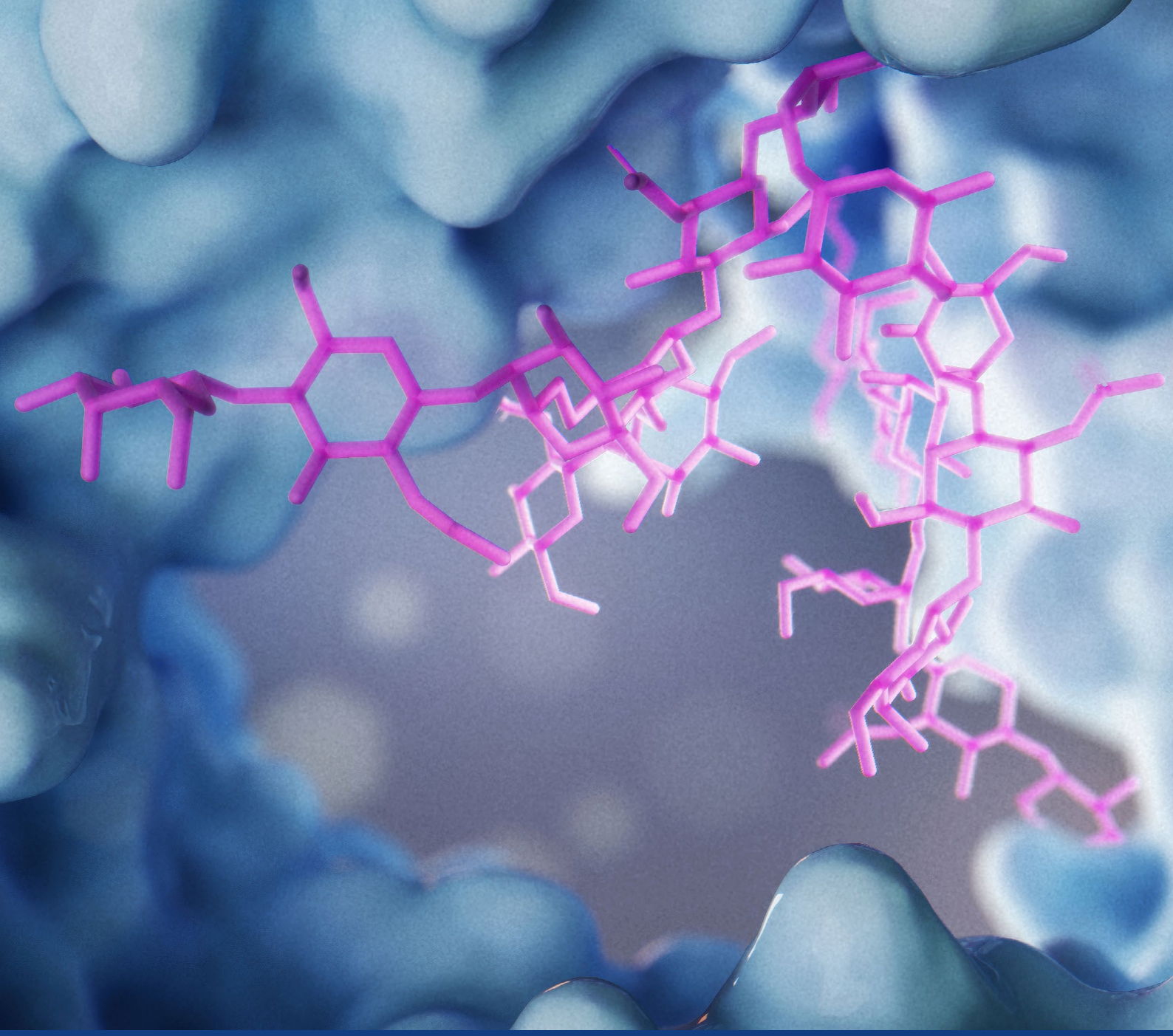

Enhancing precision in drug development and analysis of novel therapeutics

Expert Insights

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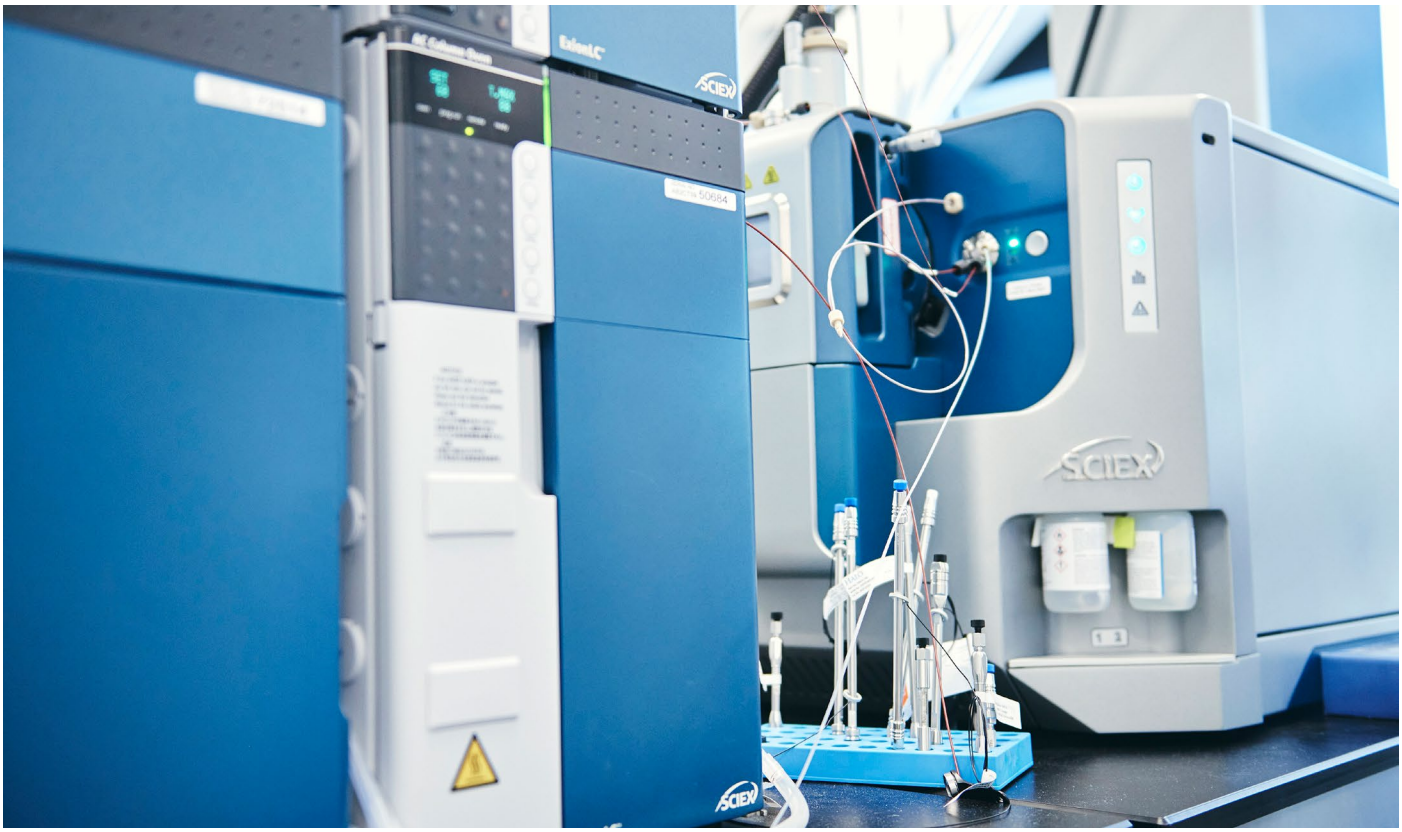


Enhancing precision in drug development and analysis of novel therapeutics

Advancements in analytical techniques are crucial for drug development and biopharmaceutical analysis. These techniques enhance the precision of structural elucidation, differentiation of complex molecules, and characterization of various novel therapeutics. By integrating multiple analytical methods, researchers can improve the accuracy and efficiency of analysis of therapeutics, ensuring drug quality and safety. This Expert Insights collection highlights the importance of these advancements and their impact on the pharma and biotech industries.

First, a study by Yao *et al*. evaluates the advantages of electron-activated dissociation (EAD) on quadrupole time-of-flight (QTOF) systems for structural elucidation of conjugation metabolites in drug development [1]. EAD provides more precise localization compared to collision-induced dissociation (CID), thereby enhancing the accuracy of conjugation metabolite structure elucidation.

Yang *et al*. then introduces a novel bottom-up LC-MS/MS approach using EAD to distinguish thio-succinimide hydrolysis isomers without reference materials [2]. EAD combines electron-capture dissociation (ECD) and hot ECD (hECD), offering advantages over traditional CID by effectively dissociating ions with various charge states.

Next, Li *et al*. utilizes capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and liquid chromatography-mass spectrometry (LC-MS) to identify glycosylated heavy chain heterogeneity in the anti-VEGFR-2 monoclonal antibody [3]. The study highlights the critical quality attributes of monoclonal antibodies and the impact of glycosylation modifications on drug safety, efficacy, and stability.

The collection features interviews with Rahul Baghla, who discusses the integration of Mass Spectrometry (MS) with other analytical techniques and its impact on the pharmaceutical industry, and Zoe Zhang, who highlights the complementary roles of MS and Capillary Electrophoresis (CE) in biopharmaceutical analysis, recent technological advancements, and future trends shaping the industry.

Finally, the technical notes provide insights into advanced analytical techniques used in characterization, identification, and quantification of analytes of interest. The first note by Duchoslav *et al*. describes an automated workflow for metabolite identification using the ZenoTOF 7600 system paired with Molecule Profiler software, leveraging electron-activated dissociation (EAD) and collision-induced dissociation (CID) for efficient metabolite identification. The second note by Al-Dulaymi *et al*. outlines a streamlined workflow for identifying and quantifying impurities in ionizable lipid ALC-0315, crucial for ensuring mRNA-LNP product quality. The last note by Santos introduces a new capillary electrophoresis sodium dodecyl sulfate (CE-SDS) method on the BioPhase 8800 system, offering higher throughput while maintaining the outstanding performance of the original method.

Overall, this Expert Insights collection focuses on the advancements in analytical techniques for therapeutic innovation and development. It explores the precision of structural elucidation, differentiation of isomers, and characterization of monoclonal antibodies. The collection emphasizes the integration of various analytical methods to enhance the accuracy and efficiency of analytes of interest, ensuring drug safety and quality.

Through the methods and applications presented in this Expert Insights, we hope to educate researchers on new technologies and techniques about analysis in pharmaceutical and biopharmaceutical development. To gain a deeper understanding of available options for improving your research, we encourage you to visit SCIEX's website.

Dr. Christene A. Smith
Editor at Wiley

References

[1] Yao, M. *et al*. (2024). Advancing structural elucidation of conjugation drug metabolites in metabolite profiling with novel electron-activated dissociation. *Rapid Communications in Mass Spectrometry*. https://doi.org/10.1002/rcm.9890.

[2] Yang, J. *et al*. (2024). Reference-free thio-succinimide isomerization characterization by electron-activated dissociation. *Rapid Communications in Mass Spectrometry*. https://doi.org/10.1002/rcm.9910.

[3] Li, M. *et al*. (2024). Using capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and liquid chromatograph mass spectrometry (LC–MS) to identify glycosylated heavy chain heterogeneity in the anti-VEGFR-2 monoclonal antibody. *Electrophoresis*. https://doi.org/10.1002/elps.202300258.

Advancing Structural Elucidation of Conjugation Drug Metabolites in Metabolite Profiling with Novel Electron-activated Dissociation

Adapted from Yao *et al.*, 2024

Introduction

This study evaluated the advantages of electron-activated dissociation (EAD) on quadrupole time-of-flight (QTOF) systems for the structural elucidation of conjugation metabolites in drug development. Traditional metabolite profiling uses conceptual "boxes" to represent potential modification sites based on MS/MS data, but EAD offers more precise localization compared to collision-induced dissociation (CID).

In drug discovery, compound characterization is vital for selecting candidates with favorable pharmacokinetic properties. Liquid chromatography with high-resolution mass spectrometry (LC-HRMS) has become essential for studying drug metabolism and pharmacokinetics. While CID remains the most commonly used fragmentation mode due to its efficiency and availability, it has limitations in locating conjugation sites due to information loss from highly selective cleavage.

EAD offers significant technological advantages over CID, featuring a tunable device that adjusts electron beam kinetic energy to suit the precursor of interest. This allows for multiple bond cleavages and rearrangements, generating abundant fragment ions without inducing neutral loss from conjugation. By rapidly adjusting electron energy, EAD can ionize and fragment neutral species—a capability beyond CID's scope.

The study examined conjugated metabolites of Diclofenac, Febuxostat, Formoterol and Raloxifene, each having multiple potential conjugation sites. These compounds were incubated with rat liver microsomes in the presence of NADPH, UDPGA, and glutathione. The resulting conjugation metabolites were analyzed using a QTOF system, collecting high-resolution tandem mass spectrometry (MS/MS) spectra via both EAD and CID fragmentations alongside time of flight (TOF) MS full scans. The metabolite profiling identified conjugation metabolites (glucuronides and glutathione [GSH] adducts) through characteristic mass shifts. Comparison of fragmentation methods revealed EAD-specific fragments for most conjugates.

EAD proves to be a powerful complementary tool to CID for metabolite profiling in drug development, particularly for identifying conjugation sites. EAD-enabled MS/MS spectra provide more comprehensive and unique structural information, significantly enhancing the precision of conjugation metabolite structure elucidation.

Despite its generally low intensity, EAD demonstrated a unique capability to break relatively stable bonds on parent drug motifs while preserving weaker conjugation bonds. This property effectively narrowed the conceptual "box" representing modification sites, providing more definitive information about conjugation locations. Molecule Profiler software was used to identify unique fragments from both EAD and CID spectra in a single results file.

Materials and Methods

Chemicals and Sample Preparation

Standard compounds (Diclofenac, Febuxostat, Formoterol and Raloxifene) were incubated with rat liver microsomes at 37 °C with a starting concentration of 10 µM with $MgCl_2$ (10mM) in the presence of cofactors involved in drug metabolism: UDPGA (1 mM), GSH (1 mM), and NADPH (1 mM). Samples were collected at 0 and 60 minutes, quenched with acetonitrile, centrifuged, and supernatant was used for LC-MS/MS analysis.

Chromatography

LC separation was performed on a C18 column at 40 °C. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a 12-minute gradient.

Mass Spectrometry Parameters

The study used a QTOF mass spectrometer with information-dependent acquisition. The instrument operated in positive ion mode with TOF MS range of 100-1000 Da and TOF MS/MS range of 50-1000 Da.

