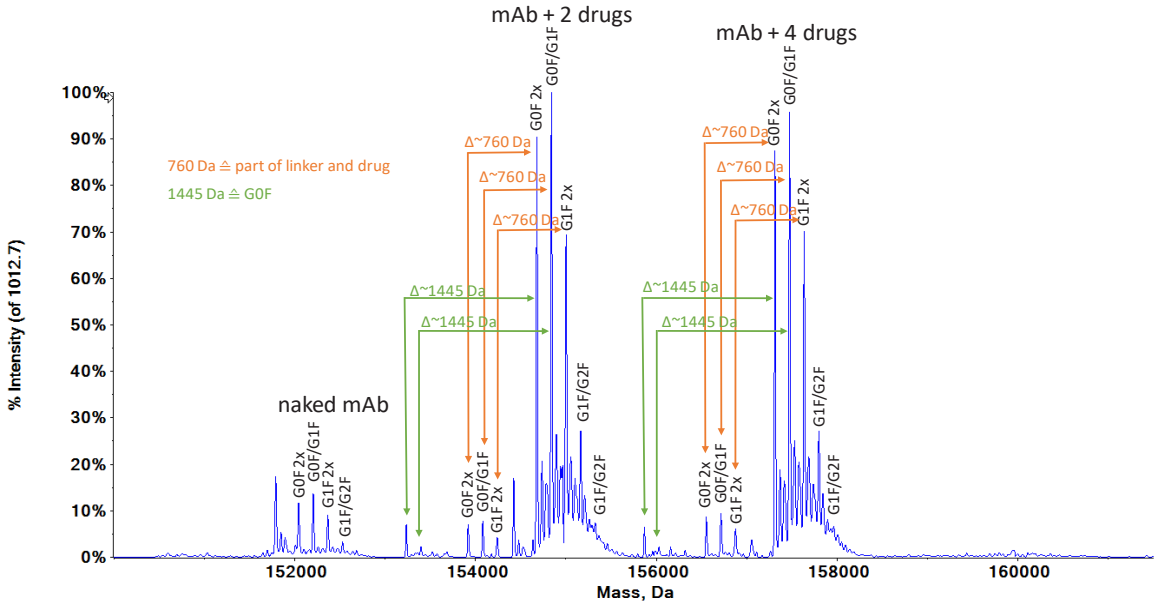

Figure 3: Reconstructed TOF-MS data. Main glycoforms of naked mAb, mAb with drug load two and four can be found. In addition delta masses of 760 Da and 1445 Da being linked to the loss of the drug and a part of the linker (orange arrows) and a loss of one G0F (green arrows) could be assigned.

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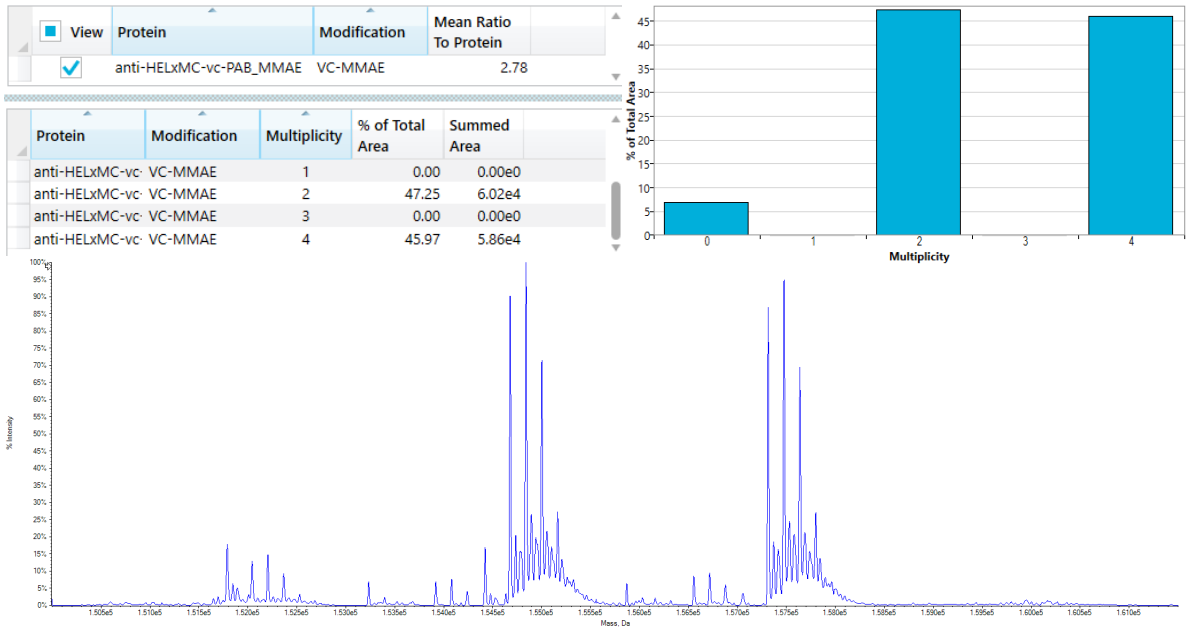

Figure 4: Automatic processing in BioPharmaView software. Automatic assignment of reconstructed peaks, DAR calculation and graphical display of drug load revealing a DAR of 2.78.

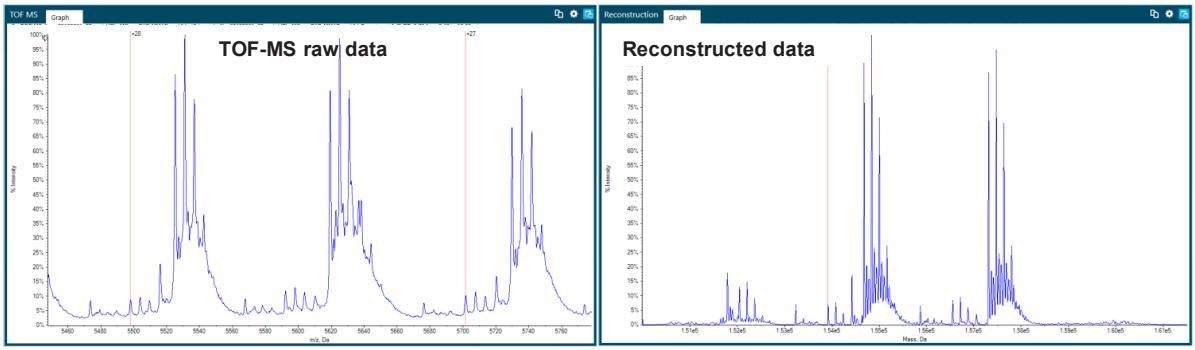

Figure 5: Hypermass feature in BioPharmaView software. Highlighting a peak in the reconstructed data (right hand side) results in a calculation of all potential charge states in the raw data (red highlights, left hand side) enabling a user to discriminate between real information and artefacts.

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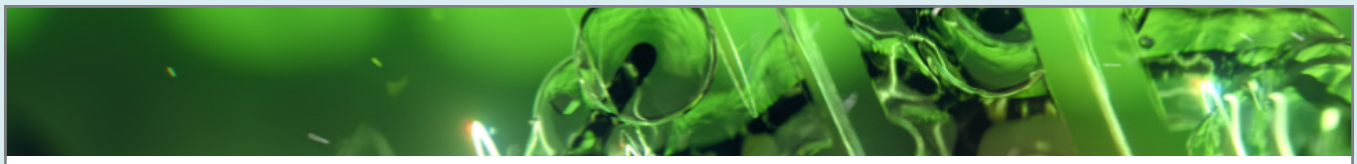

Conclusions

Native SEC-MS provides orthogonal information to other analytical tools for ADC samples during characterization. The TripleTOF 6600 system and IonDrive Turbo V source resulted in high quality data for accurate measurement of non-covalently bound samples such as cysteine-linked ADCs in an intact state under native conditions with minimal optimization. The source technology of the IonDrive Turbo V source combines an excellent desolvation of the molecule with soft ionization. The processing of the raw data with BioPharmaView software enables users to get access to critical quality attributes such as DAR with minimal hands-on time. The high quality raw data provided additional information on less abundant protein features giving further insights into the complex molecular details of ADC samples.

References

1. SCIEX Technical Note. Native Analysis of Intact Monoclonal Antibody Aggregates Using the TripleTOF® 6600 System. RUO-MKT-02-8791-A.

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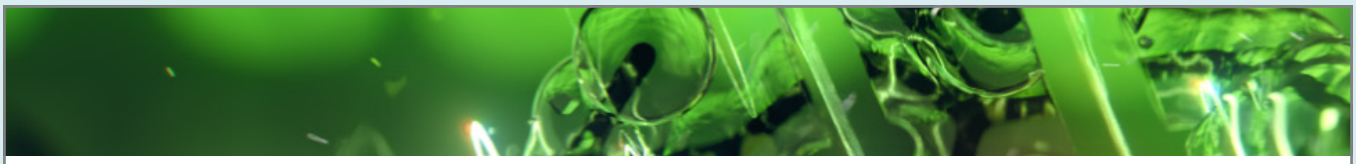

Intact Analysis of Antibody Drug Conjugates

Trastuzumab Emtansine Analysis using Benchtop X500B QTOF Mass Spectrometer

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500 Old Connecticut Path, Framingham, MA, 01701, USA

Introduction

Antibody-drug conjugates (ADCs) are an emerging class of biotherapeutics. They offer the specificity of monoclonal antibodies while incorporating cytotoxic payloads to efficiently target and kill infected cells. By their nature, ADCs are highly complex as they use an antibody backbone which must be well characterized. Adding to this complexity is conjugation of the cytotoxic payloads or drugs to the antibody. The number of drugs attached to the antibody has been shown to impact the safety and efficacy of the resulting ADC, and as such, must be characterized and monitored through development. [1-3]

Presented here is a streamlined approach for the analysis of ADCs to rapidly and accurately calculate the drug-to-antibody ratio (DAR). We will discuss the use of the new, compact X500B QToF, powered by SCIEX OS, with data processing using BioPharmaView™ 2.0.1 for routine characterization of ADCs and calculation of DAR using both the glycosylated and deglycosylated forms.

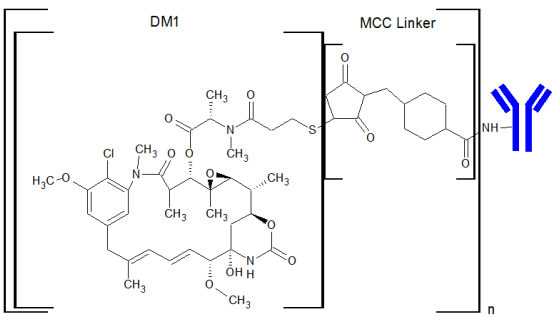

Figure 1: Trastuzumab emtasine. T-DM1 consists of a trastuzumab backbone with MCC linker binding DM1, a cytotoxic drug to the antibody via lysine residues.

Experimental

Samples were prepared either neat for the glycosylated form or using PNGase F (New England BioLabs (Ipswich, MA, USA) using their standard protocol.

LCMS analyses were conducted using a benchtop X500B QTOF mass spectrometer using SCIEX OS equipped with an Exion LC™ system. Table 1 lists the LCMS conditions used in these analyses. Data was processed using BioPharmaView™ for reconstruction of the intact protein and calculation of DAR.

Table 1 Exion LC™ conditions

Column	*Agilent Poroshell 300SB-C8 1.0 x 75mm 5 µm*
Mobile phase A	*0.1 % Formic acid in water*
Mobile Phase B	*0.1% Formic acid in acetonitrile*
Flow rate	*0.2 mL/min*
Column Temperature	*75 °C*

Table 2 X500B mass spectrometry conditions

Source parameters:	
CUR	*30*
GS1	*50*
GS2	*50*
Ion Spray Voltage	*5000 V*
Source Temperature	*400°C*
TOFMS mass range	*900 – 4000 m/z*
DP	*250 V*
Accumulation time	*0.5 s*
Time bins to sum	*80*
Intact Protein Mode (IPM)	*On*
Large Proteins (>70kDa)	*On*
Decrease Detector Voltage	*On*
DP	*250 V*

Results and Discussion

Glycosylated T-DM1

For this study, we used trastuzumab emtansine (T-DM1), a lysine conjugated ADC for the treatment of HER-2 positive metastatic breast cancer. T-DM1 is comprised of an antibody, trastuzumab, covalently linked via lysine residues to cytotoxic drug molecules which are liberated following internalization by target cells (Figure 1). As drug molecules are attached to the antibody following expression, assessment of the drug-to-antibody ratio (DAR) must be determined regularly as part of the drug development process.