Key EAD parameters were:

- Electron beam current: 5,000 nA and 6,000 nA
- Electron KE values: 10 eV, 12 eV and 14 eV
- CE value: 12 V

CID parameters were:

- CE value: 40 V
- CES value: 15 V

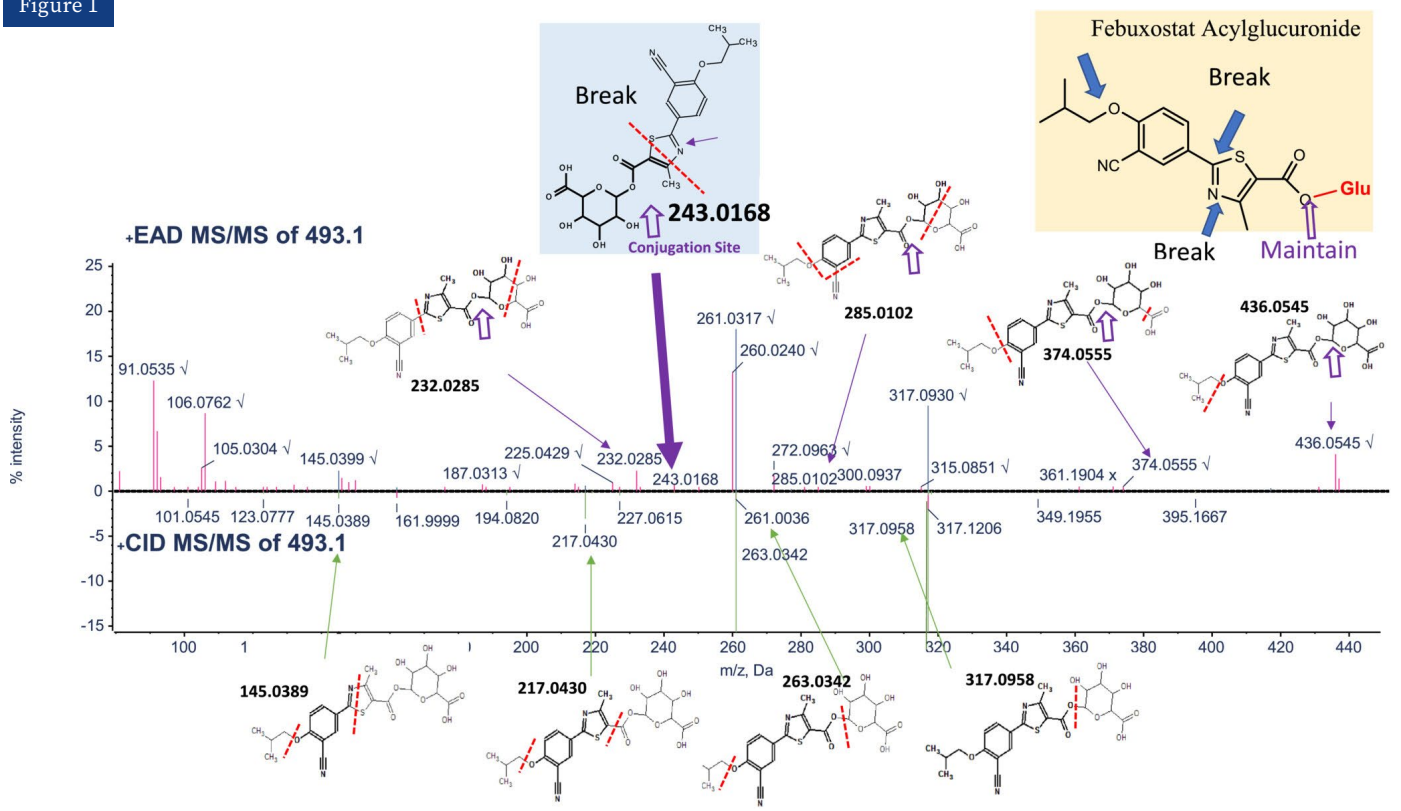
Results and Discussion

Febuxostat Glucuronide Analysis

The major glucuronide metabolite of Febuxostat (m/z 493.0545) was fragmented using both CID and EAD (Figure 1). CID fragmentation showed limited diagnostic ions (m/z 317.0958, 263.0342, 217.0430, and 145.0389), making it difficult to determine the conjugation site.

EAD revealed additional unique fragmentations (m/z 436.0545, 374.0555, 285.0102, 243.0168, and 232.0285). These fragments showed the glucuronide conjugate either intact or partially intact. A distinctive mass fragment (m/z: 243.0168) revealed the glucuronide fully maintained while the nitrogen on the thiazole ring was cleaved, confirming O-glucuronidation rather than N-glucuronidation.

Figure 1



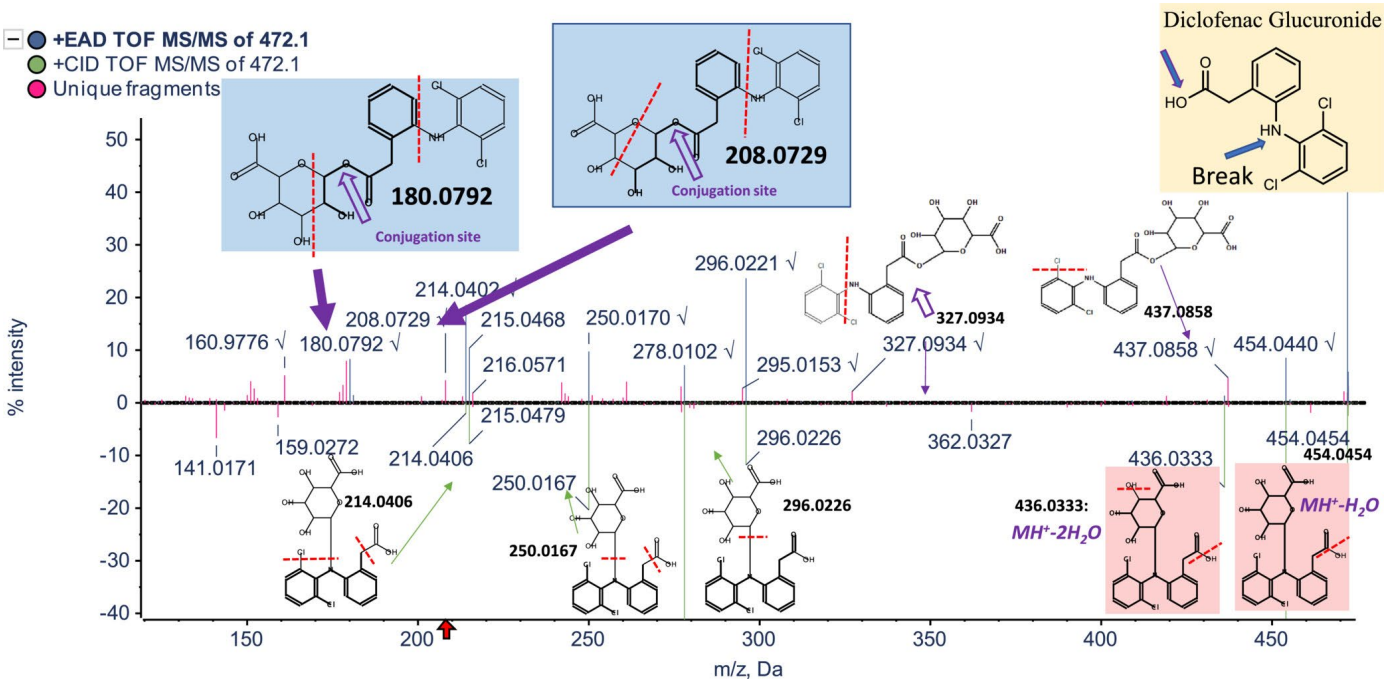
EAD vs. CID MS/MS fragmentation of Febuxostat glucuronide. CID (bottom) cleaves the weak glucuronidation bond early, making site identification challenging. EAD preserves the glucuronide partially or fully, producing distinct signature ions (magenta) that facilitate localization of the modification.

Diclofenac Glucuronide Analysis

Diclofenac can undergo either acyl (O-) glucuronidation or N-glucuronidation (Figure 2). When analyzed using CID, fragments (m/z 454.0454, 436.0346, 296.0237, 250.0183, and 214.0417) were insufficient to identify the glucuronidation site. Based on CID data, the software incorrectly predicted N-glucuronidation, contradicting published literature.

EAD revealed unique fragmentations (m/z 437.0858, 327.0936, 208.0729, and 180.0792) that precisely indicated acyl glucuronidation, contradicting the initial N-glucuronidation prediction. Two notable fragments (m/z 208.0729 and 180.0792) maintained the acyl glucuronide while showing cleavage at the nitrogen, which significantly enhanced structure elucidation.

Figure 2



CID vs. EAD MS/MS fragmentation of diclofenac glucuronide for site confirmation. Two potential glucuronidation sites were evaluated. CID-based software predicted N-glucuronidation, showing water loss, contradicting published data. EAD, however, preserved or partially broke the glucuronide, identifying acyl glucuronidation. Signature EAD fragments (m/z 180.0792, 208.0729) confirmed this by retaining the acyl glucuronide while cleaving nitrogen, correcting the initial misassignment.

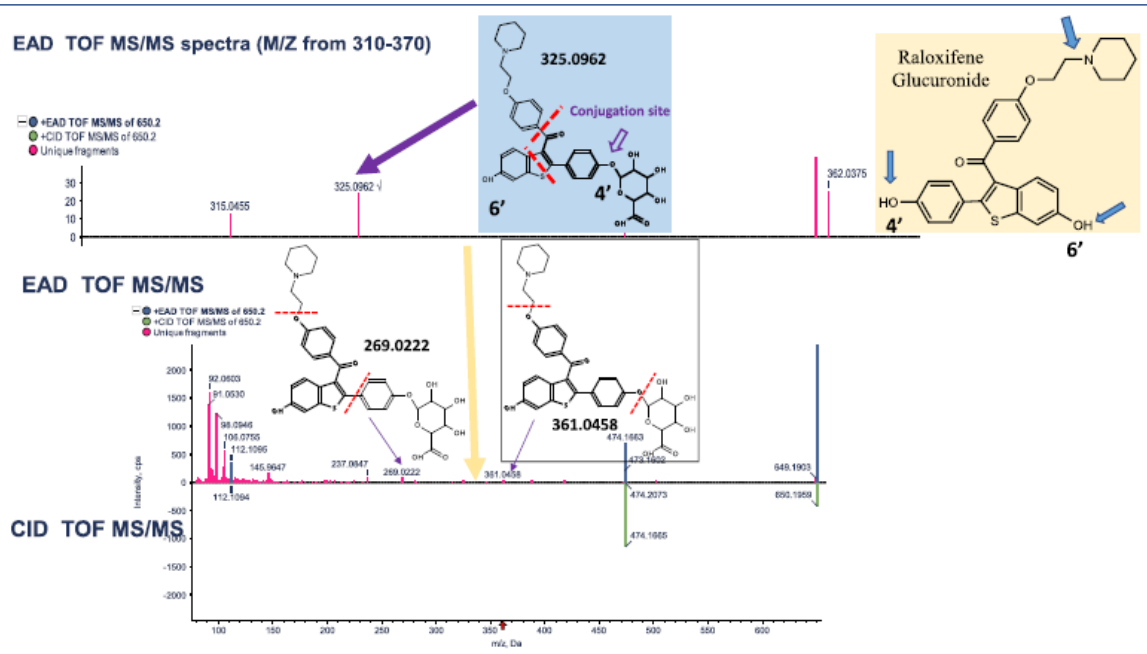
Raloxifene Glucuronidation and GSH Conjugation

Raloxifene has multiple potential conjugation sites, including phenolic hydroxyl groups for glucuronidation and multiple sites for GSH conjugation. CID of the glucuronide metabolite (m/z 650.2) showed limited information, with one major fragment at m/z 474.1665 (parent compound).

EAD identified unique fragments (m/z 269.0222, 361.0458, and 325.0962), with the latter showing the thiophene benzene ring cleaved while glucuronidation on the 4-OH of the phenol ring was maintained (Figure 3).

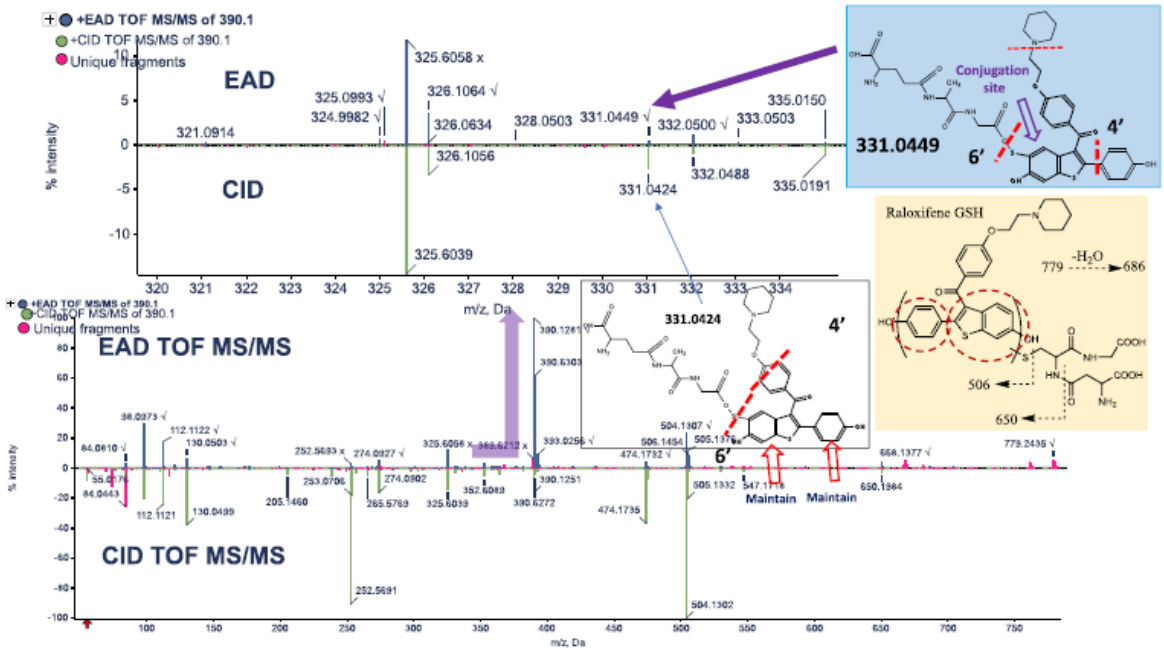
For the GSH conjugate (m/z 779.2), EAD analysis revealed a fragment (m/z 331.0449) that retained the benzo-thiophene ring and incorporated sulfur from GSH, indicating GSH conjugation on the benzo-thiophene ring, consistent with previously published NMR data (Figure 4).

Figure 3



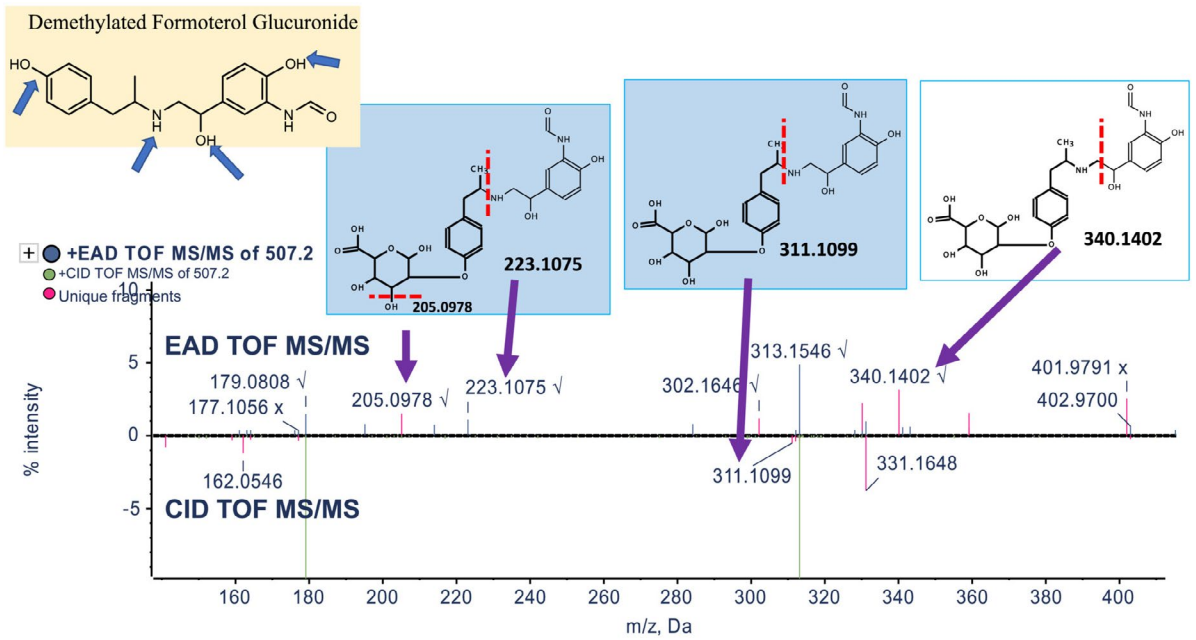
Structural characterization of Raloxifene-glucuronide via EAD and CID MS/MS. Two potential conjugation sites were identified, with a unique EAD fragment confirming glucuronidation at the 4'-OH of the phenol ring while preserving the glucuronide moiety.

Figure 4



Structural elucidation of Raloxifene-GSH conjugate via EAD and CID MS/MS. Two potential conjugation sites were identified, with a unique EAD fragment (m/z 331.0449) confirming GSH attachment at the 6'-OH of the thiophene benzene ring, preserving the GSH moiety.

Figure 5



EAD vs. CID MS/MS spectra of demethylated formoterol glucuronide from Molecule Profiler software. EAD-specific fragments (m/z 223.1075, 311.1099) indicate partial or complete glucuronide preservation, confirming O-glucuronidation.

Formoterol Demethylated Glucuronide Analysis

The Formoterol demethylated glucuronide (m/z 507.2) analyzed by CID showed a fragment at m/z 331.1648, indicating direct loss of glucuronic acid but providing limited information about the conjugation site (Figure 5).