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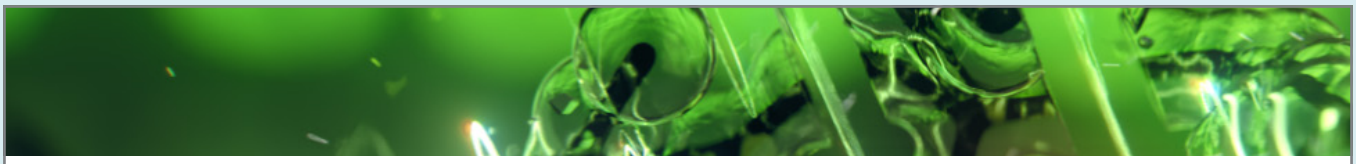


We began our study by determining the DAR of the intact ADC. As shown in Figure 2A, the resulting raw spectrum is highly complex. Using the BioPharmaView reconstruction algorithm, we generated high quality reconstructed spectra clearly showing the different DAR species as well as the glycoprofile for each DAR. The resulting reconstruction of the raw data gives us a range of between 0-8 drugs attached to the trastuzumab (Figure 2B). As expected, the glycoprofile for each DAR is consistent across each of the DAR species.

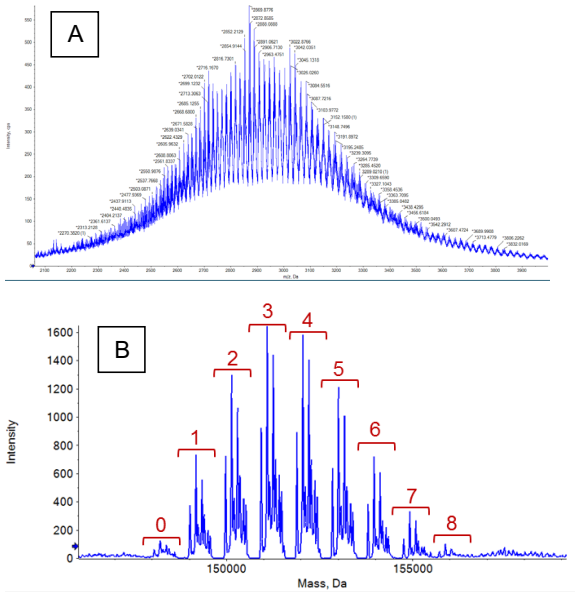


Figure 2: T-DM1 raw (A) and reconstructed (B) data. Raw data (2A) includes glycosylations and ADC which increases the normal complexity of an antibody spectrum while 2B shows the reconstruction using BioPharmaView and calculating 0-8 drugs attached to the trastuzumab.

Looking closely at each reconstructed DAR separately, we clearly see evidence for each of the main glycoforms as well as a corresponding set of species with a mass shift of 221 Da and not 219 Da which would be the mass of the linker itself. (Figure 3). This has previously been reported to be due to a reaction with lysine residues, which results in chemical crosslinking. The initial reaction of trastuzumab to the MCC linker produces an intermediate which, in some cases, due to the proximal location of lysines involves a second reaction causing the inter-chain cross linking and a linker species of 221 Da without the DM1. [2]

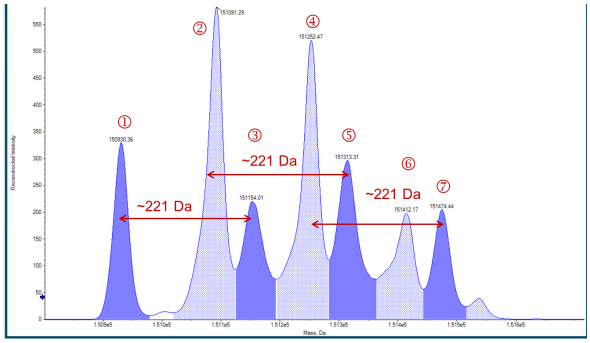


Figure 3: Trastuzumab emtansine with 3 drugs attached. A closer look at the reconstructed DAR shows evidence for the main glycoforms: 1 G0F/G0F, 2: G0F/G1F, 3:G1F/G1F and 4: G1F/G2F with corresponding additions of one MCC linker without DM1.

Using BioPharmaView, (DAR) can be calculated based on the reconstructed peak area distribution of the different drug conjugated antibodies. DAR was calculated to be 3.49 (Figure. 5), which agrees well with previously reported value of 3.5 [1-3].

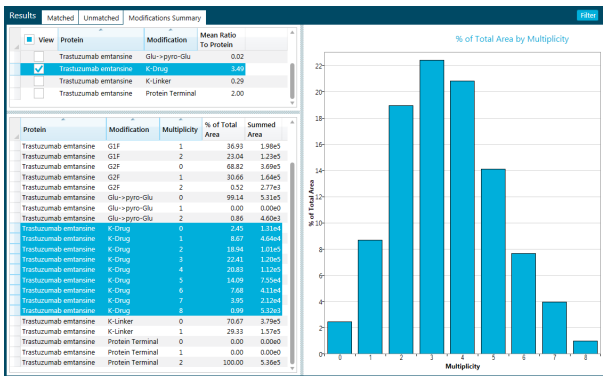
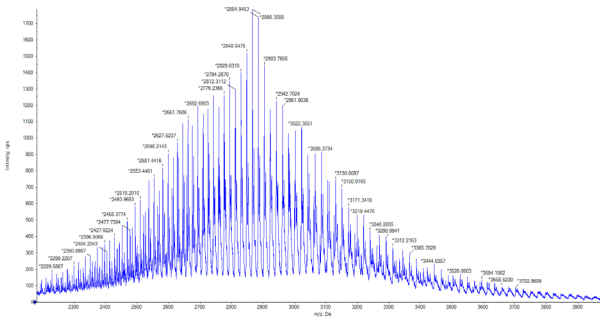


Figure 4: Calculation of DAR for trastuzumab emtasine using BioPharmaView™. Calculation of DAR using the software gives a simple table and graph, showing the median multiplicity of the ratio. Highlighted in the top right is the ratio calculated which was 3.49.

Deglycosylated T-DM1

We then removed the N-linked glycans using PNGaseF to provide a spectrum with reduced complexity as the peaks attributed to the glycoforms would be removed. As shown in Figure 5A, we can clearly see that the glycoform complexity has been reduced. In addition, the presence of the species with a 221 Da higher than each corresponding DAR species is evident confirming our previous findings.

内容提要



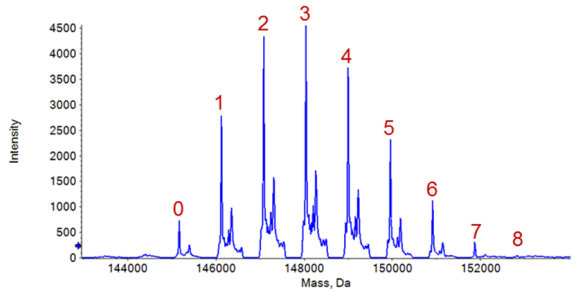


Figure 5: Deglycosylated T-DM1 raw (A) and reconstructed (B) spectrum. Raw data (5A) from the deglycosylated is used to reconstruct the intact protein mass (5B).

The reconstructed spectrum for the deglycosylated trastuzumab emtasine confirms the results observed in figure 2 of the glycosylated form. Both confirm from 0 – 8 drugs attached to the trastuzumab backbone. DAR calculations were performed using the software, and the resulting DAR value is consistent with previously reported values. [2]

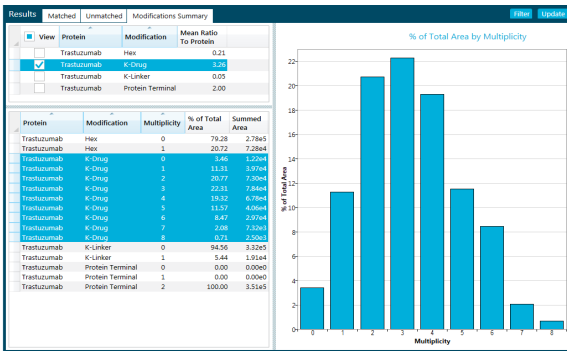


Figure 6: DAR calculation for deglycosylated trastuzumab emtansine. The 8 drug conjugates (highlighted bottom left) are then used to calculate the DAR ratio (highlighted top left) to give a DAR ratio of 3.26.

Conclusion

We have shown that the benchtop X500B QTOF system produces data with such quality that it can be used for routine analysis of such complex biologics such as ADCs which require high resolution data to identify glycosylations and the number of drugs bound to the antibody. We have shown that the mass accuracy is such that we are able to identify a prominent 221 Da mass shift that has been reported by Jacobson et al as a conjugation of the trastuzumab-MCC linker intermediate with a proximal lysine residue. Processing of the data and reconstruction was performed using BioPharmaView resulting in fast reconstruction and accurate DAR calculations. The resulting software was able to calculate the DAR ratio to be 3.46 on the glycosylated form compared to the 3.5 listed in literature. [2]

References

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[2] Yan Chen, Michael T. Kim, Laura Zheng, Galahad Deperalta, and Fred Jacobson. Structural Characterization of Cross-Linked Species in Trastuzumab Emtansine (Kadcyla). Bioconjugate Chem. 2016, 27, 2037−2047
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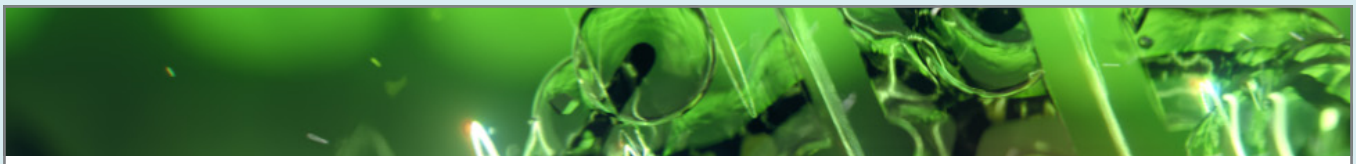

Structural Characterization of Antibody Drug Conjugates (ADCs) by a Combination of Intact, Middle-up and Bottom-up Approaches using CESI-MS

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Introduction

Antibody drug conjugates (ADCs) represent a rapidly growing class of biopharmaceuticals. ADCs are formed by the conjugation of an active drug species to a monoclonal antibody,[1] and they often result in a distribution of products containing varying numbers of active drugs bound at different locations around the antibody.

Capillary electrospray ionization (CESI) is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device (Figure 1).[2] CESI-MS operates at low nL/min flow rates and offers several advantages, including increased ionization efficiency and a reduction in ion suppression. CESI-MS separates analytes by their charge and size, and it is therefore a separation mechanism that is complementary to more traditional techniques, such as reversed-phase LC.

This document summarizes the work recently published by the research group at LSMIS at the Université de Strasbourg.3 In this application note, we will show how CESI-MS can be used to characterize a gold standard ADC, brentuximab vedotin (BV). BV is a cysteine-linked mono-methyl auristatin E (MMAE) monoclonal antibody (mAb), which was attached without disrupting the heavy and light chain linkages of the mAb.[3] We will show how CESI-MS is used as a nano-spray infusion device to identify the drug antibody ratio of the sample. We will also show how CESI-MS can be used to study the "middle-up" structure for this protein, for example, by analyzing the light chain, Fab and F(ab')2 subunits. Finally, a bottom-up analysis of a tryptic digest will be performed by CESI-MS to fully sequence the ADC.

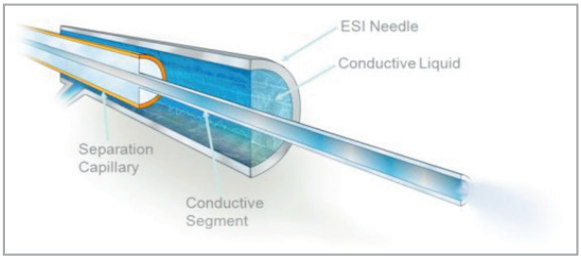

Figure 1. OptiMS - Ultra low flow ESI Interface.

Materials and methods

Chemicals: All chemicals were reagent grade and were purchased from Sigma Aldrich or alternative suppliers.[3] Brentuximab vedotin was produced by Millennium Pharmaceuticals/Takeda.

Sample Preparation: BV was buffer exchanged with 200 mM ammonium acetate buffer (pH 7.0) using Amicon filters before intact analysis.[3]

For middle-up analysis, BV was cleaved at the hinge region by proteolysis using IdeS (FabriCATOR, Genovis) to obtain Fc/2 fragments and an F(ab')2 fragment. After digestion was completed, the sample was again buffer exchanged into 200 mM ammonium acetate buffer (pH 7.0) before CESI-MS analysis.

For bottom-up analysis, a sample of desalted IdeS cleaved ADC, which had undergone buffer exchange and was diluted with RapiGest (Waters) and reduced with dithiothreitol (DTT). Before enzymatic digestion, 10% of acetonitrile was added to the sample. The reduced protein was then digested with trypsin overnight, isopropanol (40%) and formic acid (1% v/v) were added and the final solution diluted using ammonium acetate (50 mM, pH 4.0) to produce a final concentration of 2.2 µM.[3]

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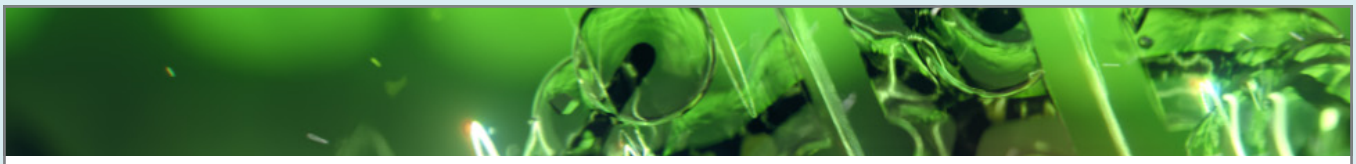

CESI-MS method: For the analysis of intact and digested samples, a bare fused silica OptiMS cartridge (30 µm ID x 91 cm) from SCIEX was used and was thermostatted using recirculating liquid coolant regulated at 20°C used. For the analysis of tryptic digests, the sample was injected hydrodynamically (10 psi, 60 s) and peptides were separated using conditions shown in Table 1 with a background electrolyte of 10% acetic acid. For MS analysis of tryptic digests, a SCIEX TripleTOF® 5600+ LC-MS/MS System was fitted with the NanoSpray® III Ion Source. Gas 1 and 2 were not used, the curtain gas was set to 5 psi and the temperature of the interface was set at 150 °C. Ionization at these very low-flow rates occurred by simply applying the ionspray voltage (1,450 V). MS data was acquired using a TOF survey scan (m/z 100–2,000 amu), which triggered MS/MS acquisition (m/z 100–2,000 amu).

Action	Time (min)	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	3.5	100	Forward	0	0.1 M NaOH
Rinse	1.5	100	Forward	0	Water
Rinse	3	100	Forward	0	0.1 M HCl
Rinse	5	100	Forward	0	10% Acetic acid
Rinse	2	75	Reverse	0	10% Acetic acid
Rinse	60s	10	Forward	0	Sample Vial
Injection	10s	5	Forward	0	10% Acetic acid
Separation	35	0.1	Forward	20	10% Acetic acid
Voltage	2	10	Forward	1	10% Acetic acid

Table 1. CESI separation conditions used for the analysis of tryptic digests.

For nano-spray infusion, the CESI capillary was flushed with 10% acetic acid (5 min, 50 psi) and then with sample (5 min, 50 psi), and MS data was acquired at 5 psi. After each analysis, the capillary was flushed with 10% acetic acid (10 min, 50 psi) to prevent carryover. For these nano-spray infusion experiments, MS data was acquired using a maXis 4G system (Bruker). The maXis system was optimized for each analysis using the actual sample and ion funnels with values that ranged from 300–400 Vpp. The electrospray voltage ranged from 1,200–1,800 V, drying gas was set at 1.5 L/min and the source temperature was set at 150 °C.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Results

When CESI is used as a nano-spray infusion device, the sample is simply pushed to the MS detector at low nL/min flow rates.[4] Desalted intact BV was analyzed in this mode to confirm the molecular weight of this ADC and measure the drug to antibody ratio (DAR), which is the drug loading on the antibody and typically ranges from 0 to 8. An example of the data achieved is shown in Figure 2, which gives an overview of the analysis of BV by CESI-MS.

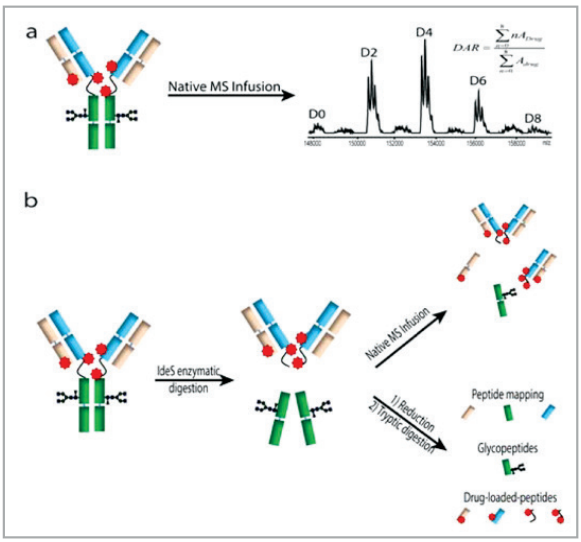

Figure 2. Overview of brentuximab vedotin structural characterization using sheathless CE-MS. (a) Native MS infusion for average DAR determination and drug loading distribution assessment. (b) Middle-up and bottom-up analysis.

Based on the charge state of the deconvoluted mass spectrum of the intact BV shown in Figure 2, the average DAR value was between 3.8 and 3.9. A distribution of baseline resolved species of BV was observed with masses that corresponded to the intact mAb linked with 0 to 8 payloads of the drug. The mass accuracies of intact BV were in total agreement with the results reported in the literature.[3]

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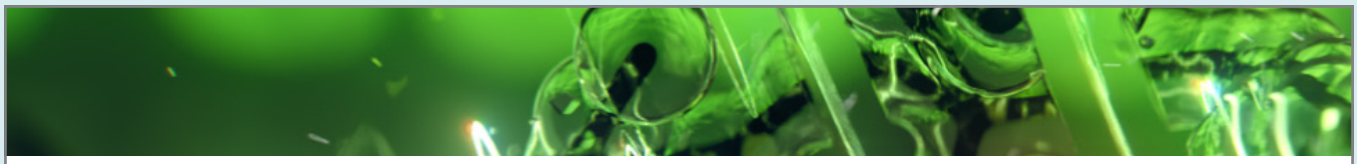

Using the same experimental conditions as the intact analysis, a middle-up sample was analyzed to elucidate the location of the drug loading on the mAb, which had been cleaved at the hinge region (Figure 2). The raw data highlighted multiply charged protein peaks in 3 separate regions across the mass range of 2,000–6,000 amu (Figure 3). When deconvoluted, the multiply charged protein envelopes depicted in Figure 3 showed that the free light chain (LC) protein subunit (MW = 25040.1 ± 0.1 Da) had 1 drug molecule linked to it as well as 2 glycation modifications. The smaller Fab subunit (approximately 48–55 kDa) had 0–4 molecules of drug linked to the protein and was present as a dimer. The F(ab')2 subunit (approximately 97–108 kDa) had 0–8 drug molecules attached to the protein subunit. As the drug units were spread across multiple regions of the mAb, the IdeS middle-up sample could not be used to calculate the DAR for ADCs, but does give information about the location of the drug molecules within BV.

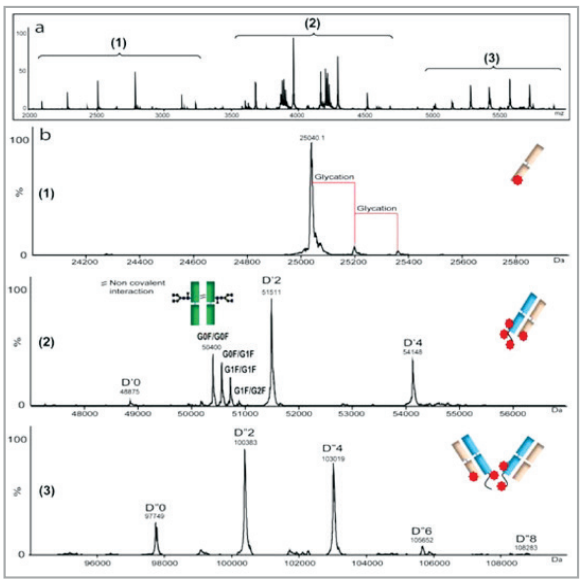

Figure 3. (a) MS spectra corresponding to native MS nano ESI infusion of middle-up BV. (b) Charge state deconvoluted mass spectra of (1) LC- drug conjugated subunit, (2) Fab subunits with the incorporation of 0 to 4 drug molecules and Fc/2 homodimers and (3) F(ab')2 subunits with the incorporation of 0 to 8 drug molecules.

CESI-MS has been previously used for the analysis of tryptic digests of mAbs.[5] The next set of experiments performed on BV were the bottom-up analysis of a tryptic digest to determine the location of post-translational modification (PTM) sites, the location of the drug linked to the mAb and the amino acid sequence. The tryptic digestion protocol had been adapted from the classical approach to improve the overall digestion, which was affected by the presence of the drug molecules bound to the mAb.[3] CESI-MS analysis took less than 35 min. Due to the sensitivity and efficiency of the CESI-MS analysis, 100% sequence coverage could be obtained for BV in a single injection with the identification of the peptides based on their accurate molecular weight as well as sequence data from MS/MS analysis. The presence of the organic solvent in the sample preparation did not seem to have an effect on the separation of the tryptic peptides demonstrated by the detection of small (3 amino acids) to large (63 amino acid) peptides.

Modified peptides (including N-glycopeptides) were detected in the analysis, for example, TKYPREEQYN[297]STYR was observed to have 11 glycoforms. Regarding drug-loaded-peptides, 4 were detected (Figure 4), which was aided by the presence of organic solvent in the sample preparation to prevent the loss of these hydrophobic modified peptides.

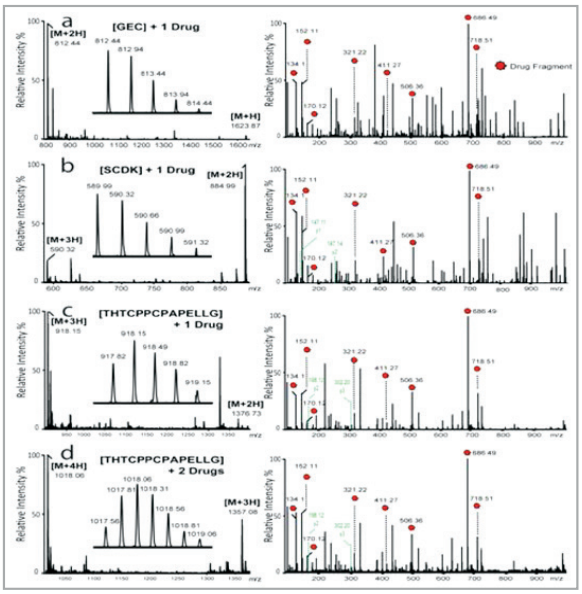

Figure 3. MS and MS/MS spectra of drug-loaded peptides. (a) [GEC] - 1 drug, (b) [SCDK] -1 drug, (c) [THTCPPCPAPELLG] - 1 drug and (d) [THTCPPCPAPELLG] - 2 drug molecules

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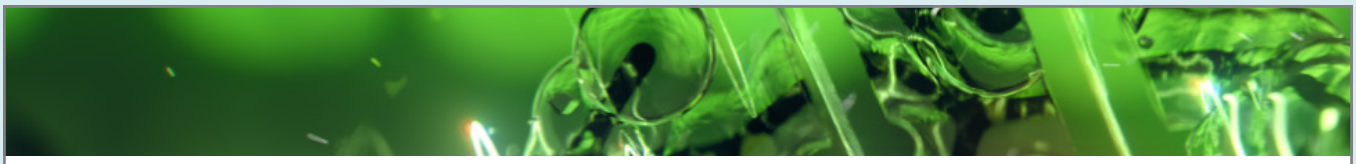

Identification of these drug containing peptides was confirmed by MS/MS data analysis, which highlighted the presence of several diagnostic drug fragment ions. A single drug loaded peptide was located on the light chain of the mAb (GEC), and the other peptides were present on the heavy chain. The THTCPPCPAPELLG peptide actually had the potential of containing 2 drug molecules, and these 2 different peptides have been separated by CESI-MS.