EAD MS/MS spectra displayed specific fragments (m/z 205.0978, 223.1075, 311.1099, and 340.1402) that indicated the glucuronide to be partially preserved or intact. These fragments supported glucuronidation at the phenolic position, aligning with previously reported studies.

Discussion

This study addressed challenges in characterizing conjugate metabolites using CID mode and explored the effectiveness of EAD in refining conjugation sites and structures. The mechanism of EAD involves free electrons being captured by ions to form a radical state, inducing fragmentation in different molecule types.

The QTOF system with EAD proved powerful for metabolite analysis. Tunable EAD technology provides various free electron-based fragmentation mechanisms, generating richer MS/MS spectra for additional structural information. However, EAD generally produces lower abundance signature peaks compared to CID, representing a trade-off between specificity and abundance.

While CID remains the primary choice for MS/MS experiments, it can sometimes result in limited or nonselective fragmentation. EAD serves as a valuable complementary approach due to its ability to offer unique fragmentation information. The integrated approach of using both EAD and CID techniques, along with specialized software, facilitates efficient analysis by combining their strengths.

Conclusion

This study demonstrates EAD's potential as a powerful tool for conjugated metabolite structure elucidation during metabolite profiling in drug development. Compared to CID, EAD offers a radical dissociation-based fragmentation technique that provides a greater variety of fragments in MS/MS spectra for most identified conjugation metabolites.

The formation of fragments with partially or entirely intact conjugated moieties leads to more comprehensive structural information for analyzing metabolic modifications. Implementing EAD in metabolite profiling studies allows the generation of unique fragments not produced by CID, significantly improving the structural elucidation of conjugation drug metabolites.

EAD and CID are complementary techniques, and by combining both technologies, more accurate and comprehensive results can be achieved. EAD enhances our understanding of drug metabolism by helping to narrow down structural possibilities, thereby contributing to the efficiency and depth of metabolite identification in drug development processes.

Reference-free Thio-succinimide Isomerization Characterization by Electron-activated Dissociation

Adapted from Yang *et al.*, 2024

Introduction

Thiol-maleimide conjugation has become widely adopted across various fields due to its specificity, selectivity, and fast kinetics. One of its most significant applications is in antibody-drug conjugates (ADCs), a promising therapeutic class with 15 approved products as of October 2023, 10 of which utilize thiol-maleimide chemistry during their conjugation.

The thio-succinimide linker site in these conjugates undergoes two competing biotransformation processes: retro-Michael reaction (causing premature linker-payload deconjugation) and ring-opening hydrolysis. The hydrolysis reaction is particularly important as it increases ADC stability by preventing deconjugation while generating two isomeric products: thio-aspartyl (thio-Asp) and thio-isoaspartyl (thio-isoAsp). While these isomers have been confirmed using fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy, their differentiation via liquid chromatography-mass spectrometry (LC-MS) has not been previously reported. Conventional approaches for identifying isomers typically require synthesized reference materials, which involve complicated synthesis and purification processes.

The authors introduce a novel bottom-up LC-MS/MS approach using electron-activated dissociation (EAD) to distinguish thio-succinimide hydrolysis isomers without reference materials. EAD, which combines electron-capture dissociation (ECD) and hot ECD (hECD), offers advantages over traditional collision-induced dissociation (CID) by effectively dissociating ions with various charge states.

The research builds on previous work with asparagine deamidation, which similarly produces isomeric products. The authors explore whether EAD could generate unique diagnostic ions specifically for thio-succinimide hydrolysis products, representing the first in-depth characterization of these isomers using LC-MS/MS without reference materials.

Methods

Materials

ADC1 and anti-idiotype (anti-ID) antibodies were generated in-house. Human and mouse plasma samples were employed for incubation studies.

Instrumentation

LC-MS/MS experiments were performed on a SCIEX ZenoTOF 7600 system mass spectrometer coupled with an Exion UHPLC system. Mass spectra were analyzed using a research version of SCIEX PeakView software (version 1.2.2.0).

ADC1 incubation, immuno-affinity enrichment, and sample clean-up

ADC1 was added to human, or mouse plasma and samples were either incubated at 37 °C for 168 h or immediately stored at –80 °C (0 h time point). Immuno-affinity enrichment was performed using biotinylated anti-ID antibody conjugated to magnetic beads. After enrichment, the ADC1 was digested using trypsin immobilized on the magnetic beads. Sample clean-up utilized reverse-phase cartridges before LC-MS/MS analysis.

LC-MS/MS parameters

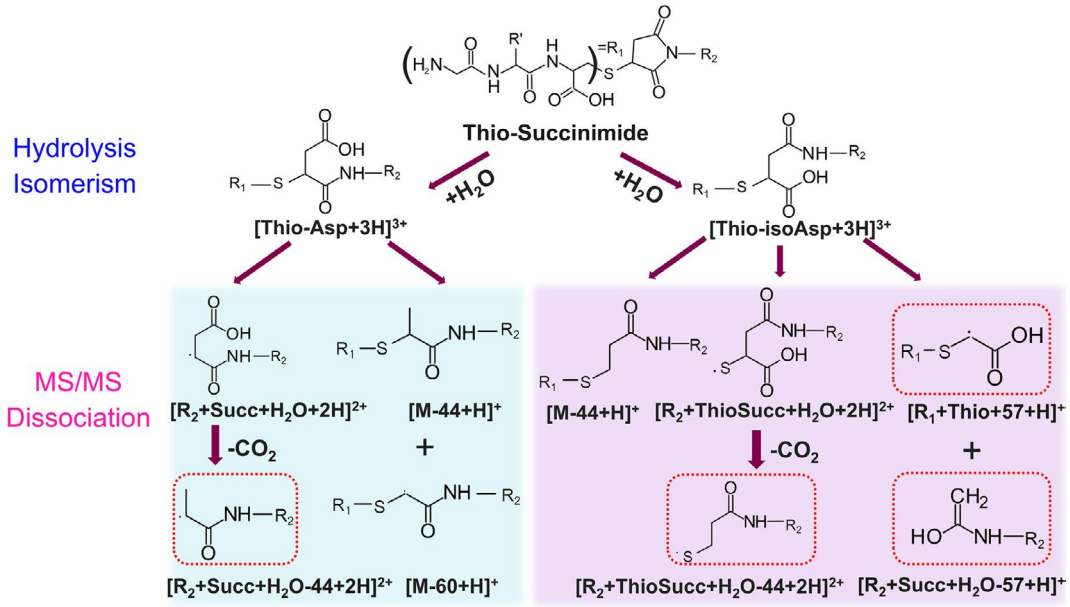
Tryptic digested peptides were separated on a C18 UPLC column. The ZenoTOF 7600 operated in full-scan MS mode (m/z 100-1500) with collision energy set at 10 V. For MS/MS, collision-induced dissociation (CID) used a collision energy of 40 V, while EAD employed a kinetic energy of 11 eV.

Results

Characterization of thio-succinimide hydrolysis

The study used a signature ADC1 tryptic digested product consisting of a tripeptide (GEC), succinimide (with or without water adduct), and the linker-payload (R_2) to demonstrate the hydrolysis process (Figure 1).

Figure 1

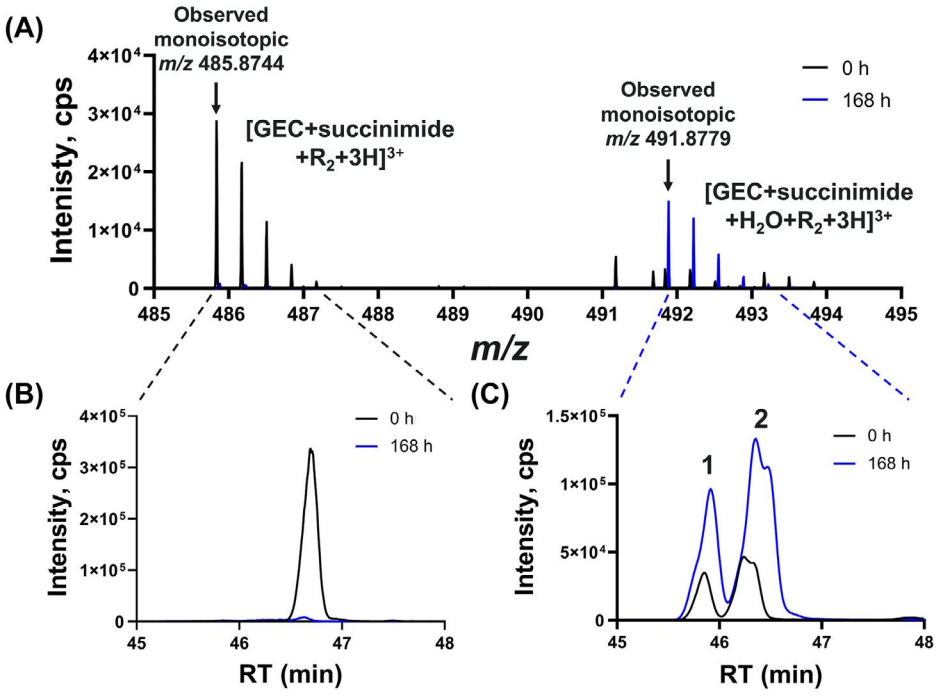


Scheme of thiosuccinimide hydrolysis mechanism and the proposed structures of product ions after electron-activated dissociation (EAD). Product ions in the red boxes are diagnostic ions with predominant charge state indicated. R_1 is a tripeptide GEC, and R_2 is linker-payload. The linker payload is covalently conjugated to the antibody via a cysteine side chain thiol. Succ + H_2O = hydrolyzed succinimide.

Mass spectra after 168 h of incubation showed a significant decrease in the signal intensity of the non-hydrolyzed species and a corresponding increase in peaks representing the species with +18 Da shift, confirming hydrolysis.

Extracted ion chromatograms of the hydrolyzed species revealed two distinctive peaks with different retention times, confirming the presence of isomers (Figure 2). These were designated as Peak 1 (RT = 45.9 min) and Peak 2 (RT = 46.4 min).

Figure 2



(A) Overlaid mass spectra of [GEC + succinimide + R_2 + 3H]$^{3+}$ and [GEC + succinimide + H_2O + R_2 + 3H]$^{3+}$ from 0 h (black line) and 168 h (blue line) plasma incubated antibody-drug conjugate 1 (ADC1) samples. Both species observed a charge state of 3. Overlaid extracted ion chromatograms of (B) [GEC + succinimide + R_2 + 3H]$^{3+}$ or (C) [GEC + succinimide + H_2O + R_2 + 3H]$^{3+}$. Black line represents the sample with no incubation, whereas the blue line is the sample incubated for 168 h.

Identification of thio-succinimide hydrolysis isomeric products

CID MS/MS failed to provide distinctive peaks for differentiating the isomers. However, EAD analysis revealed several diagnostic ions (Figure 3):

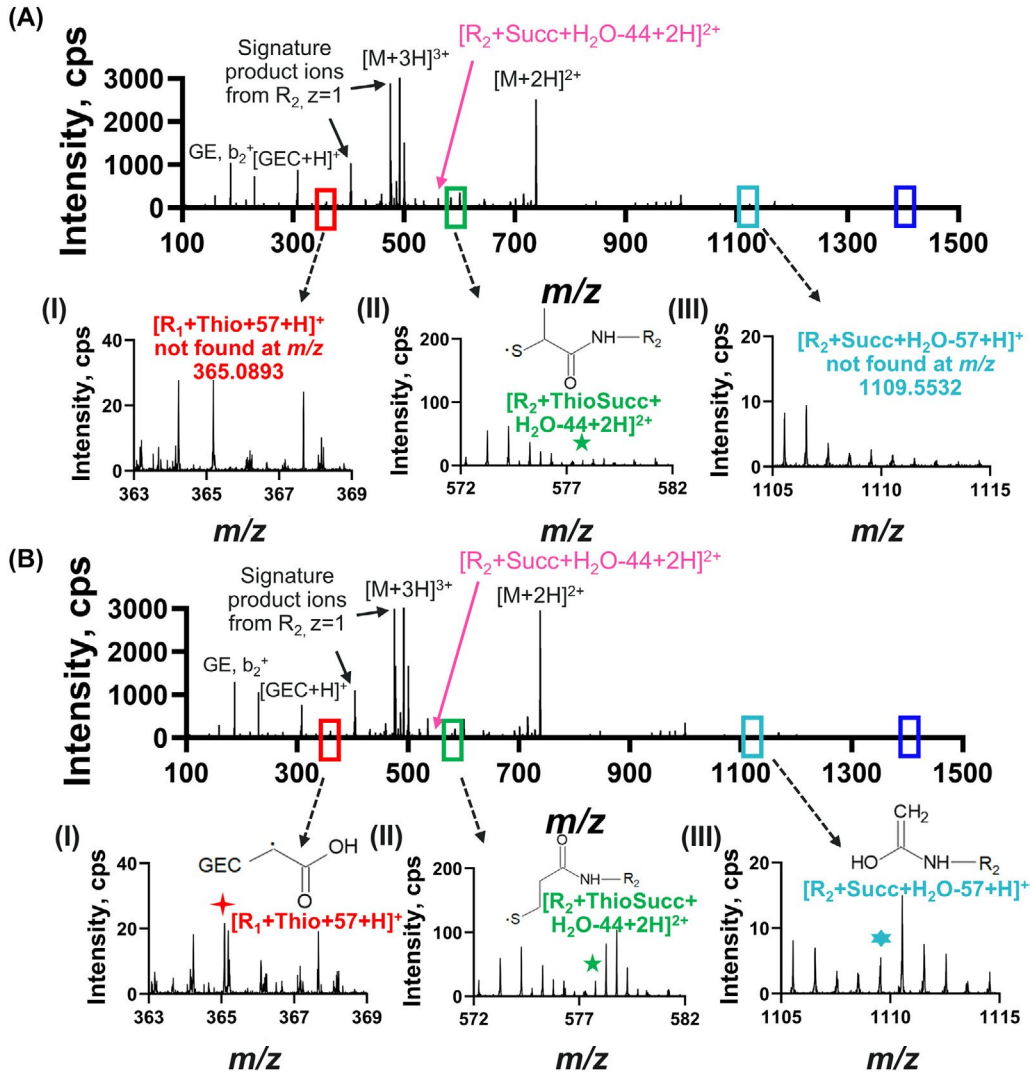
- A singly charged product ion with m/z 365.0839 was observed in Peak 2 but not in Peak 1, corresponding to $[R_1 + Thio + 57 + H]^+$ with a-15 ppm mass difference from theoretical.
- A singly charged product ion with m/z 1109.5403 was observed in Peak 2 but not in Peak 1, corresponding to $[R_2 + Succ + H_2O - 57 + H]^+$ with a-12 ppm mass difference.
- The $[R_2 + Succ + H_2O - 44 + 2H]^{2+}$ ion showed significantly higher intensity in Peak 1 compared to Peak 2.

Based on these observations, Peak 1 was identified as thio-Asp and Peak 2 as thio-isoAsp. A novel diagnostic ion, $[R_2 + ThioSucc + H_2O - 44 + 2H]^{2+}$ (m/z 561.2728), was also discovered with enhanced intensity in Peak 1 but low intensity in Peak 2.

Notably, neutral losses like $[M - 60 + H]^+$ and $[M - 44 + H]^+$ showed similar intensities in both isomers, indicating they cannot be used as diagnostic ions for differentiation.

Similar results were obtained with another tryptic peptide, SCDK, containing succinimide + R_2 from ADC1, further confirming the findings.

Figure 3



Electron-activated dissociation (EAD) MS/MS spectra (m/z 100–1500) of (A) Peak 1 and (B) Peak 2 observed in Figure 2C. Signature product ions are labeled on both spectra. Pink arrows point to a doubly charged diagnostic ion, $[R_2 + Succ + H_2O\_44 + 2H]^{2+}$. Dashed arrows point to zoomed-in EAD MS/MS spectra with different m/z ranges. (i) m/z 363–369, (ii) m/z 572–582, and (iii) m/z 1105–1115. Blue boxes are the zoomed-in spectra of m/z 1410–1440. The stars with 4, 5, and 6 arms in inset figures correspond to the monoisotopic peak of each diagnostic product ion, respectively. Their observed m/z values are 365.0839, 577.7643, and 1109.5403, respectively. $[R_2 + Succ + H_2O\_44 + 2H]^{2+}$ ion was also observed in Figure 3A (II) with-12 ppm difference to its theoretical value, although the intensity is low.