Conclusions

A CESI-MS protocol for structural characterization of ADC molecules has been developed. By using CESI-MS in 2 different modes, several properties of an ADC could be confirmed, including:

- DAR ratio calculation using native conditions
- Drug distribution on the F(ab')2 and Fc/2 using nano-spray infusion CESI-MS analysis of an IdeS digested middle-up sample using native conditions
- 100% sequence coverage of the ADC and identification of the drug location as well as the location of other PTMs
- Characterization of drug loaded peptides by analysis of MS/MS spectra

For further information on this topic we would like to refer readers to the full scientific publication on which this application note is based.[3]

References

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4. Gahoual, R., Busnel, J.M., Wolff, P., Francois, Y.-N., Leize-Wagner, E. Novel sheathless CE-MS interface as an original and powerful infusion platform for nanoESI study: from intact proteins to high molecular mass noncovalent complexes. *Anal. Bioanal. Chem.* (2014), 406: 1029–1038.
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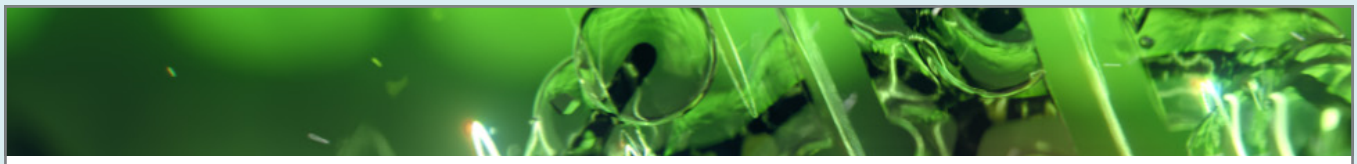

Ultra-sensitive LC-MRM analysis for trastuzumab-emtansine quantification in rat plasma

Featuring the SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP® Ready, powered by SCIEX OS Software

Zuzana Demianova and Lei Xiong
SCIEX, USA

With the adoption of immunoaffinity based sample preparation, an ultra-low LLOQ of 1 ng/mL was achieved. The assay shows high precision, accuracy, and good linearity, demonstrating the robustness and performance of the developed method.

Quantification of protein therapeutics in biological matrices, play a critical role in the drug discovery and development process. LC-MS/MS has been routinely adopted for quantification of this molecular class in bioanalytical laboratories, serving as an alternative technology to the traditional ligand binding assays (LBAs). Although mass spectrometry offers higher specificity than LBA, there still remains a need for improved sensitivity for accurate quantification of low concentration analytes in complex matrices while still maintaining high analysis throughput, instrument easy-of-use, and robustness.

In this work, the SCIEX Triple Quad 7500 System coupled with an analytical flow HPLC system is employed to quantify trastuzumab-emtansine in rat plasma. Multiple hardware improvements on the ion source and the front end of the mass analyzer significantly boost the system sensitivity.[1]

Key features of the peptide quantification workflow

- Immunoaffinity-LC-MRM workflow[2] offers solid quantification of trastuzumab-emtansine in rat plasma at 1 ng/mL, with high precision, accuracy, and linearity
- Hardware improvements on the SCIEX 7500 System provide significant gains in sensitivity for peptide quantification: the OptiFlow® Pro Ion Source with E Lens™ Technology provides improvements in ion generation and the D Jet™ Ion Guide improves ion sampling
- SCIEX OS Software—an easy to use, compliance ready, single platform for acquisition, processing and data management

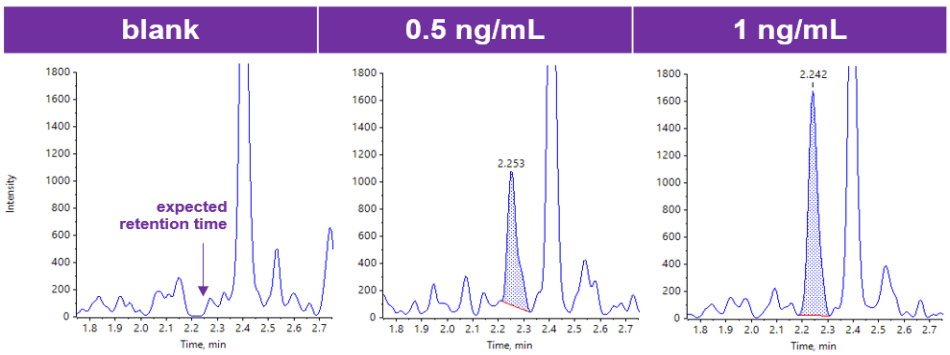

Figure 1. Extracted ion chromatograms (XICs) of selected MRM for trastuzumab-emtansine in rat plasma. From left to right are blank, 0.5 ng/mL (LOD) and 1 ng/mL (LLOQ).

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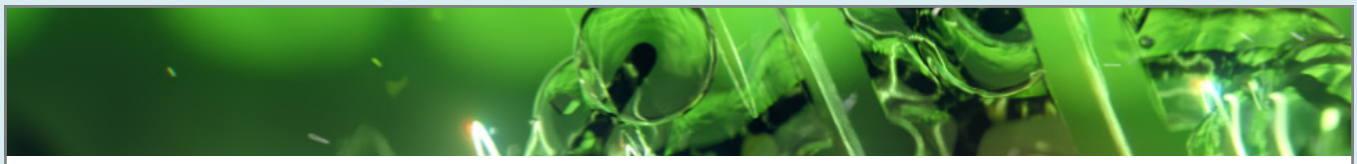

Methods

Immunocapture: A streptavidin coated immunoaffinity magnetic bead slurry was aliquoted and washed with PBS buffer (1x) three times. Biotinylated goat anti-human IgG antibody was added to the beads and incubated at room temperature for 1 hour with shaking. The conjugated beads were washed three times and re-suspended in PBS buffer. Calibration standard samples were prepared by spiking trastuzumab-emtansine and SILuMab (internal standard) into rat plasma and performing serial dilution. The concentrations of trastuzumab-emtansine in plasma ranged from 0.5 - 20,000 ng/mL. To each calibration standard sample, 250 µL of PBS Buffer (1×) and 50 µL conjugated bead slurry were added and the mixtures were incubated at room temperature for 1 hour with shaking. The beads were accumulated by magnetic stand and washed sequentially with PBS buffer and 10 mM ammonium bicarbonate. The target proteins were eluted by incubating the beads with 0.1% TFA in water and vortexing for 10 min.

Protease digestion of immuno-enriched eluents: The eluents were neutralized with 500 mM ammonium bicarbonate in water and incubated at 95 °C for 10 mins with shaking. The samples were cooled to room temperature and incubated with 1 µg of trypsin/Lys-C mix per sample overnight at 37 °C. The digestion was aborted by adding formic acid. The samples were centrifuged at 15,000×g for 5 min. The supernatants were collected and subjected to LC-MS/MS analysis.

LC-MS/MS conditions: Samples were analyzed in triplicate by a SCIEX Triple Quad 7500 System, coupled with an ExionLC System. The method details are summarized in Tables 1, 2 and 3. The data were processed using the Analytics module in SCIEX OS Software 2.0.

Table 1. Chromatographic conditions.

Parameter	Value
Column	*Phenomenex bioZen Peptide XB-C18 50×2.1 mm; 2.6 µm*
Mobile phase A	*Water with 0.1 % formic acid*
Mobile phase B	*Acetonitrile with 0.1 % formic acid*
Flow rate	*500 µL/min*
Column temperature	*40 °C*
Injection volume	*30 µL*

Table 2. Gradient information.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
Initial	*95*	*5.0*
0.7	*95*	*5.0*
0.8	*90*	*10*
3.5	*75*	*25*
4.0	*60*	*40*
4.5	*10*	*90*
6.0	*10*	*90*
6.1	*95*	*5.0*
7.7	*95*	*5.0*

Table 2. Mass spectrometric conditions.

Parameter	Value	Parameter	Value
Curtain gas	*40 psi*	*Source temperature*	*500 °C*
Ion source gas 1	*50 psi*	*Ion source gas 2*	*50 psi*
CAD gas	*11*	*Ion spray voltage*	*1700 V*

Name	*Q1*	*Q3*	*CE*	*CXP*
IYPTNGYTR1	*542.8*	*808.4*	*23*	*13*
IYPTNGYTR2	*542.8*	*405.1*	*23*	*13*
*FTISADTSK1**	*485.2*	*721.3*	*23*	*15*
FTISADTSK2	*485.2*	*608.2*	*22*	*15*
GPSVFPLAPSSK1	*593.8*	*699.4*	*31*	*15*
GPSVFPLAPSSK2	*593.8*	*846.5*	*28*	*15*
FNWYVDGVEVHNAK[H]	*562.9*	*713.3*	*23*	*15*
GPSVFPLAPSSK[H]	*597.8*	*707.4*	*28*	*15*

** MRM transition for the best sensitivity*
[H] MRM transitions of internal standard peptides from SILuMab

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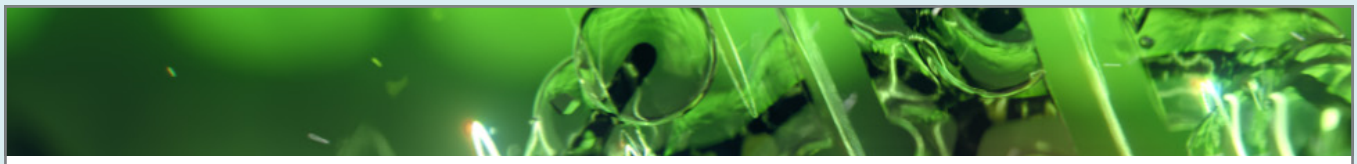

Quantification results

Component ...	Actual Co...	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
FTISADTSK1	0.00100	3 of 3	9.852e-4	9.735e-5	9.88	98.52
FTISADTSK1	0.00500	3 of 3	5.293e-3	2.785e-4	5.26	105.86
FTISADTSK1	0.01000	3 of 3	1.024e-2	7.576e-4	7.40	102.44
FTISADTSK1	0.05000	3 of 3	5.083e-2	4.477e-3	8.81	101.65
FTISADTSK1	0.10000	3 of 3	1.020e-1	6.762e-3	6.63	101.99
FTISADTSK1	0.50000	3 of 3	5.176e-1	1.101e-2	2.13	103.52
FTISADTSK1	1.00000	3 of 3	1.060e0	9.045e-2	8.53	105.99
FTISADTSK1	5.00000	3 of 3	4.989e0	3.620e-1	7.26	99.78
FTISADTSK1	10.00000	3 of 3	9.388e0	9.304e-1	9.91	93.88
FTISADTSK1	20.00000	3 of 3	1.728e1	4.775e-1	2.76	86.38

Figure 2. Quantification result summary.

The SCIEX Triple Quad 7500 System integrates innovations that provide improvements in both ion generation and ion sampling. The OptiFlow Pro Ion Source with E Lens Technology provides improvement in ion generation and the D Jet Ion Guide efficiently captures and transmits the ions in the high gas flow behind the orifice plate.[1]

With the optimized method, the presented immunocapture-LC-MRM assay demonstrated ultra-high sensitivity to quantify trastuzumab-emtansine in rat plasma, with the LLOQ at 1 ng/mL and the LOD at 0.5 ng/mL (Figure 1). As summarized in Figure 2, the assay accuracy is 86-106% and %CVs are below 10% for all tested samples. The calibration curve covered 4.5 orders of magnitude (1-20000 ng/mL) (Figure 3) and displayed a regression coefficient (r) of 0.996 using a weighting of $1/x^2$.

Conclusions

- A highly sensitive immunoaffinity-LC-MRM workflow for quantifying trastuzumab emtansine in rat plasma was demonstrated
- By using the SCIEX Triple Quad 7500 System coupled with analytical flow HPLC, trastuzumab-emtansine was solidly quantified at 1 ng/mL level
- Good reproducibility, accuracy and wide linear dynamic range as 4.5 orders of magnitude were achieved at the same time

References

1. Enabling new levels of quantification. SCIEX technical note RUO-MKT-02-11886-A.
2. Quantification of trastuzumab in rat plasma using an improved immunoaffinity-LC-MS/MS method. SCIEX technical note RUO-MKT-02-7597-A.

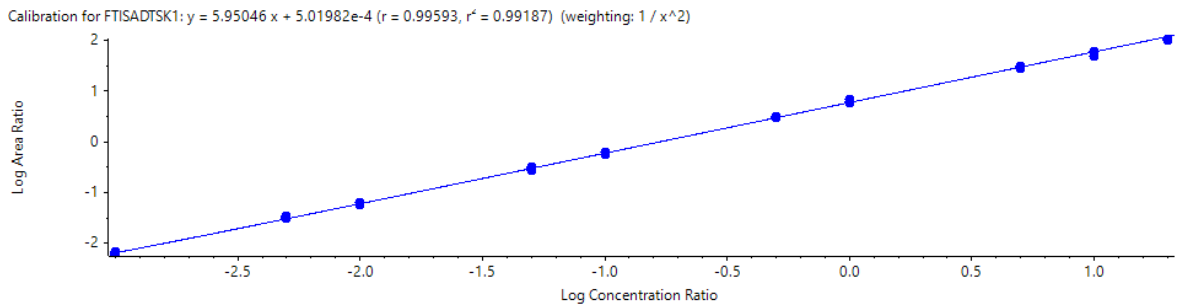

Figure 3. Calibration curve for trastuzumab-emtansine in rat plasma displayed with log-log format. Concentrations range from 1 ng/mL to 20,000 ng/mL.