Conclusions

This study demonstrated a reference material-free characterization of hydrolyzed thio-succinimide isomers using EAD on the SCIEX ZenoTOF 7600 platform. Distinctive diagnostic ions ($[R_1 + Thio + 57 + H]^+$, $[R_2 + Succ + H_2O - 57 + H]^+$ for thio-isoAsp, and enhanced $[R_2 + Succ + H_2O - 44 + 2H]^{2+}$ for thio-Asp) were identified in EAD MS/MS spectra. A unique diagnostic ion, $[R_2 + ThioSucc + H_2O - 44 + 2H]^{2+}$, was reported for the first time as a valuable tool for differentiating thio-Asp and thio-isoAsp. This ion is specific to thio-succinimide systems and has not been identified in analogous asparagine deamidation studies.

Compared with conventional approaches requiring synthesized reference materials, EAD serves as a novel tool to identify isomeric structures without references. The differentiation between thio-Asp and thio-isoAsp may ultimately benefit drug development where thiol-maleimide conjugation is employed.

The study highlights the capabilities of the SCIEX ZenoTOF 7600 system for detailed structural characterization of ADCs, demonstrating how EAD can provide insights beyond what traditional dissociation methods offer. The use of SCIEX PeakView software- now integrated in the Explore module of SCIEX OS, further enabled detailed analysis of complex spectra to identify key diagnostic ions for isomer differentiation.

This research represents a first-to-date identification of thio-succinimide hydrolysis isomers without using synthesized reference materials. The approach should be applicable to all thio-succinimide-linked molecules, potentially benefiting the development of ADCs in the future by providing deeper understanding of their structural changes during hydrolysis, which impacts stability and efficacy.

The findings underscore the value of advanced MS instrumentation like the SCIEX ZenoTOF 7600 system with EAD capability in biopharmaceutical characterization workflows, particularly for complex therapeutics like antibody-drug conjugates where subtle structural changes can significantly impact drug performance.

Using Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS) and Liquid Chromatograph Mass Spectrometry (LC-MS) to Identify Glycosylated Heavy Chain Heterogeneity in the Anti-VEGFR-2 Monoclonal Antibody

Adapted from Li *et al.*, 2024

Introduction

Size variants are critical quality attributes of monoclonal antibodies (mAbs), with capillary electrophoresis sodium dodecyl sulfate (CE-SDS) being one of the principal analytical techniques for size-variant analysis. This technique has been extensively applied to characterization, batch release, and stability studies of mAbs. Reduced CE-SDS analysis is especially valuable because it fully breaks intrachain and interchain disulfide bonds of mAbs, enabling characterization of light chains, glycosylated heavy chains, and their degradation fragments, while accurately quantifying non-glycosylated heavy chain content.

Anti-vascular endothelial growth factor receptor 2 (VEGFR-2) mAb is an antagonist of human VEGFR-2 that blocks interactions with its ligands (VEGF-A, VEGF-C, and VEGF-D), thereby inhibiting VEGFR-2 activation and its downstream signaling pathways. This suppresses endothelial cell proliferation and migration. Several drugs targeting this pathway have entered clinical trials or been approved for gastric cancer, non-small cell lung cancer, and colorectal cancer.

Reduced CE-SDS was used in this study to analyze the anti-VEGFR-2 antibody's critical quality attributes. The antibody exhibited a typical IgG1-antibody reduced CE-SDS profile, with the main distinction being that the glycosylated heavy chain presented as a distinct split peak, indicating significant glycosylated heavy chain heterogeneity. This heterogeneity has potential impacts on safety, efficacy, and stability of drugs for clinical use, making its characterization critical for risk evaluation.

Importantly, CE-SDS size heterogeneity can be difficult to identify by MS/MS, creating a significant obstacle in mAb development and quality control across the biopharmaceutical industry. For mAbs, there are two common sources of heavy chain heterogeneity: cleavage of peptide bonds in the heavy chain, and post-translational modifications, primarily glycosylation.

Therefore, a multi-step approach was used to identify the source of glycosylated heavy chain heterogeneity. Reduced MS analysis was combined with MS-based glycosylation profiling and CE-SDS analysis after glycosidase digestion to preliminarily confirm the role of glycosylation. Reference mAbs with specific glycoforms were then prepared and integrated additional analyses were performed to conclusively verify that glycosylation modifications caused the observed heterogeneity.

Materials and Methods

Reagents

The IgG Purity and Heterogeneity Kit (SCIEX) was used to assess the purity and heterogeneity of IgG molecules.

Analytical Methods

Multiple analytical approaches were used in this study:

- Reduced molecular weight analysis was performed using ultra-high-performance liquid chromatography system with a high-resolution mass spectrometer.
- Reduced CE-SDS analysis was performed on a PA800 Plus system (SCIEX) equipped with a photodiode array detector, using bare-fused silica capillaries with effective lengths of 20 and 40 cm.
- N-Glycan content was determined using high-performance liquid chromatography with fluorescence detection and MS.
- N-Glycan enzymatic digestion and modification reactions involved PNGase F digestion, galactosidase reactions, and N-glycan transglycosylation.
- IdeS protease enzymatic digestion was performed to obtain single chain Fc (scFc) fragments.
- Intact molecular weight analysis was conducted to characterize whole antibodies and fragments.
- Non-reduced CE-SDS analysis was performed for analysis of fragments without reduction of disulfide bonds.

Results and Discussion

Glycosylation Profile and CE-SDS Analysis

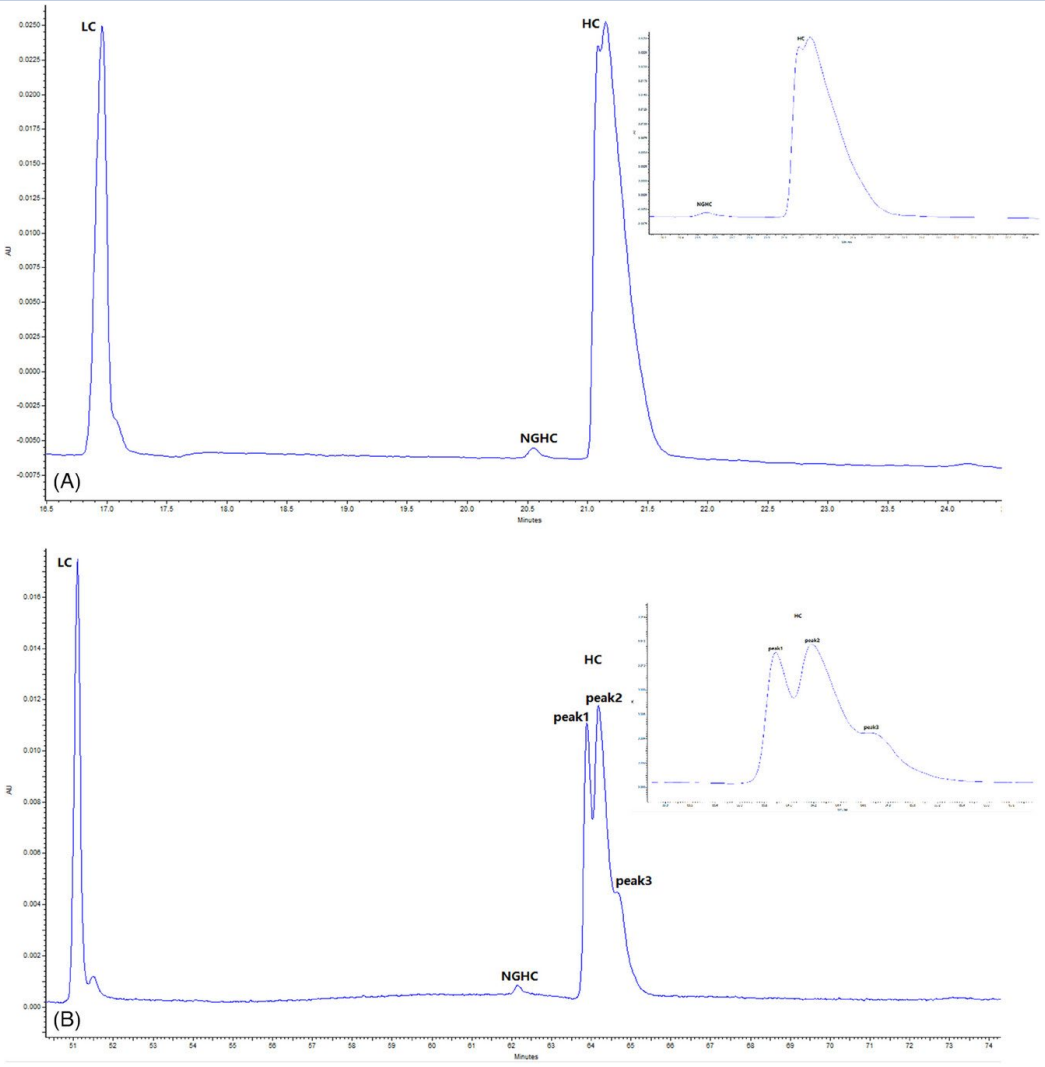
The glycosylated heavy chain contained three major glycoforms (G0F, G1F, and G2F) as determined by MS analysis. With standard CE-SDS conditions (20-cm effective capillary length), the glycosylated heavy chain formed only two peaks (Figure 1). When the method was optimized using a 40-cm effective length capillary, three peaks were resolved. The area normalization method showed that the trend of peaks 1, 2, and 3 content was similar to that of G0F, G1F, and G2F glycoforms, with the following distribution (Figure 2):

- G0F glycoform: 36.07%; Peak 1: 27.58%
- G1F glycoform: 48.80%; Peak 2: 54.72%
- G2F glycoform: 12.05%; Peak 3: 17.69%

Enzymatic Treatments to Confirm Glycosylation Role

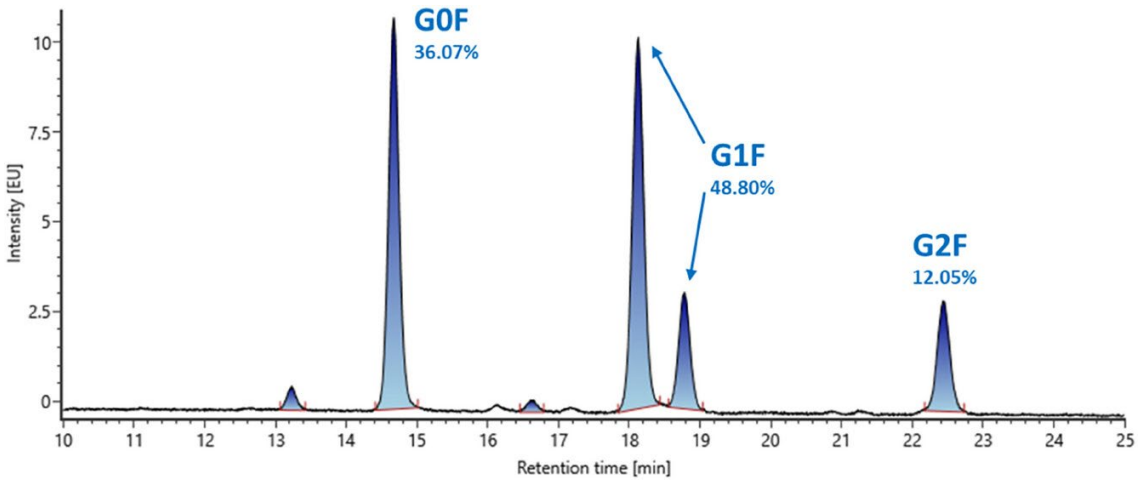
To prove that peaks 1, 2, and 3 were associated with glycosylation modifications, N-glycan cleavage was performed using PNGase F, and both peaks of the glycosylated heavy chain were completely transformed into a non-glycosylated heavy chain (Figure 3). Next, β1-4-galactosidase cleavage followed by reduced CE-SDS analysis was performed. After galactosidase treatment, both peaks of the glycosylated heavy chain were consolidated into a single peak, indicating that the heterogeneity was due to β-galactose modifications.

Figure 1



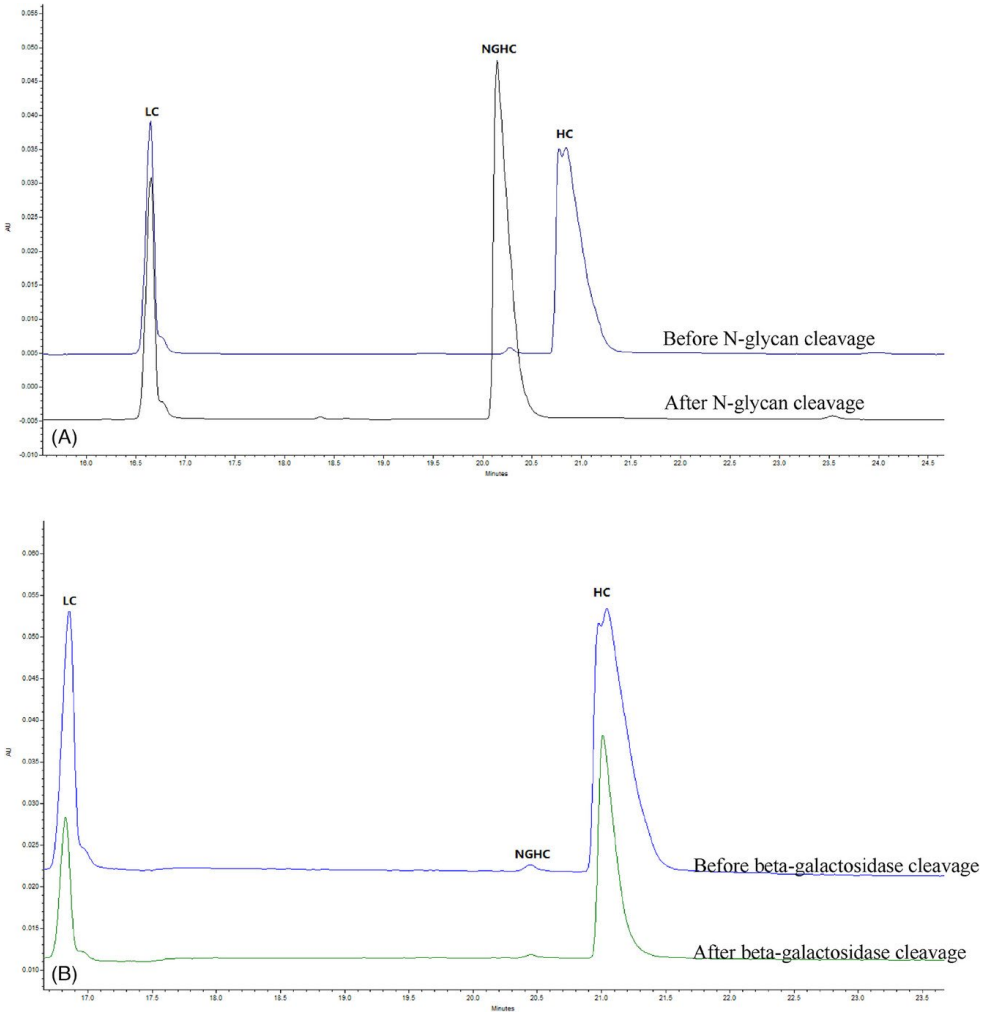
Reduced CE-SDS electropherograms of the anti-VEGFR-2 mAb. (A) Reduced CE-SDS separation was performed using a capillary with an effective length of 20 cm, and glycosylated heavy chains formed 2 peaks, with an enlarged view of glycosylated heavy chains shown in the upper right corner. (B) Reduced CE-SDS separation was performed using a capillary with an effective length of 40 cm, and glycosylated heavy chains formed three peaks (labeled peaks 1, 2, and 3), with an enlarged view of glycosylated heavy chains shown in the upper right corner.

Figure 2



Fluorescence liquid chromatogram of the wild-type glycan profile of the anti-VEGFR-2 mAb labeled with RapiFluor and separated by UPLC. The content of G0F was 36.07%. The total content of the two peaks of G1F was 48.80%. The content of G2F was 12.05%.

Figure 3



Reduced CE-SDS electropherograms of the anti-VEGFR-2 mAb (effective length 20 cm). (A) The upper figure shows the reduced CE-SDS profile before PNGase F digestion, the lower figure shows the reduced CE-SDS profile after PNGase F digestion, and both peaks of glycosylated heavy chain after PNGase F digestion were transformed into a non-glycosylated heavy chain. (B) The upper figure shows the reduced CE-SDS profile before galactosidase digestion, the lower figure shows the reduced CE-SDS profile after galactosidase digestion, and both peaks of glycosylated heavy chain after galactosidase digestion were consolidated into a single peak of glycosylated heavy chain without galactose

Preparation of Glycoform-Specific mAb References

To further confirm the identity of the CE-SDS peaks, mAb references with specific glycoforms (G0F, G1F, and G2F) were prepared by transglycosylation. The purity of G0F, G1F, and G2F in the 3 reference products was 93.44%, 95.96%, and 92.35%, respectively.

These references were then subjected to reduced CE-SDS analysis, allowing peak identification (Figure 4):

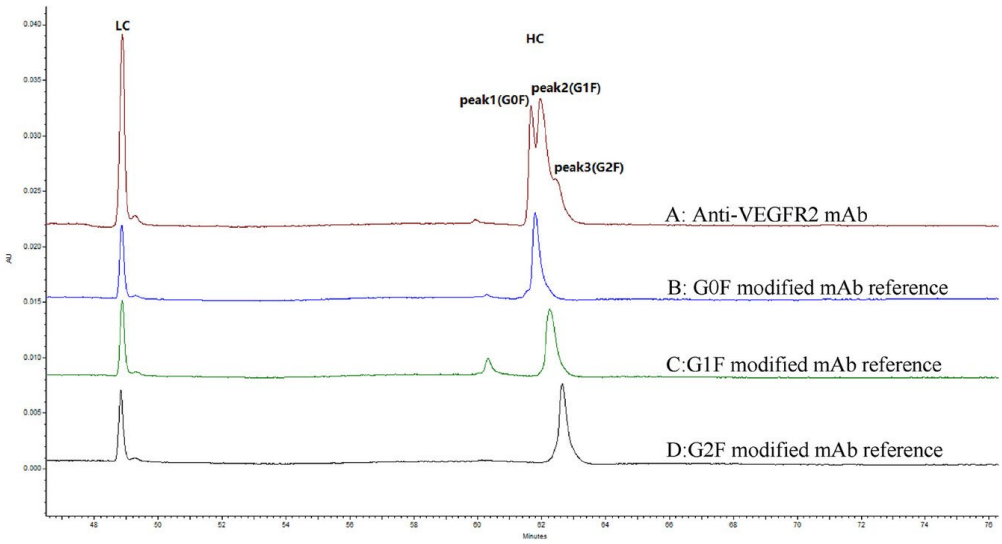
- Peak 1 was identified as G0F-modified heavy chain
- Peak 2 was identified as G1F-modified heavy chain
- Peak 3 was identified as G2F-modified heavy chain

Enhanced Resolution with scFc Fragments

To further identify peaks 1, 2, and 3 and quantify glycosylated heavy chain content more accurately, IdeS protease was used to cleave the anti-VEGFR-2 mAb samples and reference mAbs. This yielded single chain Fc (scFc) fragments, which were characterized by MS and then analyzed by non-reduced CE-SDS. The molecular weights of the scFc fragments matched theoretical values:

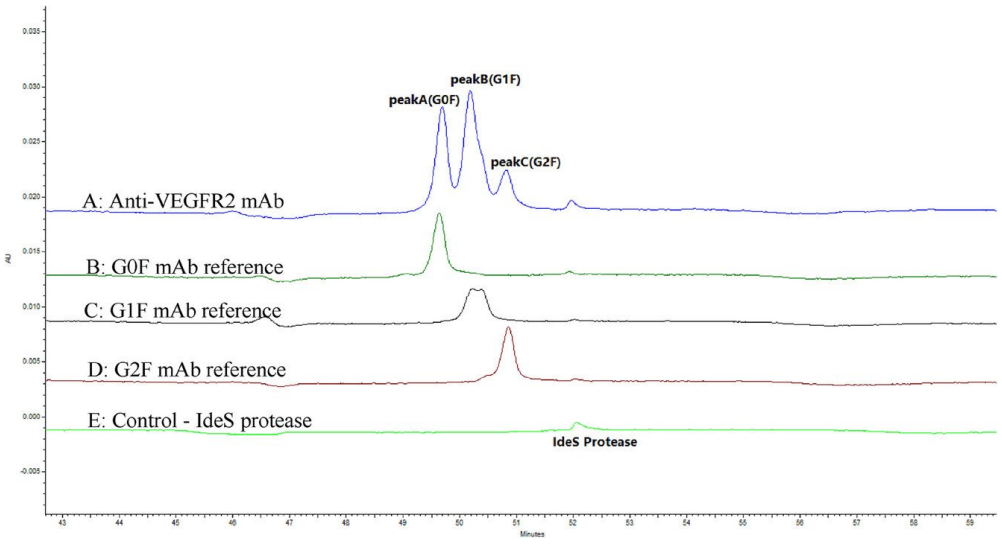
- G0F scFc: 25,231 Da (theoretical: 25,232 Da)
- G1F scFc: 25,393 Da (theoretical: 25,394 Da)
- G2F scFc: 25,555 Da (theoretical: 25,556 Da)

Figure 4



Reduced CE-SDS electropherograms of anti-VEGFR-2 mAb samples, and G0F-, G1F-, and G2F-modified mAb references (effective length 40 cm). Trace A represents the anti-VEGFR-2 mAb, and three peaks (peaks 1, 2, and 3) are separated from the glycosylated heavy chain. Trace B represents the G0F mAb reference; Trace C, the G1F mAb reference; Trace D, the G2F mAb reference. Therefore, peak 1 can be preliminarily identified as a G0F-modified heavy chain; peak 2, as a G1F-modified heavy chain; and peak 3, as a G2F-modified heavy chain.

Figure 5



Non-reduced CE-SDS electropherograms of anti-VEGFR-2 mAb and G0F/G1F/G2F mAb references following IdeS protease cleavage (effective length 40 cm). Trace A represents the scFc fragment of the anti-VEGFR-2 mAb, and three peaks (peaks A, B, and C) are separated from the scFc fragment. Trace B represents the scFc fragment of G0F mAb reference. Trace C represents the scFc fragment of G1F mAb reference. Trace D represents the scFc fragment of G2F mAb reference. Trace E represents the blank-IdeS protease. Therefore, peak A can be preliminarily identified as a G0F-modified scFc fragment; peak B, as a G1F-modified scFc fragment; and peak C, as a G2F-modified scFc fragment.

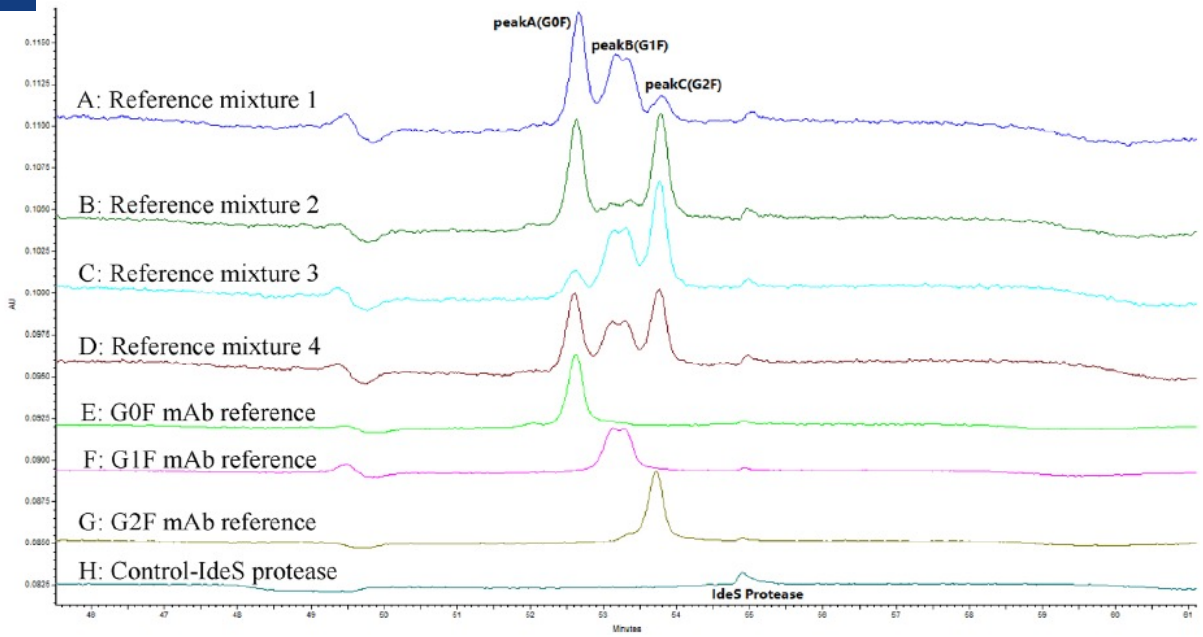
The separation of scFc fragments with different glycosylations showed much better resolution than heavy chains (resolution values for scFc fragments: 1.10 between peaks A-B and 1.35 between peaks B-C, compared to 0.51 and 0.18 for heavy chains). This allowed accurate identification (Figure 5):

- Peak A was confirmed as G0F-modified scFc fragment
- Peak B was confirmed as G1F-modified scFc fragment
- Peak C was confirmed as G2F-modified scFc fragment

Reference Mixture Analysis

To validate the method, reference mixtures of G0F, G1F, and G2F mAbs were prepared in different proportions. The measured contents of each glycoform correlated well with the theoretical mixture ratios, with recovery rates ranging from 96.6% to 106.7%, demonstrating that the method could accurately characterize the purity of scFc fragments with different galactose modifications (Figure 6).

Figure 6



Non-reduced CE-SDS electropherograms of G0F/G1F/G2F mAb references and mixed reference following IdeS protease cleavage (effective length 40 cm). Trace A represents the single chain Fc (scFc) fragment of reference mixture 1. Trace B represents the scFc fragment of reference mixture 2. Trace C represents the scFc fragment of reference mixture 3. Trace D represents the scFc fragment of reference mixture 4. Trace E represents the scFc fragment of the G0F mAb reference. Trace F represents the scFc fragment of the G1F mAb reference. Trace G represents the scFc fragment of the G2F mAb reference. Trace H represents the control-IdeS protease.

Conclusion

This study demonstrated that the heavy chain heterogeneity observed in CE-SDS analysis of anti-VEGFR-2 mAb was induced by different levels of galactosylation modifications, which potentially impact the efficacy of antibody drugs (particularly complement-dependent cytotoxicity).

The SCIEX PA800 Plus system, along with the IgG Purity and Heterogeneity Kit, proved to be a powerful tool for analyzing mAb size variants as critical quality attributes. By optimizing the CE-SDS method using longer capillaries (40 cm effective length versus standard 20 cm), improved resolution was achieved that enabled separation of heavy chains with different galactosylation levels.

Furthermore, CE-SDS offers a comprehensive approach for investigating and identifying heavy chain heterogeneity in reduced CE-SDS. This methodology is particularly effective in assessing galactosylation modification, providing a feasible strategy for mAb quality control and evaluation across the biopharmaceutical industry.

Advancing Metabolite Identification: Exploring the Role of Mass Spectrometry in Drug Development

Interview with Rahul Baghla

In this interview, Rahul Baghla, Sr. Manager, Global Scientific Marketing & Echo CoE at SCIEX, discusses the advancements in metabolite identification using mass spectrometry (MS). With over 15 years of experience in mass spectrometry, Rahul shares insights on how MS technology is transforming drug development by providing more precise structural elucidation of metabolites. He also highlights the integration of MS with other analytical techniques and the impact of these technologies on the pharmaceutical industry.

Can you tell us about your role at SCIEX and your journey in the field of mass spectrometry?

I am the Senior Manager of Global Scientific Marketing and Echo MS Center of Excellence at SCIEX. My team drives scientific collaborations worldwide, helping the scientific community solve analytical challenges using SCIEX technology. Additionally, I manage our global center of excellence for Echo MS technology, which focuses on high-throughput solutions using innovative acoustic ejection mass spectrometry. My journey with mass spectrometry began in college with competitive metabolism studies. This experience paved the way for my first role in the industry supporting clinical pharmacokinetics followed by generating clinical claims for consumer health products using mass spectrometers. In 2013, I joined SCIEX as a scientist, further advancing my expertise in the field of mass spectrometry.

How does mass spectrometry complement other analytical techniques in metabolite identification?

Mass spectrometry (MS) is a highly effective tool for identifying metabolites. Its sensitivity and specificity make it particularly valuable for detecting low-abundance metabolites in biological matrices. When coupled with liquid chromatography, mass spectrometry's capabilities are further enhanced, providing additional resolving power and sensitivity.

Can you share an example of a workflow that integrates MS with other techniques for drug development, and the benefits it provides?

Echo MS+ is another cutting-edge technology that integrates acoustic ejection with mass spectrometry, providing a high-throughput solution for drug discovery and development. This technology offers the flexibility to couple with SCIEX nominal mass and high-resolution accurate mass spectrometers. The system can achieve a sample acquisition speed of 1 second per sample for multiple drug discovery assays, significantly reducing the time required for sample analysis. Additionally, the option to use the ZenoTOF 7600 system provides further flexibility to analyze new modalities, including large proteins at lightning speed.

How have recent technological advancements in MS impacted the field of drug metabolism and pharmacokinetics?

Recent advancements in MS have greatly improved metabolite identification and drug metabolism studies. One key innovation is Electron Activated Dissociation (EAD), a fragmentation technique that enables more precise structural elucidation of drug metabolites. Unlike traditional methods, EAD uses tunable electron energy to selectively cleave bonds while preserving fragile functional groups, which is especially useful for metabolites with labile modifications. This preserves key structural features, enhancing the accuracy of metabolite identification and reducing ambiguities in biotransformation pathways.

What future trends do you foresee in the application of MS in pharmaceutical research and development?

MS will continue to play a pivotal role in pharmaceutical research and development, driven by advancements in sensitivity, selectivity, automation, and data analytics. MS can dramatically speed up research projects, opening the door to groundbreaking medicines that enhance human health. Imagine mass spectrometers becoming even more sensitive and selective, empowering studies that focus on creating more potent and highly effective drug products.

Metabolite Profiling

What are the general benefits of using MS for metabolite identification?

Mass spectrometers, when coupled with liquid chromatography, have significantly improved the identification and characterization of drug metabolites in complex biological matrices. The high resolving power of liquid chromatography enhances the capability of mass spectrometers to detect and characterize low levels of drug metabolites. Mass spectrometers provide information-rich MS/MS spectra, which support the structure elucidation of drug metabolites, ultimately aiding in the development of more effective and safer drugs.

How does MS enhance the accuracy and comprehensiveness of metabolite profiling in drug development?

MS enhances the accuracy and comprehensiveness of metabolite profiling in drug development by providing precise molecular characterization and structural elucidation. High-resolution mass spectrometry (HRMS) coupled with tandem MS (MS/MS) allows for accurate mass determination and fragmentation pattern analysis, enabling the identification of metabolites with high confidence. Advanced fragmentation techniques, such as electron-activated dissociation (EAD), complement traditional collision-induced dissociation (CID) by preserving labile bonds, facilitating more detailed structural insights. These capabilities help pinpoint metabolic soft spots and elucidate biotransformation pathways.

In what ways does the ability of MS to ionize and fragment neutral species improve the overall process of metabolite identification?

MS enhances metabolite identification by converting neutral molecules into charged ions, enabling their analysis. Once ionized, metabolites can be fragmented using techniques like CID or EAD, generating distinct fragment patterns. These fragments provide valuable structural insights, helping to determine metabolic modifications and biotransformation pathways.

Electron Activated Dissociation & Collision-Induced Dissociation

In your technical note "*Comprehensive metabolite identification with electron-activated dissociation (EAD) and collision-induced dissociation (CID)*" you discuss the use of EAD and CID in metabolite profiling. How does MS improve the structural elucidation of metabolites compared to other analytical methods?

CID and EAD are complementary fragmentation techniques, each generating unique fragment ions that aid in the structural elucidation of drug metabolites. This technical note highlights the use of both techniques on the ZenoTOF 7600 system to produce distinctive fragments. Additionally, it introduces an innovative semi-automated software solution, Molecule Profiler software, which utilizes both EAD and CID spectra for more confident identification and characterization of drug metabolites.

What are some typical analytical challenges faced in metabolite profiling using MS, particularly with EAD and CID, and what strategies does SCIEX employ to overcome them?