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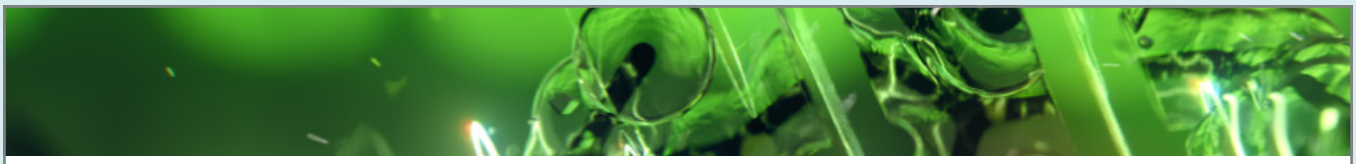


Sensitive and Accurate Quantitation of the Antibody-Drug Conjugate Ado-Trastuzumab Emtansine in Rat Plasma

High-Sensitivity Bioanalysis of ADC using BioBA with M3 MicroLC and QTRAP® 6500+ LC-MS/MS system

Khatereh Motamedchaboki,[1] Remco van Soest[1] and Ian Moore[2]
[1]SCIEX, USA, [2]SCIEX, Canada

Overview

Who Should Read This: Senior Scientists, Lab Directors

Focus: Improved quantitation of total antibody-drug conjugates (ADCs) using BioBA solution and microflow liquid chromatography-mass spectrometry (LC-MS/MS).

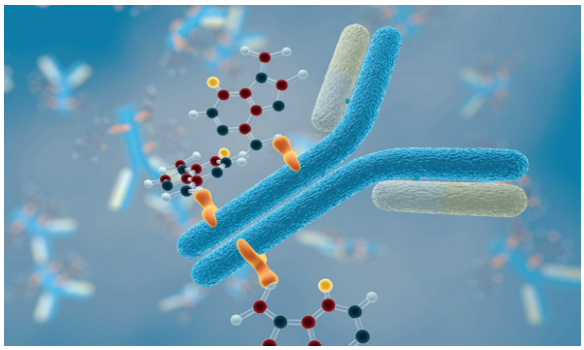
Goal: Develop an accurate and sensitive trap-and-elute microflow LC-MS/MS method to improve sensitivity and linear dynamic range of the BioBA High Capacity Sample Enrichment method for quantitation of antibody-drug conjugates in complex matrices.

Problem: Traditional LC-MS/MS methods for the quantitation of antibody-drug conjugates and similar biotherapeutics often deliver insufficient sensitivity. While we have seen improvement in sensitivity for the antibody-drug conjugate ado-trastuzumab emtansine quantitation in rat plasma using direct injection microflow LC-MS/MS,[1] this approach results in long sample loading times, and MS contamination as a divert valve could not be used.

Results: The SCIEX M3 MicroLC with a QTRAP 6500+ mass spectrometer provides up to 5X lower limit of quantitation for the antibody-drug conjugate ado-trastuzumab emtansine in rat plasma. Quantitation over 5 orders of magnitude linear dynamic range was achieved with r value of 0.995.

Key Challenges:

- Ligand binding assays (LBAs) like ELISA have been utilized in the past for the quantitation of biotherapeutics such as monocolonal antibodies (mAbs) and ADCs. These methods have limited linear dynamic range, cross reactivity and poor reproducibility
- Quantitation of mAbs over a wide dynamic range at low nanogram levels often requires greater sensitivity than can be achieved with LC-MS/MS with traditional LC flow rates
- Scientists who are familiar with quantitation of traditional small molecule therapeutics and new to analysis of these large and heterogeneous proteins conjugates require a robust and sensitive work flow which is easy to implement and reproducible in different laboratories



Key Features:

- The SCIEX BioBA Kit includes a generic method and all the reagents necessary for immunocapture and digestion to quantitate ADCs in plasma
- Microflow LC-MS/MS allows quantitation over a wide linear dynamic range of 5 orders of magnitude (1ng/ml-100ug/ml)
- Up to 4X improvement in s/n ratio enables quantitation at 5x lower concentration than can be achieved with traditional flow LC-MS/MS
- Robust on-line trap-and-elute sample loading and desalting increases column lifetime and reduces solvent consumption and costs

Fast-Growing Field: Protein biotherapeutics like immunoglobulin G (IgG)-derived monoclonal antibodies and antibody-drug conjugates occupy a rapidly increasing share of the pharmaceutical industry due to their lower toxicity, higher potency and target specificity. Ado-trastuzumab emtansine (Kadcyla) is the first HER2-targeted treatment for metastatic breast cancer. It is made of two cancer-fighting drugs, a monoclonal antibody trastuzumab (Herceptin) which targets HER2 and a lysine conjugated chemotherapy drug. It is made to bring chemotherapy inside HER2-positive cancer cells and kill them with less harm to normal cells.

Due to the heterogeneous nature of lysine conjugated adotrastuzumab emtansine, several bioanalytical assays,

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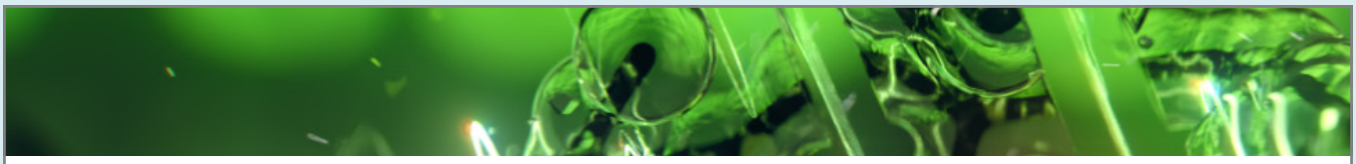

including ligand binding assays (LBAs), are required during the drug development process. Ligand binding assays have limited dynamic range, which makes hybrid LBA LC-MS/MS assays a more attractive choice for quantitation of the total antibody. These hybrid assays are based on quantitation of signature peptides which provide a wide linear dynamic range.

The BioBA High Capacity Sample Enrichment Kit provides a hybrid LBA LC-MS/MS workflow with a sample preparation protocol, and a generic method for pharmacokinetic studies of biotherapeutics during pre-clinical or phase I-IV studies. The BioBA workflow (Figure 1) includes the enrichment of mAbs from plasma using magnetic streptavidin beads conjugated with an anti-Human IgG for enrichment and quantitation of the total antibody with a generic or specific signature peptide. A target specific immunocapture strategy can also be employed with a recombinant target protein or an anti-idiotype antibody.

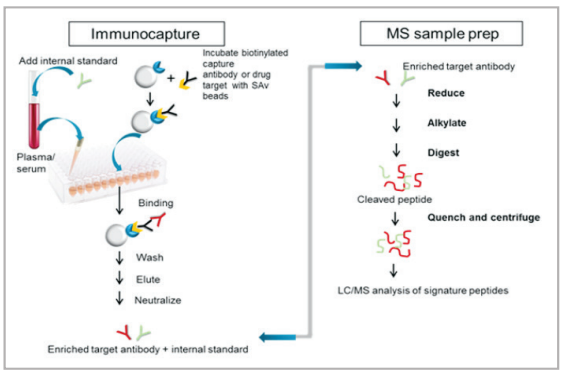

Figure 1. BioBA Immuno-Enrichment and Sample Processing Workflow. BioBA workflow includes immuno-enrichment of mAbs and ADCs, reduction, alkylation, digestion and a generic traditional flow LC-MS/MS method for the quantitation of the mAbs and ADCs in plasma.

BioBA magnetic beads offer several advantages including: ease of handling, scalability, improved sample recovery, parallel processing of samples using a variety of magnetic stands and use in high-throughput formats with robotics.[3] Following enrichment, the proteins are eluted, reduced, alkylated, and digested before detection using MRM based LC-MS/MS analysis of generic or signature peptides of mAbs of interest. The trypsin/lys-C enzyme mixture allows for the most efficient digestion. Digestion can be completed within three and half hours, enabling the workflow to be completed in a single day.[1]

In this application note we describe a robust and sensitive workflow for total antibody quantitation of ado-trastuzumab emtansine in rat plasma using a hybrid LBA LC-MS/MS approach. The BioBA sample preparation kit and BioBA generic immunocapture strategy is employed, and then followed by analysis of signature peptides on a M3 MicroLC coupled to a QTRAP 6500 mass spectrometer. This method takes advantage of a trap-and-elute strategy for fast sample injection and desalting to increase throughput of lower flow rate microflow LC analysis.

Experimental Design

Sample Preparation: 10x spiking solutions of ado-trastuzumab emtansine were first prepared in 1X BioBA bind/wash buffer containing 0.01% BSA (bovine serum albumin), then spiked into Sprague-Dawley rat plasma, K2EDTA (BioreclamationIVT) at the final concentrations of 0.5-100000 ng/ml. SILuMab (Sigma-Aldrich), was used as internal standard (IS) and was added to the plasma samples prior to BioBA immunocapture processing.

A blank and double blank sample was also prepared. The double blank only had 1X BioBA bind/wash buffer containing 0.01% BSA and the blank sample had rat plasma with additional internal standard. Spiked plasma samples (50 µL) were mixed with internal standard and processed based on the BioBA generic method[2] with some modified steps to reduce background signal and improve signal to noise (s/n) ratio in order to utilize the advantage of higher sensitivity provided by microflow LC. The modified steps include; an extra hour of incubation of conjugated beads with sample (2 hrs vs 1 hr in generic method) followed with two wash steps with 500 µL of BioBA bind/wash buffer and a 500 µL of 50 mM ammonium bicarbonate instead of the two wash steps with 200 µL of BioBA bind/wash buffer in generic method. Also a 1:10 dilution of stock BioBA digestion buffer (500 mM ammonium Bicarbonate) was used for preparing iodoacetamide, Trypsin/Lys-C and a mass spec compatible surfactant was used, with 1ug total Trypsin/Lys-C for each sample digestion.

Traditional Flow LC		Microflow LC	
Time (min)	%B	Time (min)	%B
0	5	0	3
0.7	5	0.7	5
0.8	10	0.8	10
3.5	25	3.5	25
5	40	5	40
5.1	95	5.1	95
5.9	95	10.0	95
6	3	10.1	3
7	3	15	3

Table1. Gradients used for Traditional and Microflow LC-MS/MS.

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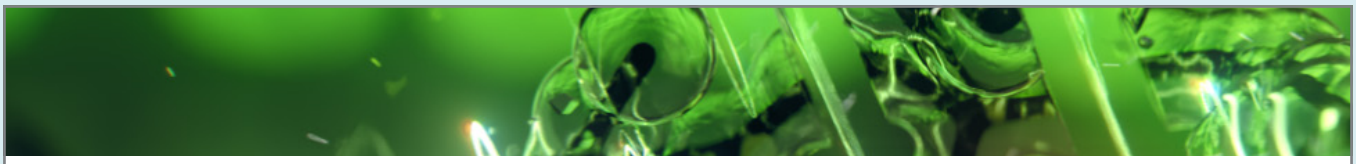

Traditional Flow Liquid Chromatography: A Shimadzu Prominence HPLC system with two LC-20AD pumps, CTO-20A column oven and a SIL-20AC autosampler was used. The column was a 100 x 2.1 mm Kinetex C18 2.6 µm 100 Å column (Phenomenex). Mobile phase A, water with 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid was used at a flow rate of 0.5 ml/min. Wash solvent for the autosampler was 20/20/60 methanol/acetonitrile/IPA. Injection volume was 25 µL, and the column was kept at 40° C.The gradient method used is listed in Table 1.

Microflow Liquid Chromatography: A SCIEX M3 MicroLC-TE system, with two microLC gradient pumps and an integrated autosampler was used in combination with a source mounted column oven (SCIEX). A 10 x 0.3 mm trap column packed with 5 µm 120 Å ChromXP C18 CL and an analytical column 50 x 0.3 mm HALO Peptide ES-C18 2.7 µm 160 Å column was used (SCIEX). Mobile phase A in the analytical gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid (Table 1) with flowrate of 10 µL/min. The column temperature was set to 40° C. Injection volume was 25 µL, and the autosampler needle and valve wash consisted of two cycles using mobile phase B, followed by one cycle using mobile phase A. For trapping conditions, mobile phase A in the loading gradient was water with 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid. Sample was loaded from the injection loop onto the trap column using 100% A for two and half minute at 50 µL/min flow rate. The trap was then washed with 95% B followed by 100% A each at 50 µL/min for 5 minutes after every injection (Table 2).