Two major challenges in metabolite profiling using MS are sensitivity at MS/MS levels and interpreting complex data. The ZenoTOF 7600 system addresses sensitivity with the Zeno trap, enhancing cycle time by over 90% and increasing signal up to 20-fold at the MS/MS level, crucial for characterizing low-abundant drug metabolites. Additionally, SCIEX's Molecule Profiler software integrates CID and EAD data, enabling more confident and faster identification and characterization of drug metabolites compared to manual data interpretation.

The integration of MS with other analytical techniques, as highlighted in the application note, has shown significant benefits in metabolite profiling. Can you discuss a case study where this integration provided unique insights?

The integration of MS with liquid chromatography (LC) is highly beneficial for identifying low-abundant metabolites. Separating these low-abundant metabolites from high-abundant peaks in the chromatogram is crucial for accurate identification and structural elucidation. Utilizing LC column chemistries that offer greater retention for polar metabolites significantly enhances resolution, thereby improving the overall sensitivity of the system. This improvement is essential for effective metabolite profiling studies.

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Rahul Baghla is a scientist with 15 years of expertise in mass spectrometry applications within the pharmaceutical industry. He specializes in integrating mass spectrometry technologies to advance drug discovery efforts. As a team leader, Rahul oversees a group of scientists dedicated to producing high-quality scientific content and fostering global collaborations with the pharmaceutical community.

Unlocking Bioanalysis: Exploring the Integration of MS and CE in Biopharmaceutical Analysis

Interview with Zoe Zhang

In this interview, Zoe Zhang, Senior Manager at SCIEX, shares her extensive knowledge on the integration of Mass Spectrometry (MS) and Capillary Electrophoresis (CE) in biopharmaceutical analysis. With over 10 years of research experience in analyzing protein therapeutics and small-molecule drugs, Zoe discusses the complementary roles of MS and CE, the impact of recent technological advancements, and the future trends shaping the industry. She also delves into the challenges and benefits of these techniques, providing valuable insights from her work at SCIEX. This interview offers an in-depth look at the transformative impact of MS and CE on biopharmaceutical development.

Can you tell us a bit about your role at SCIEX and your journey in the field of biopharma?

I joined SCIEX in 2017 as a Biopharma Application Scientist. At that time, I focused on developing methodologies to support biopharma workflows. Later, I took on the role of manager to lead the Scientific Marketing, Biopharma Protein Group. My responsibility was to guide my team in fostering collaboration with key thought leaders in the biopharma industry and building strong relationships that are essential for groundbreaking advancements. This experience has been highly rewarding.

In addition to close collaborator engagement, my team actively develops innovative workflows to ensure we stay at the forefront of cutting-edge methodologies. Each year, the team works to deliver high quality technical notes, peer reviewed publications, posters, and conference presentations from SCIEX technical marketing, along with supporting the development of collaborator presentations and peer reviewed publications. SCIEX as a company is constantly innovating in the field of analytical technologies. With new instrumentation and technology features being released, the technical marketing team is heavily engaged in supporting the development of workflows to highlight the real-world capabilities of next-generation instrumentation and software.

How do mass spectrometry (MS) and capillary electrophoresis (CE) complement each other in biopharmaceutical analysis?

Throughout biopharmaceutical development, MS and CE are essential and complementary technologies for biopharmaceutical analysis. Characterization needs change throughout the pipeline, and so each method takes the spotlight for different analytical workflows as a drug progresses from discovery through to manufacturing and QC. CE offers a rapid high resolution means for detecting biomolecules, while MS provides identification capabilities, allowing for more in-depth and detailed characterization of complex biomolecules. CE is widely used throughout the entire biopharma pipeline, but with its speed and flexibility is often utilized in the early development phases, for higher throughput screening for protein stability, purity, and integrity with assays such as CE-SDS and ciEF. CE is reproducible, with simple method transfer and so is commonly utilized in scale up and compliant environments for lot release assays. High-resolution mass spectrometry (HRMS) is more commonly used in research, discovery and development for biomolecule characterization, where more in-depth knowledge of a molecule structure is required, and is used to define the critical quality attributes of a particular drug moving through the pipeline. With the improved sensitivity of mass spec, and more straightforward instruments entering the market, there is a noticeable trend of HRMS being adopted in QC as well. When unexpected peaks are detected in CE assays, MS assays are necessary to identify the cause of those peaks.

Can you share an example of an integrated workflow that utilizes both MS and CE for biopharmaceutical analysis, and the benefits it provides?

icIEF is the gold standard assay for charge variant analysis in biopharma. It happens frequently that the relative abundance of variant peaks in icIEF does not align with historical data or unexpected peaks appear. In such cases, an investigation using MS is necessary to determine which species lead for the changes in abundance or contribute to the unknown peaks.

How have recent technological advancements in MS and CE impacted the field of biopharma?

We can continue the conversation from the last question. Usually, when an unexpected peak occurs, fractionation of that peak is necessary to collect samples for LCMS analysis. This process typically takes several days to weeks, because multiple rounds of fractionation are needed to generate sufficient samples for LCMS analysis. After fractionation, verification is required to ensure that the collected sample accurately represents the peak of interest. The entire process is labor and resource intensive and also remains open for undesired modifications to occur.

Recently, SCIEX launched a new platform that addresses this challenge, and introduces a fully integrated icIEF-UV/MS workflow that eliminates the need for fractionation and provides a direct mass identity of proteoforms. By maintaining the same icIEF separation, variant peaks can be directly detected and identified through HRMS system. This approach provides quick answers on proteoform ID in around an hour, while minimizing the risk of undesired modifications that may be introduced during fractionation.

What future trends do you foresee in the application of MS and CE in biopharmaceutical research and development?

HRMS and CE will remain essential technologies for monitoring and characterizing the complex structures of biotherapeutics for PTMs and critical quality attributes (CQAs). The flexibility of both platforms lends itself to the evolving therapeutic landscape, with new and more complex modalities being introduced. I anticipate an increased integration of MS and CE, like the iciEF-UV/MS technology SCIEX offers, to enhance the characterization of complex biotherapeutics and expedite the development process.

Mass Spectrometry for Biopharmaceuticals

How does the streamlined workflow for identifying and quantifying impurities improve the quality control of biopharmaceutical raw materials?

A streamlined workflow for identifying and quantifying impurities in biopharmaceutical raw materials significantly enhances quality control. It enables faster, more accurate, and efficient testing, ultimately leading to improved product quality and safety. Typically, MS and chromatography are employed for rapid impurity analysis, thereby reducing turnaround times, and increasing throughput.

Various assays are employed for quality control; for instance, monitoring components in cell culture media (CCM) during the development phases of biotherapeutic production is one. To be more specific, CCM undergoes consistent compositional changes throughout the manufacturing process. To ensure optimal CCM conditions and maintain the overall quality of the biotherapeutic, it is necessary to develop analytical assays that are sensitive, accurate, and robust. These assays should allow for comprehensive profiling of CCM components.

Conventional platforms such as biosensor analyzers, NMR, and Raman spectroscopy often lack a single-platform qualitative and quantitative method for CCM analysis in a matrix with high sensitivity, selectivity, and throughput. In comparison, CCM MS analysis methods for triple quadrupole and high-resolution QTOF systems offer high sensitivity, selectivity, and robustness. For example, the SCIEX method enables the identification and quantitation of over 110 compounds in a single analytical method in under 20 minutes.

What are some typical analytical challenges faced in MS for biopharmaceutical analysis, and what strategies does SCIEX employ to overcome them?

In biopharma MS analysis, there are a few challenges scientists face daily, including the need for a straightforward approach to comprehensively characterize complex and heterogeneous biotherapeutic

drugs, as well as the requirements for high sensitivity, selectivity, and quick answers to questions. At SCIEX, our strategy is to support the entire biopharma development pipeline with a streamlined workflow.

For the characterization of complex biotherapeutics, we offer cutting-edge technology, such as electron-activated dissociation (EAD), which provides accurate characterization and localization of critical quality attributes (CQAs), including glycosylation, isomerization, etc., compatible with data-dependent acquisition (DDA). Additionally, our Zeno Trap technology, also on the same MS platform, delivers high sensitivity for MS/MS spectra, ensuring data with high confidence.

To address the need for quick answers, we provide icIEF-UV/MS technology, which can save weeks in identifying charge variants without requiring fractionation.

Can you elaborate on the advantages of EAD for MS characterization of complex biopharmaceutical samples?

Electron-activated dissociation (EAD) is an approach for tandem mass spectrometry (MS/MS) applications. EAD extends beyond the industry standard collision induced dissociation (CID). CID can leave gaps in our understanding of molecular structure, but EAD provides a unique fragmentation pattern that closes this gap. In the development of protein therapeutics for example, EAD has proven useful in confirmation on positional isomers, enhancing sequence coverage for subunit analysis, and significantly enhancing in depth characterization of glycosylation profiles.

Capillary Electrophoresis for Biopharmaceuticals

There are other analytical methods available for biotherapeutic analysis such as SDS-PAGE or HPLC-based method. What are some advantages of using CE-SDS over other methods?

Compared to conventional methods like SDS-PAGE and HPLC, CE-SDS offers faster analysis, higher resolution, and better precision, making it particularly suitable for biopharma workflows.

When compared to SDS-PAGE, CE-SDS provides significantly improved separation resolution, linearity, and throughput. In addition, simple sample preparation means that CE-SDS workflows can be automated, allowing for even faster time to answer. In comparison to HPLC-based methods, CE-SDS can effectively resolve closely sized protein variants and differentiate between non-glycosylated and glycosylated heavy chains, where separation can be challenging for LC-based methods.

What are some of the common analytical challenges you encounter in CE for biopharmaceutical analysis, and how does SCIEX address them?

Different challenges, arise at certain parts of the development pipeline. In the upstream bioprocessing environment for example, throughput is one such challenge. At this stage of development, thousands of samples are screened annually, leading to a focus on sample analysis throughput. Traditional analytical technologies, like conventional CE-SDS or SDS-PAGE, often struggle to meet these throughput requirements due to their inherently low capacity. Scientists face the challenge of improving throughput while maintaining the resolution needed to accurately identify different peaks.

To address this challenge, my team worked to move the SCIEX CE-SDS workflow from a single capillary instrument, do one that can process 8 samples in parallel. The team developed an ultrafast CE-SDS separation method that achieves a throughput of just 1.8 minutes per sample. This innovative strategy leverages the multi-capillary environment of the BioPhase 8800 system, allowing for the screening of 96 samples in half a working day.

If we move a bit further down the pipeline to early cell line development, there is a need to screen clones to identify the best and most stable candidate to move forward in development. This is accomplished by screening for charge heterogeneity. Historically, charge variants are separated using icIEF, followed by characterization and identification of the charge variant through offline fraction collection and LC-MS analysis. This process is time-consuming, taking days or even weeks, and typically requires the collaboration of multiple analytical teams.

However, SCIEX offers an icIEF-UV/MS workflow through the Intabio ZT system, which provides on-line MS analysis and identification of charge variants separated by icIEF. This advancement significantly reduces the time needed for comprehensive charge heterogeneity analysis, shortening it from weeks to just hours.

The BioPhase 8800 system is multi-capillary system with 8 capillaries. The technical note on the efficient transfer of the high-speed CE-SDS method discusses transitioning from the PA 800 Plus system to the BioPhase 8800 system. What are the main challenges and benefits of this transition?

One of the challenges facing the biopharma industry today across various analytical platforms is the adoption of new technology while ensuring compliance with existing assays that have been validated, integrated, and approved by regulatory authorities. CE-SDS is widely used in biotherapeutic analyses for lot release, stability testing, formulation buffer screening, process development, cell line development, and product characterization. The CE-SDS assays on the PA 800 Plus system are considered the gold standard in the industry. These methods have been adopted, validated, transferred, and utilized in analytical development and quality control for numerous commercial molecules.

Although the BioPhase 8800 system offers eight times the throughput of the PA800 Plus system, it poses challenges for deployment without demonstrating comparable data quality and profiling. To address this, my team developed a new CE-SDS-based method for the BioPhase 8800 system that matches the performance of the IgG HS CE-SDS method on the PA800 Plus system. This development allows scientists to benefit from increased throughput while ensuring that the data is consistent with their historical data.

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Zoe Zhang is the Senior Manager of Biopharma applications and technical marketing at SCIEX. She brings over 15 years of research experience in analyzing protein therapeutics and small-molecule drugs within the biopharma industry, with a strong background in GMP environments.

In her role, she guides her team in fostering collaboration with scientific thought leaders and building strong relationships that are essential for groundbreaking advancements. In addition to close collaborator engagement, her team actively develops innovative workflows, ensuring they remain at the forefront of cutting-edge methodologies. They are also involved in next-generation instrumentation and software development. Overall, her team conducts in-depth mass spectrometry and capillary electrophoresis characterization and quantitation across various modalities and next-generation therapies.

Comprehensive metabolite identification with electron-activated dissociation (EAD) and collision-induced dissociation (CID)

Automated metabolite identification on the ZenoTOF 7600 system using Molecule Profiler software

Eva Duchoslav[1], Disha Thakkar[1], Yves Le Blanc[1], Jason Causon[1], Rahul Baghla[2] and Eshani Nandita[2]
[1]SCIEX, Canada; [2]SCIEX, USA

This technical note describes a streamlined, software-aided automated workflow to study metabolite identification using the ZenoTOF 7600 system paired with Molecule Profiler software. Confident metabolite structure assignments were performed using both CID and EAD data. The more informative MS/MS spectra provided by EAD lent higher confidence to the software-based identification of drug metabolites to support drug development.

The qualitative capabilities of accurate mass spectrometry, such as automated LC-MS/MS workflows using CID, have been important for investigating the metabolism of candidate modalities in the early stages of pharmaceutical drug development.

Recent accurate mass spectrometry advancements, including improvements in the duty cycle, enabled the application of EAD on LC time scales and the integration of EAD into LC-MS/MS workflows, providing more confident characterizations of compounds of interest.[1-3]

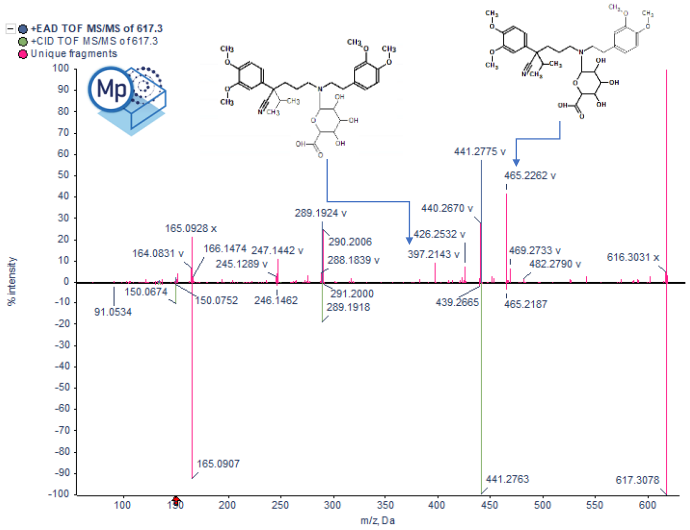


Figure 1. Panel view of the MS/MS spectra and structure assignments in Molecule Profiler software. The software displayed an inverted overlay of the EAD and CID spectra, highlighting unique fragments (pink) and putative structure assignments based on spectra weightage. Diagnostic fragments such as m/z 397 and 465 support the N-glucuronide conjugation of verapamil. EAD TOF MS/MS fragment mass accuracy was within 5 ppm, enabling confident metabolite confirmation and identification.

Here, data were generated and analyzed using a single SCIEX OS software platform to test metabolite identifications. The data were acquired using an advanced metabolite identification workflow using the ZenoTOF 7600 system and processed with Molecule Profiler software. Molecule Profiler software now supports the consolidation and ranking of structures based on EAD and CID data (Figure 1), making it an ideal tool for comparing MS/MS spectra to identify unique fragments in a single results file.

Key features for metabolite identification using the ZenoTOF 7600 system and Molecule Profiler software

- **Confident structure assignments:** Analyze EAD and CID spectra from a single results file to achieve more confident structure assignments
- **Enhanced structure assignment:** EAD preserves fragile modifications to easily localize phase II conjugates. EAD also provides information-rich MS/MS spectra that enable more confident identification of phase I metabolites than CID.
- **Efficient metabolite identification:** Perform fast and efficient software-aided identification of drug metabolites using Molecule Profiler software with the ZenoTOF 7600 system
- **Streamlined data acquisition and processing workflow:** Utilize a quick and easy-to-use workflow from data acquisition to analysis in SCIEX OS software with the integration of Molecule Profiler software

Methods

Sample preparation: Verapamil, buspirone and nefazodone were incubated in rat hepatocytes at a 1µM starting concentration. Samples were removed from incubation and quenched with acetonitrile at 0-, 30- and 120-minute time points.