Mass Spectrometry and Data Processing: A SCIEX 6500 QTRAP with IonDrive™ Turbo V source was used. For the microflow LC experiments, the standard electrode was replaced with a 25 µm ID electrode (SCIEX). The transitions and MS parameters were optimized using DiscoveryQuant software (SCIEX) and kept constant for both the traditional flow and microflow LC experiments. The source and gas parameters are listed in table 3. The MS parameters are listed in table 4, and MultiQuant™ 3.0.2 software (SCIEX) was used for data analysis. Sample for both microflow and traditional flow LC-MS/MS analysis was prepared on the same day to exclude variations in response due to sample preparation. Three replicate LC-MS/MS injections were acquired for both the traditional flow and trap-and-elute microflow LC analysis.

Time (min)	%B
0	0
1	0
2.5	0
2.6	95
8	95
8.1	0
15	0

Table2. Gradient used for the Trap Wash Workflow.

MS Parameters	Traditional Flow LC	Microflow LC
Electrode ID	100 µm	25 µm
Curtain Gas	30	20
Collision GAS	High	High
IonSpray Voltage	5500	5000
Temperature	600	250
Ion Source Gas 1	50	20
Ion Source Gas 2	65	20

Table3. Source Parameters

Results and Discussion

Improved Sensitivity: The calibration curve of Ado- trastuzumab Emtansine standards in rat plasma matrix (0.5-100,000 ng/ml) was generated using MultiQuant Software (Table 5, 6) for data acquired by traditional and microflow LC using signature tryptic peptides IYPTNGYTR and FTISADTSK. Figure 2 shows the calibration curve for signature peptide IYPTNGYTR using Microflow LC. Figure 3 shows the extracted ion chromatograms (XIC's) of the signature peptide (FTISADTSK) used for quantitation of trastuzumab in both methods at the 5 ng/ml and 10 µg/ml level. S/N ratio was improved by 4 fold for both peptides using microflow LC. The LLOQ's for both methods were determined using the requirements of precision < 20% and accuracy between 80 and 120% at LLOQ, and at any higher concentration a precision <15% and accuracy between 85% and 115%. LLOQ improved by a factor of 5 using the microflow LC trap-and-elute method using both signature peptides and the generic peptide DTLMISR. For the traditional LC method the limit of quantification (LOQ) was 5 ng/ml where the LOQ of 1 ng/ml was achieved by microflow LC. Both the traditional flow and microflow LC methods showed good linearity with r >0.99.

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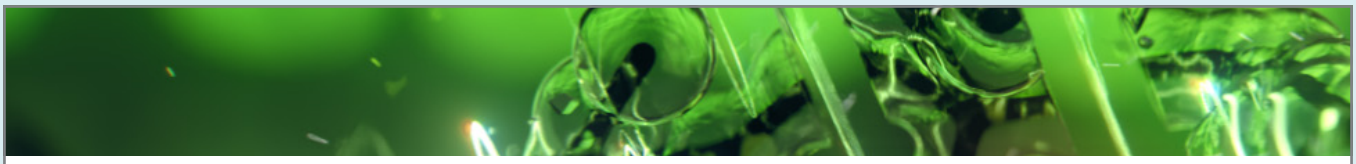

Transitions	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	Signature Peptide Sequences	DP (volts)	CE (volts)	CXP (volts)
1	**423.2**	629.4	20	**S-DTLMIS[R].heavy 1**	40	24	18
2	423.2	516.3	20	S-DTLMIS[R].heavy 2	60	22	17
3	**418.2**	619.3	20	**G-DTLMISR.1**	60	22	15
4	418.2	506.2	20	G-DTLMISR.2	40	20	18
5	542.8	405.8	20	S-IYPTNGYTR.1	120	23	10
6	**542.8**	808.2	20	**S-IYPTNGYTR.2**	60	16	11
7	485.2	608.2	20	S-FTISADTSK.1	50	25	25
8	**485.2**	721.3	20	**S-FTISADTSK.2**	90	20	15

Table 4. Transitions and MS Parameters for Signature Peptides of Ado-Trastuzumab Emtansine and Internal Standard. Peptide transitions in bold were used for quantification and the 2nd peptide transitions were used for confirmation with S-DTLMIS[R].heavy 1 transition used for internal calibration.

	Microflow LC			Traditional Flow LC		
Actual Concentration	Mean Measured Concentration (ng/ml)	Accuracy (%)	CV (%)	Mean Measured Concentration	Accuracy (%)	CV (%)
1	1.00	100.42	3.24	-	-	-
5	4.87	96.92	7.61	4.63	92.63	7.48
10	10.43	104.29	9.37	11.50	115.00	7.30
50	46.94	93.89	6.35	49.65	99.29	8.05
100	89.38	89.38	2.41	99.52	99.52	1.62
1000	921.6	92.16	4.38	921.6	92.16	0.71
10000	10930	109.27	0.56	10120	101.24	4.00
25000	27720	110.86	3.04	26270	105.06	2.04
50000	54780	109.57	3.04	52720	105.44	1.41
100000	93250	93.25	4.35	89650	89.65	2.93

Table 5. Quantitation Curve for Standard Ado-Trastuzumab Emtansine using Traditional Flow and Microflow LC-MS/MS. MultiQuant analysis based on peptide IYPTNGYTR resulted in an accurate quantitation with single digit CV% and r value of 0.995. Micro LC-MS/MS provides 5X increased in sensitivity with wider linear dynamic range as compared to Traditional LC-MS/MS.

	Microflow LC			Traditional Flow LC		
Actual Concentration (ng/ml)	Mean Measured Concentration (ng/ml)	Accuracy (%)	CV (%)	Mean Measured Concentration (ng/ml)	Accuracy (%)	CV (%)
1	0.9694	96.94	8.04	-	-	-
2	2.109	105.43	6.80	-	-	-
2.5	2.552	102.10	4.65	-	-	-
5	4.73	94.71	8.04	4.15	83.12	19.83
10	10.44	104.35	3.27	10.44	104.42	15.65
50	49.91	99.82	2.14	52.25	104.49	2.23
100	112.6	112.59	6.81	122.4	122.41	4.11
1000	1052	105.18	1.18	1017	101.67	0.91
10000	10150	101.49	3.68	10430	104.25	4.00
25000	23040	92.15	3.59	26230	104.93	3.63
50000	42620	85.24	3.94	48300	96.60	2.68

Table 6. Quantitation Curve for Standard Ado-Trastuzumab Emtansine using Traditional Flow and Microflow LC-MS/MS. MultiQuant analysis based on peptide FTISADTSK resulted in an accurate quantitation with single digit CV% for micro LC data and CV of 20% at LLOQ for Traditional LC data with r value of 0.995 and 0.998 for Microflow and traditional flow LC. Micro LC-MS/MS provides 5X increased in sensitivity with wider linear dynamic range as compared to Traditional LC-MS/MS.

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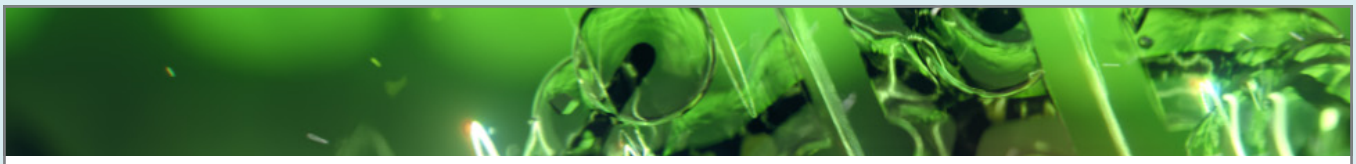

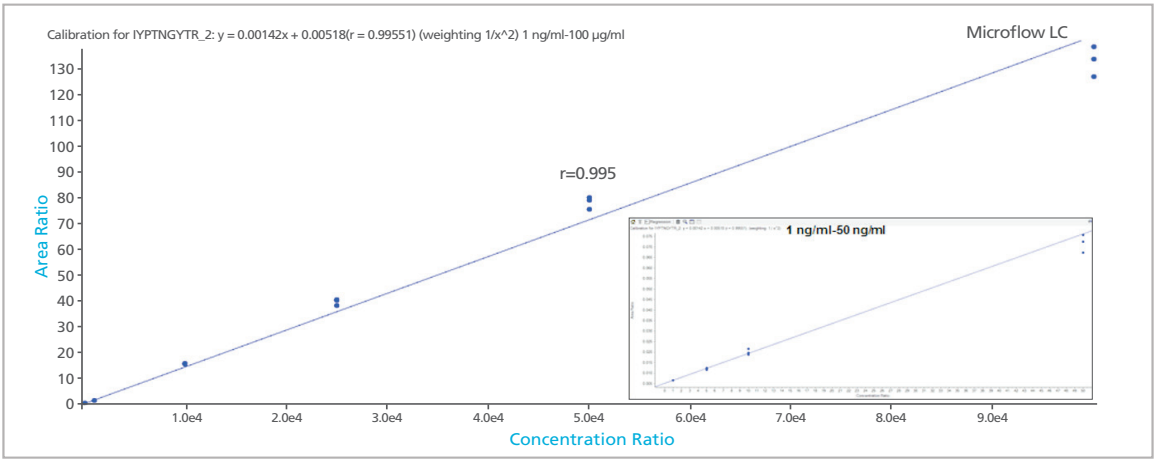

Figure 2. Quantitation Curve for Standard Ado-Trastuzumab Emtansine using Signature Peptide IYPTNGYTR. MultiQuant quantitation curve using peptide IYPTNGYTR resulted in an accurate quantitation with single digit CV% and r value of 0.995 using peak area and 1/x^2 weighting.

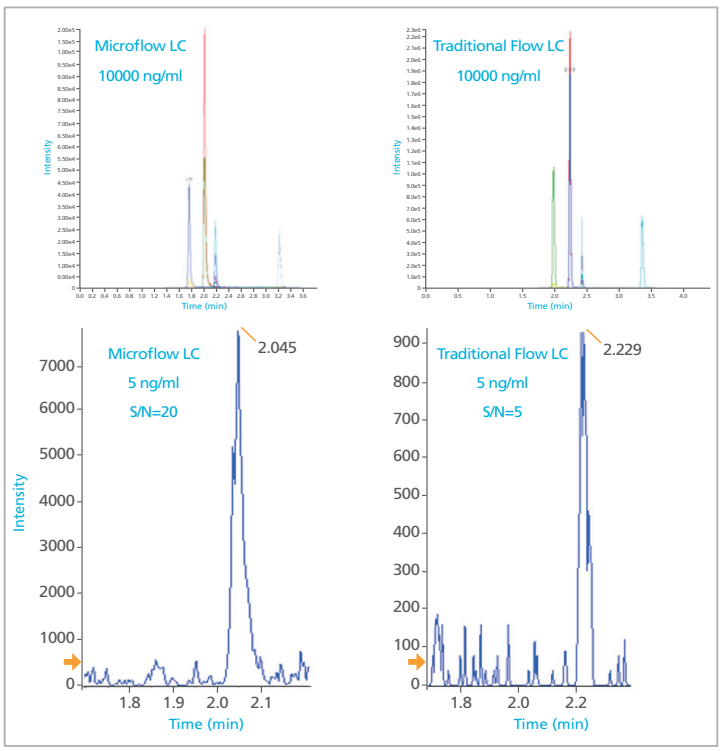

Figure 3. Signal Intensity Improvement using M3 MicroLC. A 4 fold improvement in s/n ratio was achieved using micro flow LC. XIC data for micro flow and traditional flow LC for 10 µg/ml (Top) and 5ng/ml (Bottom) is shown here with improved s/n.

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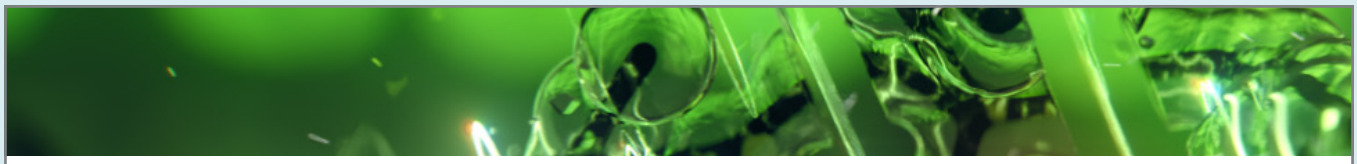

Column Carryover: Carryover was determined by injecting the digest of the immunocaptured extract from a blank+IS plasma sample after an injection of the ULOQ of 100,000 ng/ml using traditional flow LC-MS/MS, and The trap-and-elute microflow LC method. Observed carryover was 0.0075 and 0.007 respectively. The carryover was 1/3 of the response at the LLOQ (1 ng/ml) which is slightly higher than required 20%. Additional washing of trap and column is required to reduce the carryover if wider dynamic range is required.