Chromatography: Separation was performed on a Phenomenex Luna Omega Polar C18 (2.1 x 150 mm, 3 µm, 100 Å) column at 40°C. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. An injection of 5 µL was used for analysis.

The chromatographic gradient conditions used are summarized in Table 1.

Table 1. Chromatographic gradient.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	95	5
0.5	95	5
1.5	85	15
3.5	50	50
4.75	5	95
5.75	5	95
5.8	95	5
6.5	95	5

Mass spectrometry: The samples were analyzed using the data-dependent acquisition (DDA) method with Zeno CID DDA and Zeno EAD DDA on the ZenoTOF 7600 system. The source and gas conditions used are summarized in Table 2. The method conditions used are summarized in Table 3.

Table 2. Source and gas conditions.

Parameter	Setting
Curtain gas	35 psi
Ion source gas 1	55 psi
Ion source gas 2	55 psi
CAD gas	7
Ion spray voltage	5500 V
Source temperature	550°C

Table 3. Zeno DDA parameters.

Parameter	Setting	
	Zeno CID DDA	Zeno EAD DDA
Method duration	6.5 min	
TOF MS start-stop mass	100–1000 Da	
Maximum candidate ions	8	
Accumulation time (TOF MS)	0.05 s	
TOF MS/MS start-stop mass	40–1000 Da	60–1000 Da
Accumulation time (TOF MS/MS)	0.06 s	0.09 s
Collision energy (CID)	35 V	
Collision energy spread (CID)	15 V	
Electron kinetic energy (EAD)		10 eV
Electron beam current (EAD)		5000 nA

Data processing: SCIEX OS software was used for data acquisition. Molecule Profiler software was used to predict biotransformation sites using Zeno CID DDA and Zeno EAD DDA data.[4]

Metabolite identification using Molecule Profiler software

Metabolites from the incubation of verapamil, buspirone and nefazodone in rat hepatocytes were analyzed using Zeno CID and Zeno EAD. Molecule Profiler software enabled the processing and analysis of Zeno CID and Zeno EAD data in a single results file.

Interpretation of the site of metabolism was enabled by the automated assignment of the structures, based on the relative weighting of Zeno EAD and Zeno CID spectra on a scale of 1% to 100%. The interpretation panel in the software allows users to modify structures and the total score for the modified structures. Figure 2 shows the overview of the results panel, in which users can view the list of potential metabolites, assigned structures and scoring information. A potential metabolite is scored based on the mass defect, isotope pattern, MS/MS data and mass accuracy. The data can be viewed using TOF MS or MS/MS spectra and XICs. The software also displays the mass defects and isotope patterns of the metabolites.

Using Zeno CID and Zeno EAD MS/MS data can reduce the ambiguity in identifying positional isomers (Figure 1). For glucuronide conjugates, the EAD data provided more specific and unique MS/MS fragments than CID, aiding in the correct assignment of the site of metabolism. Figure 3 shows an example of verapamil metabolites identified in rat hepatocytes (t = 30 minutes). Based on the EAD data, 15 metabolites and cleavages were identified. The Molecule Profiler software enables rapid metabolite identification from automated data processing.

Metabolites were assigned rank values of 1, 2, 3 or >3 based on the probability of occurrence (Figure 4). Metabolites assigned rank 1 have the highest probability of occurrence based on the total score derived from the mass defects, isotope patterns, MS/MS data and mass accuracy. Based on the datasets acquired from verapamil, nefazodone and buspirone, EAD provided more informative MS/MS spectra that

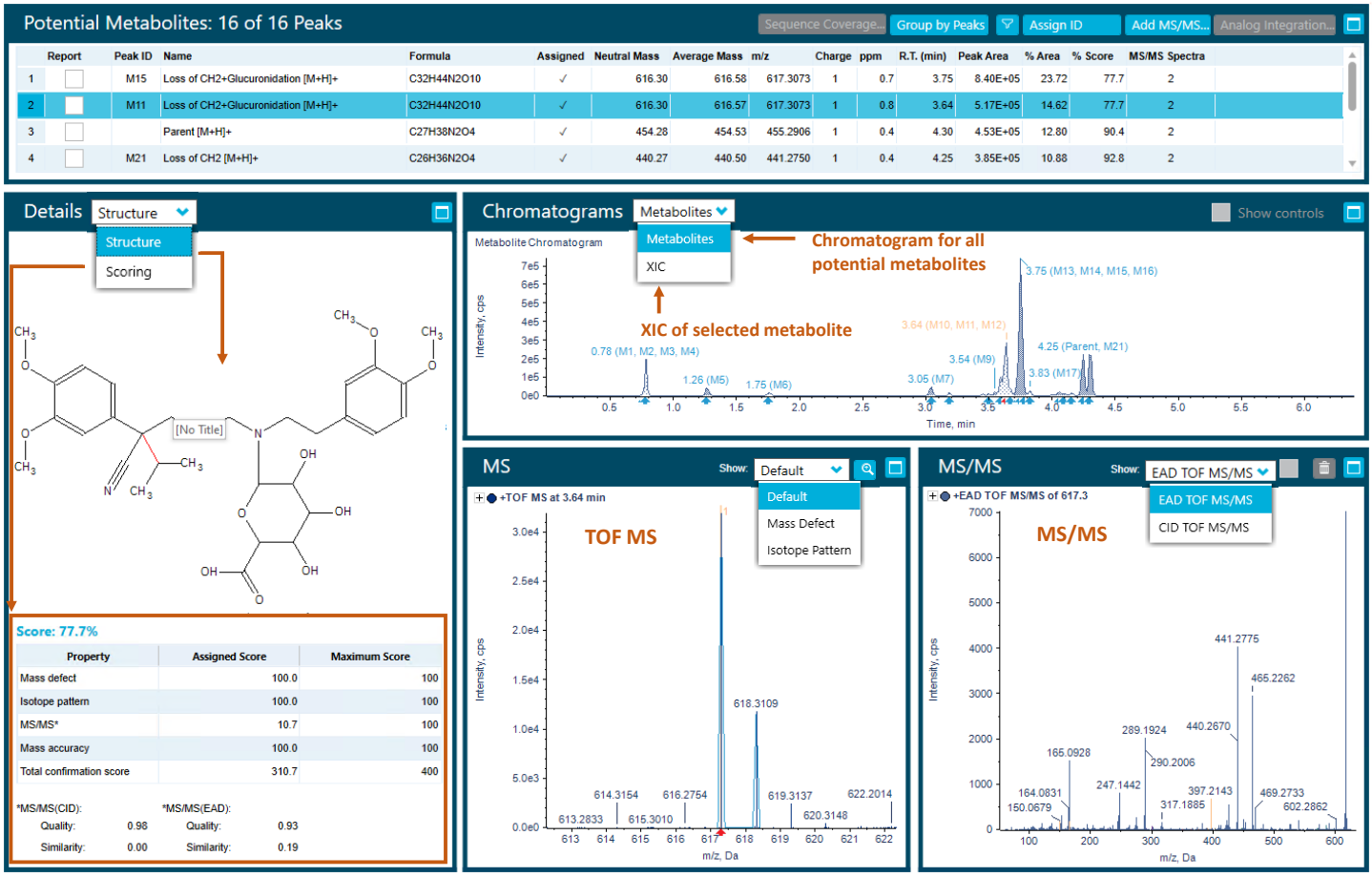
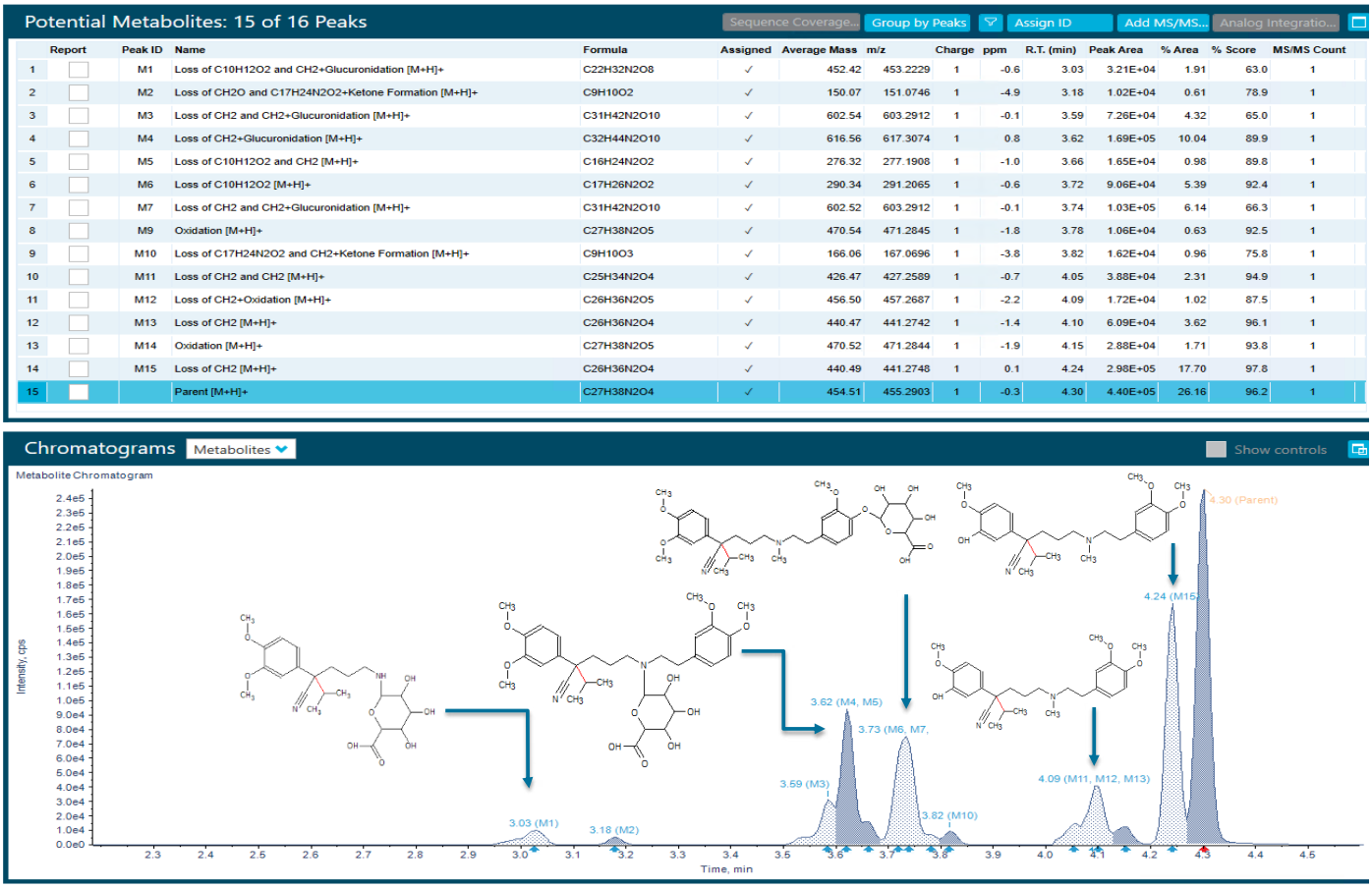


Figure 2. Results view in Molecule Profiler software. The software shows the number of potential metabolites with detailed information about the structure and scoring, options to view the XICs of selected or all metabolites and TOF MS spectra. Molecule Profiler software also displays the mass defect, isotope pattern, and CID and EAD spectra of the selected metabolite.

provided greater confidence in metabolite identification. For example, 40% of glucuronide metabolites from verapamil were ranked 1 based on the CID data, whereas 60% of the glucuronide metabolites were ranked 1 based on the EAD data (Figure 4). Similarly, in the buspirone dataset, 70% of oxidative metabolites were ranked 1 based on the CID data, while the remaining metabolites were ranked 2 or 3. Based on the EAD data, however, >80% of the oxidative metabolites were ranked 1 in the buspirone dataset. Metabolite confirmations were further supported by CID data. In the verapamil dataset, cleavage and oxidative metabolites were equally confirmed by CID and EAD data, with 80% of metabolites placed as rank 1 (Figure 4).



	Report	Peak ID	Name	Formula	Assigned	Average Mass	m/z	Charge	ppm	R.T. (min)	Peak Area	% Area	% Score	MS/MS Count
1		M1	Loss of C10H12O2 and CH2+Glucuronidation [M+H]+	C22H32N2O8	✓	452.42	453.2229	1	-0.6	3.03	3.21E+04	1.91	63.0	1
2		M2	Loss of CH2O and C17H24N2O2+Ketone Formation [M+H]+	C9H10O2	✓	150.07	151.0746	1	-4.9	3.18	1.02E+04	0.61	78.9	1
3		M3	Loss of CH2 and CH2+Glucuronidation [M+H]+	C31H42N2O10	✓	602.54	603.2912	1	-0.1	3.59	7.26E+04	4.32	65.0	1
4		M4	Loss of CH2+Glucuronidation [M+H]+	C32H44N2O10	✓	616.56	617.3074	1	0.8	3.62	1.69E+05	10.04	89.9	1
5		M5	Loss of C10H12O2 and CH2 [M+H]+	C16H24N2O2	✓	276.32	277.1908	1	-1.0	3.66	1.65E+04	0.98	89.8	1
6		M6	Loss of C10H12O2 [M+H]+	C17H26N2O2	✓	290.34	291.2065	1	-0.6	3.72	9.06E+04	5.39	92.4	1
7		M7	Loss of CH2 and CH2+Glucuronidation [M+H]+	C31H42N2O10	✓	602.52	603.2912	1	-0.1	3.74	1.03E+05	6.14	66.3	1
8		M9	Oxidation [M+H]+	C27H38N2O5	✓	470.54	471.2845	1	-1.8	3.78	1.06E+04	0.63	92.5	1
9		M10	Loss of C17H24N2O2 and CH2+Ketone Formation [M+H]+	C9H10O3	✓	166.06	167.0696	1	-3.8	3.82	1.62E+04	0.96	75.8	1
10		M11	Loss of CH2 and CH2 [M+H]+	C25H34N2O4	✓	426.47	427.2589	1	-0.7	4.05	3.88E+04	2.31	94.9	1
11		M12	Loss of CH2+Oxidation [M+H]+	C26H36N2O5	✓	456.50	457.2687	1	-2.2	4.09	1.72E+04	1.02	87.5	1
12		M13	Loss of CH2 [M+H]+	C26H36N2O4	✓	440.47	441.2742	1	-1.4	4.10	6.09E+04	3.62	96.1	1
13		M14	Oxidation [M+H]+	C27H38N2O5	✓	470.52	471.2844	1	-1.9	4.15	2.88E+04	1.71	93.8	1
14		M15	Loss of CH2 [M+H]+	C26H36N2O4	✓	440.49	441.2748	1	0.1	4.24	2.98E+05	17.70	97.8	1
15			Parent [M+H]+	C27H38N2O4	✓	454.51	455.2903	1	-0.3	4.30	4.40E+05	26.16	96.2	1

Figure 3. Major verapamil metabolites in rat hepatocytes (t = 30 minutes). Fifteen significant metabolites were assigned putative structures during the automated processing with Molecule Profiler software.