Conclusion

Up to 5x lower LLOQ was achieved using a trap-and-elute microflow LC-MS/MS method at 10 µL/min, compared to using a direct inject traditional flow LC-MS/MS method at 500 µL/min for the quantitation of ado-trastuzumab emtansine using the signature tryptic peptides IYPTNGYTR and FTISADTSK in samples prepared using BioBA magnetic bead based immunocapture.

The trap-and-elute method ensures similar throughput while injecting the same 25 µL of extracted sample, while protecting the column and MS from contamination. Similar results were observed for infliximab using M3 MicroLC with SCIEX 6500 QTRAP.[4,5]

Optimized BioBA sample preparation protocol in combination with microflow LC provides wider linear dynamic range of 4.5 for traditional flow and linear dynamic range of 5 for microflow as compared to the data obtained from generic method optimized for traditional LC using IYPTNGYTR peptide transitions.[1]

This workflow offers a solution for applications where mAb's need to be quantified in low concentrations and/or when sample volumes are limited.

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Who is SCIEX? SCIEX company's global leadership and world-class service and support in the capillary electrophoresis and liquid chromatography-mass spectrometry industry have made it a trusted partner to thousands of the scientists and lab analysts worldwide who are focused on basic research, drug discovery and development, food and environmental testing, forensics and clinical research.

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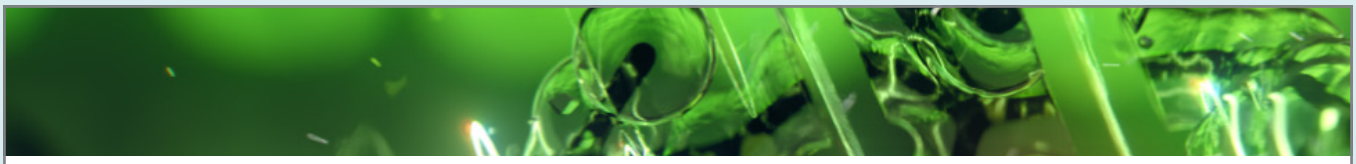

Metabolite Identification of Payload Species of Antibody Drug Conjugates with Noncleavable Linkers using MetabolitePilot™ 2.0 Software and TripleTOF® 6600 System

MetabolitePilot™ 2.0 Software and SCIEX TripleTOF® 6600 System

Ian Moore and Yuan-Qing Xia
SCIEX, Concord, ON, Canada

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.

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

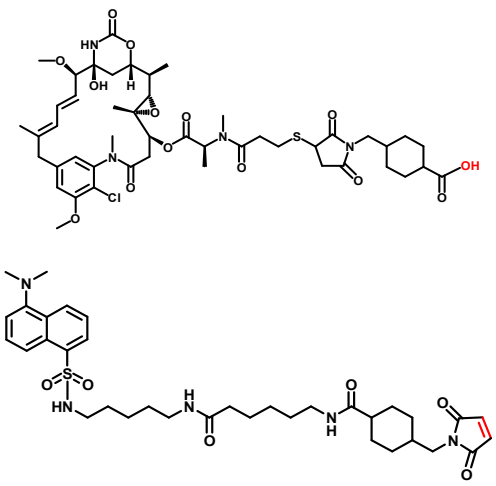

Figure 2A and Figure 2B. The structures of the linker plus payload of trastuzumab emtansine (T-E) and SigmaMAb Antibody Drug Conjugate mimic (MSQC8), sites of conjugation to the mAb are highlighted in red.

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

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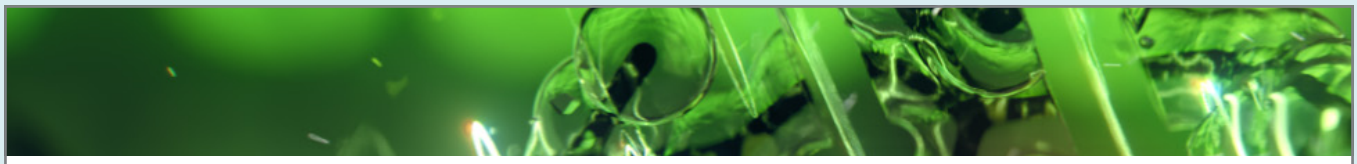

components: the antibody used for selective targeting, the cytotoxic drug and a linker to connect the two. The goal in design and development of ADCs is to maximize delivery to the targeted tissue and minimize delivery to untargeted tissues. There are currently two FDA approved ADC therapies on the market 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

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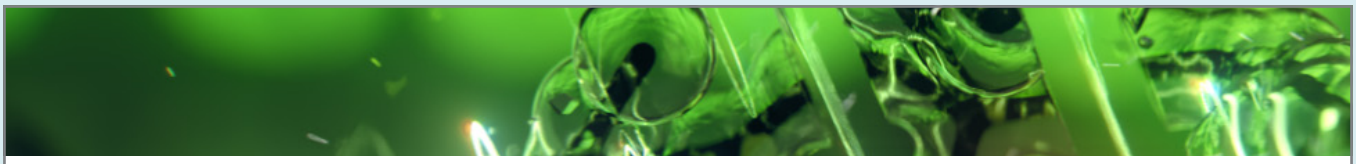

only: loss of water, demethylation, internal hydrolysis and the parent (payload plus linker). Phase I and II metabolism of the parent was not considered for this *in vitro* system. A reference MS/MS spectrum was available for DM1+MCC and used to designate characteristic product ions and neutral losses.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

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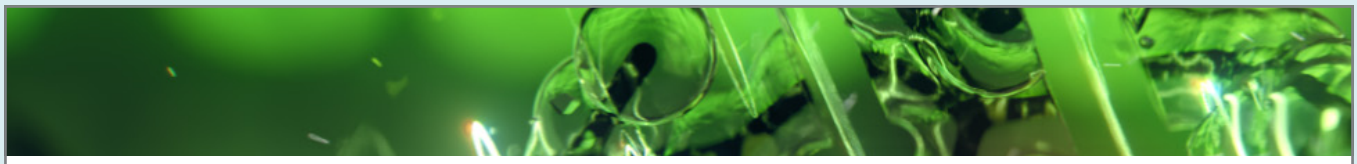

payload and linker is loaded into the fragment interpretation workspace and the putative peptide sequence is loaded into the sequence workspace and the residue of conjugation is assigned. Next the assign fragments feature is used to calculate potential structures for the fragments and neutral losses 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 ±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 ±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 ±2.0 mDa and three of the four ions contained the dansyl ring.

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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

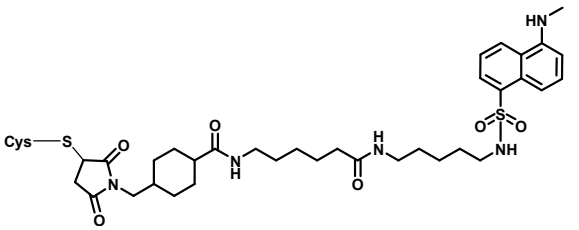

Figure 6. Structural assignment of the demethylated MSQC8 released payload species.

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

Thanks to Chris Bohl of Xenotech for helpful discussions.

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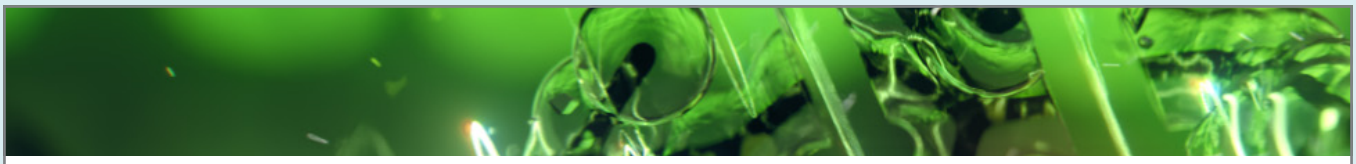

CE-SDS和CIEF方法对半胱氨酸偶联的抗体偶联药物的分析

Heterogeneity analysis of cysteine-linked antibody-drug conjugate by CE method

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Key Words: Antibody-drug conjugate, Cysteine-linked, CE-SDS, cIEF, Heterogenecity analysis

Abstract: Antibody drug conjugates (ADCs) are innovative biopharmaceutical emerging in recent years. And this kind of biotherapeutics show unprecedented intrinsic heterogeneity, so separation technologies play a critical role in their characterization. In this tech note, heterogeneity of a Cys-linked ADC is analyzed by using CE-SDS and cIEF. In CE-SDS, we established the reduced and non-reduced method to evaluate the purity of the ADC and explain the meaning of each peak in CE-SDS. In cIEF method, we compared the change of charge variant distribution between ADC and the monoclonal antibodies. Both two method can give us the reference of quality control assessment to the ADCs.

前言

抗体偶联药物（Antibody-drug conjugates, ADC）是一种将化学药物通过连接物（linker）与抗体偶联形成的药物。它既有抗体的靶向性又有化学药物的强杀伤力，成为肿瘤治疗用药的研究与发展热点。2011年8月和2013年2月，ADC药物brentuximabvedotin（商品名Adcetris）和ado-trastuzumabemtansine（商品名Kadcyla）分别获FDA批准上市。该两种ADC药物上市仅仅是ADC研发热潮的缩影，目前，世界范围内有约60种ADC药物处于临床阶段[1-2]。

ADC常见的偶联方式有赖氨酸偶联和半胱氨酸偶联。赖氨酸是抗体中最常见的连接位点，其氨基可以与连接物的羧基反应，得到较为稳定的酰胺键。一般的，抗体中约有80个赖氨酸，其中10个赖氨酸可作为连接位点。对于半胱氨酸偶联的方式，由于抗体表面没有游离的巯基，可以通过对断裂重链或者轻链间的二硫键，在抗体编码中加入半胱氨酸，另一方面，可以通过用还原剂打断抗体链间已有的二硫键，形成游离的巯基，实现偶联[3]。图1简单描述了赖氨酸和半胱氨酸可能的位点的结构示意图[4]。

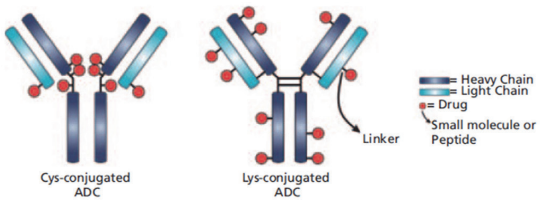

图1. 半胱氨酸和赖氨酸偶联位点及连接方式示意图。

ADC药物的质量控制与单克隆抗体类药物分析类似，主要针对其异质性进行分析，包括大小异质性及电荷异质性分析。而毛细管电泳技术近年来以其分离度高、分析速度快及自动化程度高成为了生物制药质控中不可或缺的分析技术，其中主要包括毛细管凝胶电泳（CE-SDS）进行纯度分析（大小异质性）和毛细管等电聚焦（cIEF）进行等电点和电荷异质性分析。

本研究采用Sciex公司的PA800 Plus生物制药分析系统，以通过还原剂打开二硫键后通过半胱氨酸偶联的ADC为例，建立和完善了CE-SDS和cIEF对该种ADC药物的分析方法，对其大小异质性和电荷异质性进行了分析，为ADC药物的质量控制提供参考。

2、仪器和试剂

2.1 仪器

毛细管电泳仪（PA800 Plus生物制药分析系统，PDA和UV检测器，Sciex）；5430型台式高速冷冻离心机（Eppendorf，Germany）；MA3涡旋混合器（IKA，Germany）；Thermomixer compact型恒温混匀仪（Eppendorf，Germany）。

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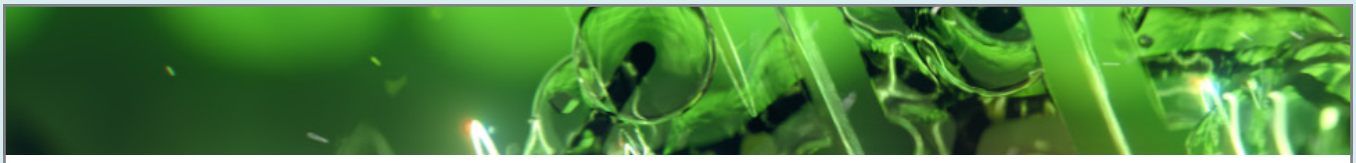

2.2 试剂

IgG Purity/Heterogeneity Assay Kit（PN A10663）和Advanced cIEF Kit（PN A80976）采购自Sciex。两性电解质（PN 17-0456-01）采购自GE Healthcare。其余所有试剂均为分析纯。巯基乙醇（PN M6250）、碘乙酰胺（PN I-1149）、磷酸（PN 345245）、氢氧化钠（PN 221465）、氢氧化铵溶液（PN 221228）、亚氨基二乙酸（PN 220000）、精氨酸（PN A5006）冰醋酸（PN 537020）和尿素（U0631）均采购自Sigma-Aldrich。实验所需用水均采用二次去离子水（Double Deionized Water, DDI Water）。Ultracel YM-10 滤膜（Millipore PN A11530）。

1）CE-SDS：

碘乙酰胺溶液：取46 mg 碘乙酰胺与1.5 mL EP管中，加入1 mL DDI Water溶解混匀。现用现配，避光保存。

2）cIEF：

阳极液、 阴极液、化学迁移剂、酸性冲洗液、4.3 M尿素溶液、阳极稳定液、阴极稳定液、3 M Urea-cIEF Gel溶液的配制方法参照Sciex cIEF试剂盒说明书[5]。

2.3 样品及前处理

裸抗和其通过半胱氨酸偶联的ADC样品均来自某合作单位。

取 400 µL 样品入超滤管（截留分子量10 kD）中，14000 rpm，离心10 分钟；加入300 µL DDI water，14000 rpm，离心10 分钟；再加入300 µL DDI water，14000 rpm，离心10 分钟。调整样品最终浓度为5.0 mg/mL，盐离子浓度低于50 mM。

1）CE-SDS：

取两支1.5 mL EP管，分别取20 µL样品于两支EP管中，然后各加入75 µL的Sample buffer使得体积为95 µL。然后再向两支EP管中分别加入5 µL 2-巯基乙醇（还原法）和碘乙酰胺溶液（非还原法）。盖上瓶盖，充分混合后离心1 分钟。然后置于70 ℃水浴10 分钟。随后离心去除气泡，吸取100 µL置于样品管中。

2）cIEF：

分析多个样品的时候，推荐预先配制样品混合物母液（Master Mix），如表1。Master Mix 可简化样品配制并且使误差最小化。在表格中输入样品的数量，然后样品数量加1 计算，按照计算的各个溶液的体积进行配制Master Mix。

随后，取10 µL样品与240 µL上述溶液进行混合，涡旋混匀。取出200 µL置于样品管中。

表1. 分析多个样品时配制的 cIEF Master Mix。

试剂	每个样品需要的量（µL）	样品数量	总体积（µL）
3 M 尿素-cIEF 胶	180	×（____+1）	
3-10 两性电解质	12	×（____+1）	
阴极稳定剂	20	×（____+1）	
阳极稳定剂	2	×（____+1）	
pI 标准品1	2	×（____+1）	
pI 标准品2	2	×（____+1）	
pI 标准品3	2	×（____+1）	

3、方法

3.1 CE-SDS：

分离条件：毛细管：非涂层毛细管、50 µm内径、30 /20 cm总/有效长度；进样：-5 kV，20 s；分离：-15 kV， 40 min；数据采集频率：4 Hz；样品室温度：25 ℃；毛细管温度：25 ℃；窗口狭缝：2号（100×200 µm）；PDA检测器，检测波长：220 nm；

新毛细管的预平衡：碱性冲洗液，20 psi 压力冲洗10分钟；酸性冲洗液，20 psi 压力冲洗5分钟；DDI Water，20 psi 压力冲洗2分钟；Gel Buffer，70 psi 压力冲洗10分钟。最后，以-15 kV电压平衡10 分钟。

每针之间冲洗：碱性冲洗液，70 psi 压力冲洗3分钟；酸性冲洗液，70 psi 压力冲洗1分钟；DDI Water，70 psi 压力冲洗1分钟；Gel Buffer，70 psi 压力冲洗10分钟。

3.2 cIEF：

分离条件：毛细管：中性涂层毛细管、50 µm内径、30 /20 cm总/有效长度；进样：25 psi，99 s；聚焦：采用200 mM磷酸和300 mM氢氧化钠作为阳极液和阴极液，25 kV，15分钟；化学迁移：用350 mM醋酸来代替阳极液，30 kV，30分钟。数据采集频率：4 Hz；样品室温度：10 ℃；毛细管温度：25 ℃；窗口狭缝：2 号（100×200 µm）；UV检测器，检测波长：280 nm。

新毛细管的预平衡：酸性冲洗液，50 psi 压力冲洗5分钟；DDI Water，50 psi压力冲洗2分钟；cIEF Gel，50 psi压力冲洗5分钟。

每针之间冲洗：4.3 M尿素溶液，50 psi压力冲洗3分钟；DDI Water，50 psi压力冲洗2分钟。

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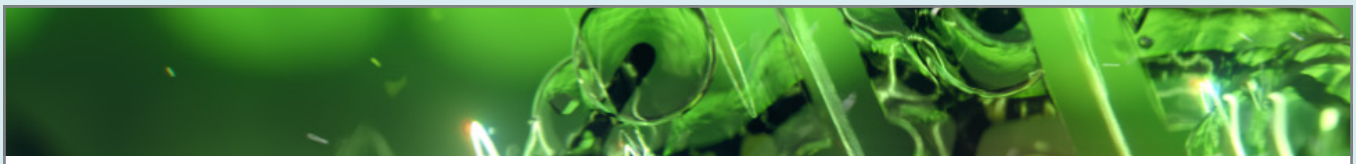


4、结果与讨论

4.1 CE-SDS法进行纯度（大小异质性）分析

CE-SDS因其分析速度快，重现性好，分辨率高，稳定性强等特点，已经成为检测蛋白类药物分析大小变异体的首选方法。药典中要求对单抗药物的还原（Reduced, R）和非还原（Non-Reduced, NR）样品进行分别检测[6]。两种分析方法中样品均需要与十二烷基硫酸钠（sodium dodecyl sulfate, SDS）络合，但还原单抗纯度分析时需要使用巯基乙醇等还原剂将单抗结构中的二硫键断裂，形成轻链（Light Chain，LC）和重链（Heavy Chain，HC）。而非还原单抗纯度分析则需要保持二硫键稳定不断裂，故需加入巯基封闭剂对裸露的巯基进行封闭[7]，防止游离巯基介导的链间二硫键断裂而产生碎片[8,9]。

对于ADC药物的纯度分析，采用同药典中对单抗分析相同的分析策略，即对ADC进行还原和非还原法的纯度进行测定[3]。

还原CE-SDS分析：

通过CE-SDS还原法谱图（图2），可推断1号峰为未偶联的轻链（含量8.03%），2号峰为偶联的轻链（含量23.13%），3号峰为偶联和未偶联的重链之和（含量 64.73%），4号峰在此条件下，视为杂质（含量4.12%）。由结果可知，通过还原CE-SDS分析，能够将偶联和未偶联药物小分子的轻链实现分离[10]。而重链上可能会偶联1至4个药物小分子，而在还原法CE-SDS谱图中并未发现偶联和未偶联重链的分离[11]。通过1和2号峰含量的变化，可以在一定程度上反应出轻链的偶联效率，并对二者进行准确的定量。通过方法的结果我们可知，1和2号峰所代表的片段之所以能够实现分离，正是源于其偶联药物分子后分子量的差异，二者的分离度达到了1.4，能够实现对1和2号峰的准确定量。而并未发现偶联和未偶联的重链得以分离，可能是由于重链分子量较大，偶联前后分子量变化在重链中体现不明显所致。

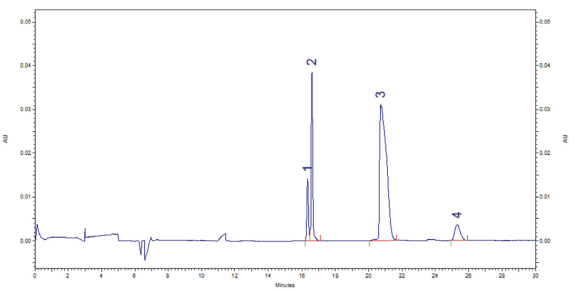


图2. 某ADC药物的还原法CE-SDS电泳谱图。电泳条件见第3节。

非还原CE-SDS分析：

由于该ADC药物是通过半胱氨酸偶联的，在进行偶联时，需利用还原剂打开抗体结构中的二硫键。因此，ADC 药物会解离成多种碎片组分，且碎片类型与小分子药物的偶联位点相关。其非还原CE-SDS分析谱图见图3。根据分子量的估算，1-5号峰分别为经过偶联的轻链(LC)，重链(HC)，重轻链（HL）、重重链（HH）、重重轻链（HHL），6号峰为完整的裸抗[1]。

对还原和非还原谱图的峰进行分析，不仅可监测各个成分的纯度，还可反映出药物的偶联效率[11]。如，在非还原CE-SDS中（图3），1号轻链峰为单峰，说明在非还原条件下，游离的轻链已全部偶联，还原谱图中的未偶联轻链（图2中峰1）来源于HL、HHL和完整裸抗的还原产物（图3中的3、5和6号峰）。

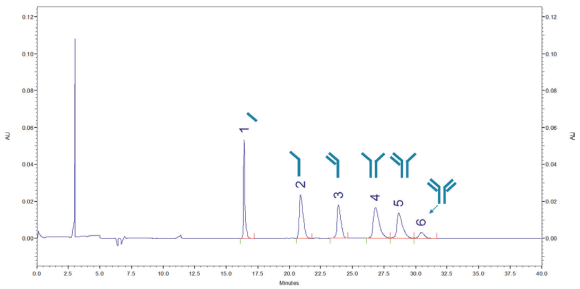
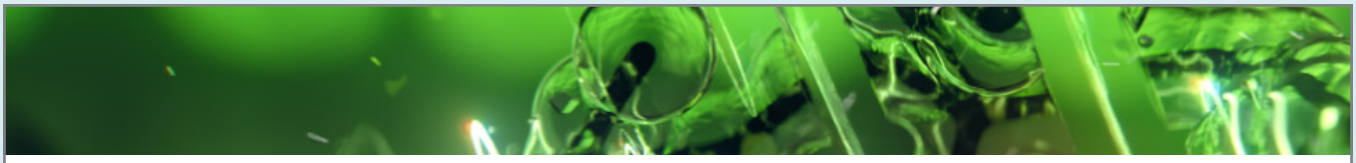


图3. 某ADC药物的非还原法CE-SDS电泳谱图。电泳条件见第3节。

4.2 cIEF法进行等电点及电荷异质性分析

如图4所示，（A）和（B）分别代表了裸抗及其对应的ADC药物。采用3个pI Marker（10.0、9.5、5.5）进行等电点的线性计算（R^2>0.999），获得裸抗主峰的实测值为8.67，与根据氨基酸序列计算的理论值8.73十分接近。当通过半胱氨酸偶联药物小分子后，由于药物分子的引入以及偶联后分子结构发生了变化[8]，所以ADC药物的等电点和电荷变异体相对于裸抗也发生了变化。以峰面积百分比含量最高的峰为主峰，则该ADC药物主峰的等电点由裸抗的8.67变化至8.10，电荷变异体的pI值范围整体向酸性移动（裸抗：8.12-8.76；ADC药物：7.48-8.89）。

内容提要



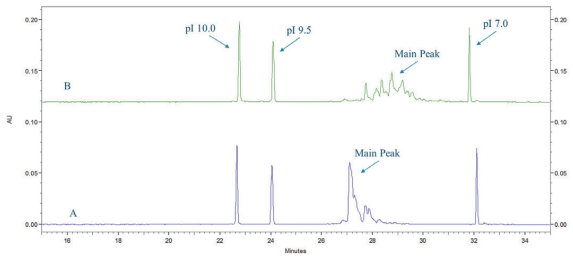


图4. 某ADC药物偶联前后的cIEF电泳谱图。（A）裸抗；（B）ADC药物。电泳条件见第3节。

5、结论

本研究建立了基于半胱氨酸偶联的ADC药物的异质性分析方法。使用CE-SDS和cIEF方法分别对其大小异质性和电荷异质性进行分析。通过还原和非还原CE-SDS的方法，对该样品进行了纯度的评价。1）在还原条件下，偶联和未偶联的轻链可以实现高分离度的分离，并可以对这两个片段进行准确的定量分析。2）在非还原条件下，对该类半胱氨酸偶联的ADC样品，可实现六种片段的分离和定量。3）通过还原和非还原CE-SDS的对比分析，能够在一定程度上反映出药物的偶联效率。4）在裸抗及其对应的ADC药物的cIEF方法分析中，通过其主峰的等电点及其电荷变异构体的变化，能够有效的监控其等电点的变化并分离ADC药物的酸碱修饰。

由于ADC药物高度复杂的异质性，我们更需要组合多种理化分析技术对其进行检测。本研究通过毛细管电泳方法的建立，实现了ADC类药物大小异质性和电荷异质性分析，对该类生物技术药物的研究和质量控制提供了参考。

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