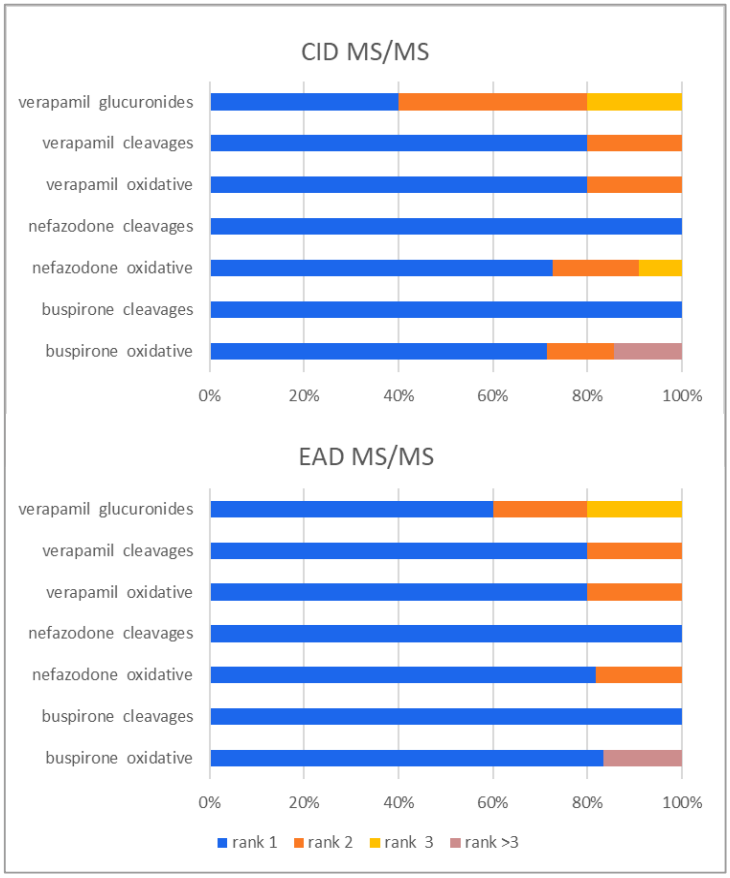


Figure 4. Summary of the automated structure assignment using CID and EAD MS/MS data. Glucuronides, cleavages and oxidative metabolites were evaluated in verapamil, nefazodone and buspirone. Each assignment was based on software rankings of 1, 2, 3 and >3. Metabolite structures with the highest total scores were assigned as rank 1.

Conclusions

- Accurate and comprehensive CID and EAD MS/MS data were generated on the ZenoTOF 7600 system using a fast LC gradient workflow
- An innovative feature in Molecule Profiler software was used to identify unique fragments from EAD and CID spectra to achieve more accurate metabolite structure assignments
- The enhanced sensitivity provided by the Zeno trap supported confident identification and characterization of low-abundant metabolites
- Data acquisition and processing were streamlined in a single software platform to expedite data management and analysis for drug metabolism studies

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2. Comprehensive metabolite characterization using orthogonal MS/MS data. SCIEX poster, RUO-MKT-10-14711-A
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Acknowledgment

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Streamlined identification and quantitation of impurities of the ionizable lipid ALC-0315 for rapid and confident vendor-to-vendor raw material assessment to ensure mRNA-LNP product quality

Featuring the ZenoTOF 7600 system and ionizable lipid workflow in Molecule Profiler software

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[1]SCIEX, Canada; [2]SCIEX USA; [3]Acuitas Therapeutics Inc, Canada

This technical note describes an automated workflow for the identification, relative quantitation and structural elucidation of impurities in the ionizable lipid, ALC-0315, sourced from 3 different vendors. This method uses the ZenoTOF 7600 system equipped with electron activated dissociation (EAD) capabilities and paired with Molecule Profiler software (Figure 1). The workflow enables users to confidently identify impurities at abundances as low as 0.01%. The workflow also allows the differentiation of unwanted impurities by in-depth structural elucidation with EAD and interpretation with Molecule Profiler software. Analytical scientists can utilize this workflow to ensure lot/vendor quality for raw materials and to increase confidence in the efficiency and safety of formulated lipid nanoparticle (LNP) products.

The use of LNPs as a nucleic acid delivery vehicle is gaining momentum, following their use in the COVID-19 vaccines that changed the course of the SARS-CoV-2 pandemic. The purity of the lipid raw materials in LNP formulations is important because it could impact the efficacy of the resulting drug product. A recent study suggests that oxidation and subsequent hydrolysis of the tertiary amine in ionizable lipids lead to the formation of reactive species, such as N-oxide, that covalently bind to the nucleobase of mRNA and cause its loss of activity.[1] Hence, a sensitive, robust and streamlined analytical workflow is required to monitor lot/vendor variability in ionizable lipids and to ensure the quality of these key raw materials. While impurity identification can rely on high-resolution mass spectrometry, confident structure confirmation requires comprehensive MS/MS spectrum annotation based on signature fragment ions. These diagnostic fragment ions are produced by EAD MS/MS using the Zeno trap to achieve high sensitivity. Traditional (Electron-transfer dissociation) ETD-based fragmentation does not work efficiently with low charge state ions that are related to lipids.[2,3] The identification of these species requires (i) information-rich MS/MS data that allow for explicit structural elucidation and site-specific localization and (ii) powerful and intuitive processing software capable of overcoming time-consuming manual interpretation of highly symmetrical lipid structures. In this technical note, Molecule Profiler software leveraged the wide interscan dynamic range of the ZenoTOF 7600 system to identify impurities present in ALC-0315 at concentrations as low as 0.01%. Furthermore, the software used the EAD-generated MS/MS diagnostic fragment ions to achieve automatic structure interpretation of impurities.

Key features of LNP impurity profiling

- **Efficient impurity identification:** Molecule Profiler software and the ZenoTOF 7600 system achieved identification of low-abundance ionizable lipid impurities (0.01%) and enabled thorough characterization of vendor-to-vendor variation
- **Confident structure assignments:** Comprehensive structural elucidation of ALC-0315 and its impurities was enabled by the information-rich EAD MS/MS data and the strong MS/MS spectrum interpretation ability of Molecule Profiler software
- **Streamlined end-to-end solution:** Sensitive and rapid data acquisition by the ZenoTOF 7600 system was accompanied by easy-to-use data analysis and automatic structural assignment using Molecule Profiler software, all integrated into SCIEX OS software

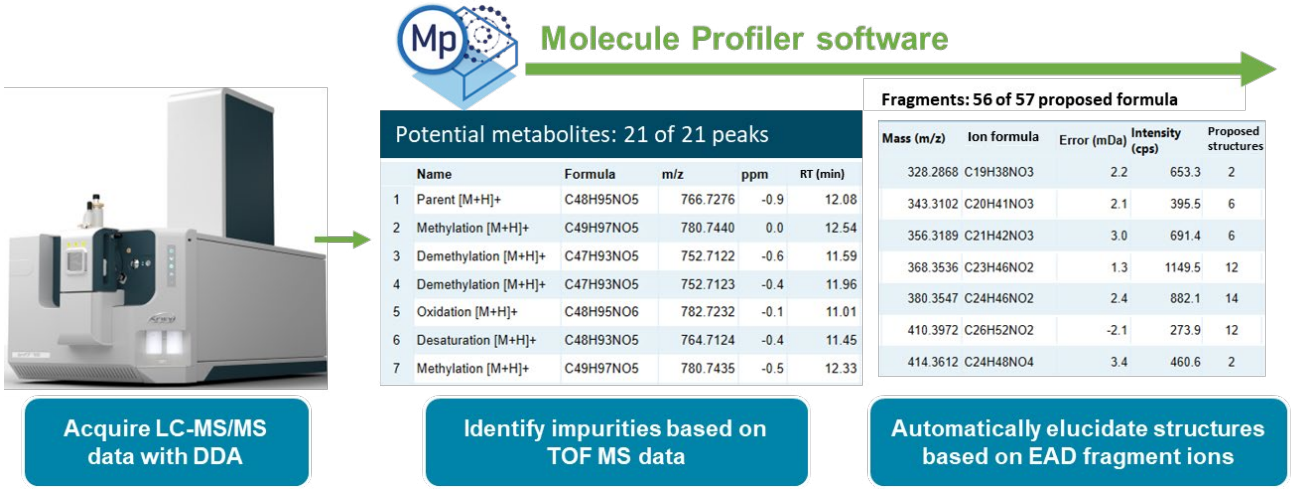


Figure 1. Workflow representation for the relative quantitation and structural elucidation of ALC-0315 using EAD-based MS/MS.

Methods

Sample preparation: Samples of ALC-0315 from 3 different suppliers were provided by Acuitas Therapeutics. Stock solutions of the 3 ionizable lipids were diluted 100-fold using mobile phase B and then were further diluted 100-fold using mobile phase A. Mobile phase B was 60:40 (v/v), acetonitrile/MeOH with 10mM ammonium acetate. Mobile phase A was a solution containing by volume 15% water, 30% MeOH and 55% acetonitrile with 10mM ammonium acetate. The resulting samples were analyzed directly by UPLC-MS/MS.

LC-MS/MS analysis: Samples were analyzed by a UPLC-ESI-MS/MS system equipped with a Waters H-class UPLC system and a ZenoTOF 7600 system. The samples were separated using a UHPLC Peptide BEH C18 column (2.1 × 150 mm, 1.7 µm, 300 Å, Waters, P/N: 186003687) using the gradient conditions shown in Table 1. The flow rate was set to 0.3 mL/min and the column temperature was set to 70°C. The injection volume was set to 2 µL.

Table 1. LC gradient.

Time (min)	A (%)	B (%)
0	100	0
2	100	0
11	0	100
21	0	100
21.1	100	0
27	100	0

LC-MS/MS data were acquired using the data-dependent acquisition (DDA) scan mode. The parameters used in these experiments are shown in Table 2.

Data processing: Structural elucidation and relative quantitation were performed using the Molecule Profiler software modules of SCIEX OS software. A self-built raw material impurities list was integrated into the processing parameters (Table 3). The maximum C-C bond to break was set to 1 and the number of EAD fragment peaks selected for the assignment was set to 60 under MS/MS parameters. The rest of the parameters were set to the default.

Table 2. TOF MS and EAD MS/MS parameters.

Parameter	MS	MS/MS
Scan mode	TOF MS	DDA
Polarity	Positive	
Gas 1	60 psi	
Gas 2	80 psi	
Curtain gas	35 psi	
Source temperature	450°C	
Ion spray voltage	5500 V	
Declustering potential	60 V	
Collision energy	10 V	12 V
CAD gas	7	
Workflow	Small molecule	
Maximum candidate ion	2	
Intensity threshold	1000 cps	
Exclusion time	5 s after 3 occurrences	
Start mass	300 m/z	50 m/z
Stop mass	1000 m/z	1000 m/z
Electron KE	N/A	15 eV
Electron beam current	N/A	5000 nA
ETC	N/A	100
Reaction time	N/A	30 ms
Zeno trap	N/A	ON
Accumulation time	0.1 s	
Time bins to sum	6	

Table 3. Impurities list used in data processing parameters setting.

Name	Mass shift	Description
Oxidation	-15.9949	+O
Loss of 2 H and CH2	-16.0314	-CH2 and -H2
Loss of CH2	-14.0157	R-CH3 to R-H
Loss of CH2, addition of 2 H	-12.0000	-CH2 and +2H
Ketone formation	13.9793	R-CH2-R_1 to R-CO-R_1
Desaturation	-2.0157	R_1CH2-CH2R_2 to R_1CHCHR_2
Hydrogenation	2.0157	+2H
Loss of 4 CH2	-56.0626	NA
Addition of CH2	14.0157	R-H to R-CH3
Loss of 3 CH2	-42.0470	NA
Addition of 2 CH2	28.0313	+2 CH2
Di-oxidation	31.9898	+2O

Purity of the ALC-0315 sample and the detection of impurities across vendors

ALC-0315 is an ionizable lipid used in the preparation of the LNP-mRNA formulations for COVID-19 vaccines. In this work, ALC-0315 samples from 3 different suppliers were evaluated to assess variations in their impurity profiles. A main peak of the pure ALC-0315 was observed at a retention time (RT) of 12.1 min and its relative abundance varied across the 3 evaluated samples (Figure 2). The highest purity was observed in the sample from Vendor 1, with a peak area of 98.20%, followed by the samples from Vendor 3 (96.86%) and Vendor 2 (94.99%). Several low-abundance impurities, with concentrations ranging from 1.37% down to 0.01%, were detected (Figure 2), demonstrating the wide interscan dynamic range of the ZenoTOF 7600 system and the ability of Molecule Profiler software to identify low-abundance impurities.

Molecule Profiler software automatically identified several impurities in samples from the 3 vendors. These results are summarized in Table 4, including their relative abundance peak areas and abundance rank within each vendor based on TOF MS peak areas. The type and abundance of impurities varied across the 3 vendors. For example, a methylated impurity eluting at RT 12.3 min showed relative abundances of 0.24%, 0.46% and 1% in the samples acquired from Vendors 2, 1 and 3, respectively. Whereas the loss of 4 CH2 was a common impurity in the sample from Vendor 3, this impurity was not observed in the samples from Vendors 1 or 2. The ability of this workflow to capture such variation in impurity content between the samples will provide a streamlined solution for lipid raw material control.

The high resolution of the ZenoTOF 7600 system allowed for excellent mass accuracy for MS and MS/MS data. Analyzed impurities demonstrated mass accuracy of the monoisotopic peak within 2.5 ppm. Molecule Profiler software also performed thorough data analysis even with low-abundance species.

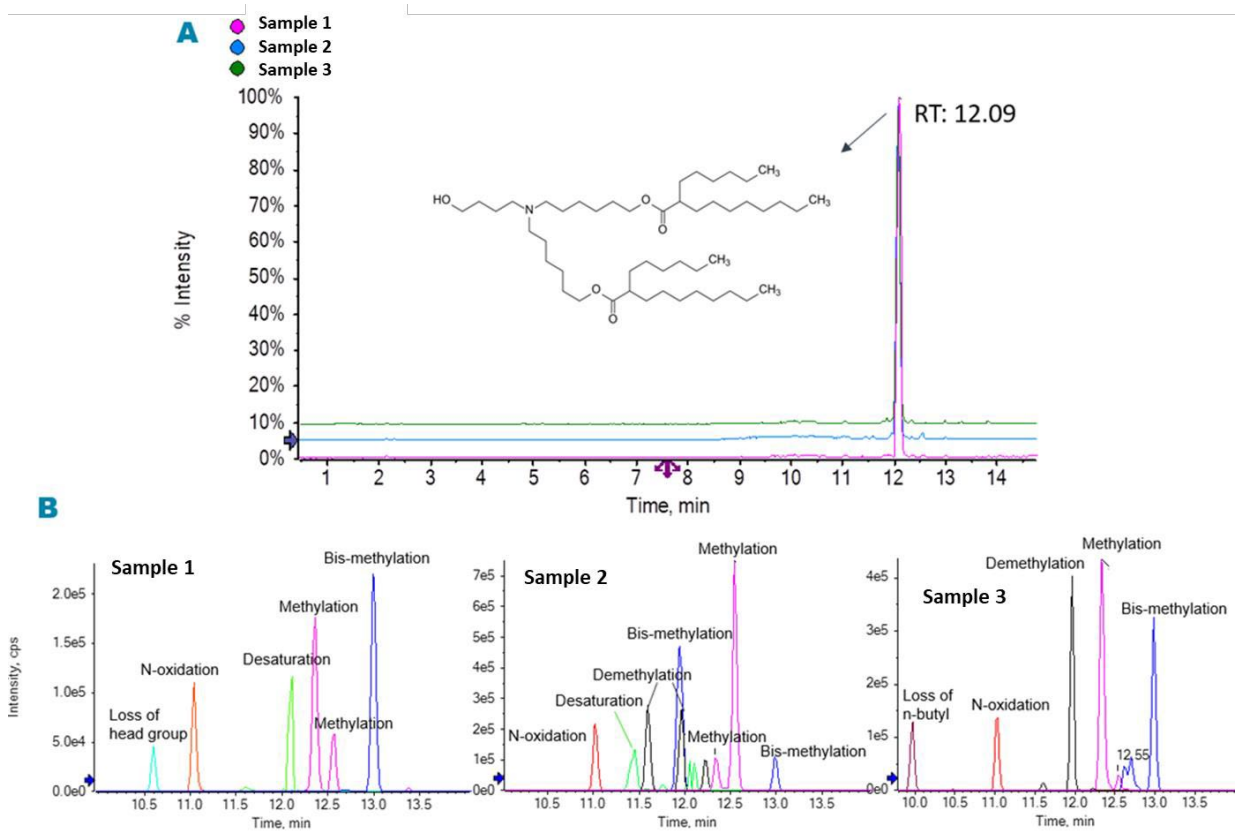


Figure 2. Total ion chromatogram (TIC) and extracted ion chromatogram (XIC) of the main impurities in samples of ALC-0315 from 3 vendors. The TIC (A) shows the major peaks that eluted at 12.1 min, corresponding to ALC-0315. Different types and levels of impurities were observed in the XIC (B).

Table 4. Main impurities identified automatically by Molecule Profiler software in samples of ALC-0315 from 3 vendors. The relative abundance peak areas and abundance rank are included for each vendor.

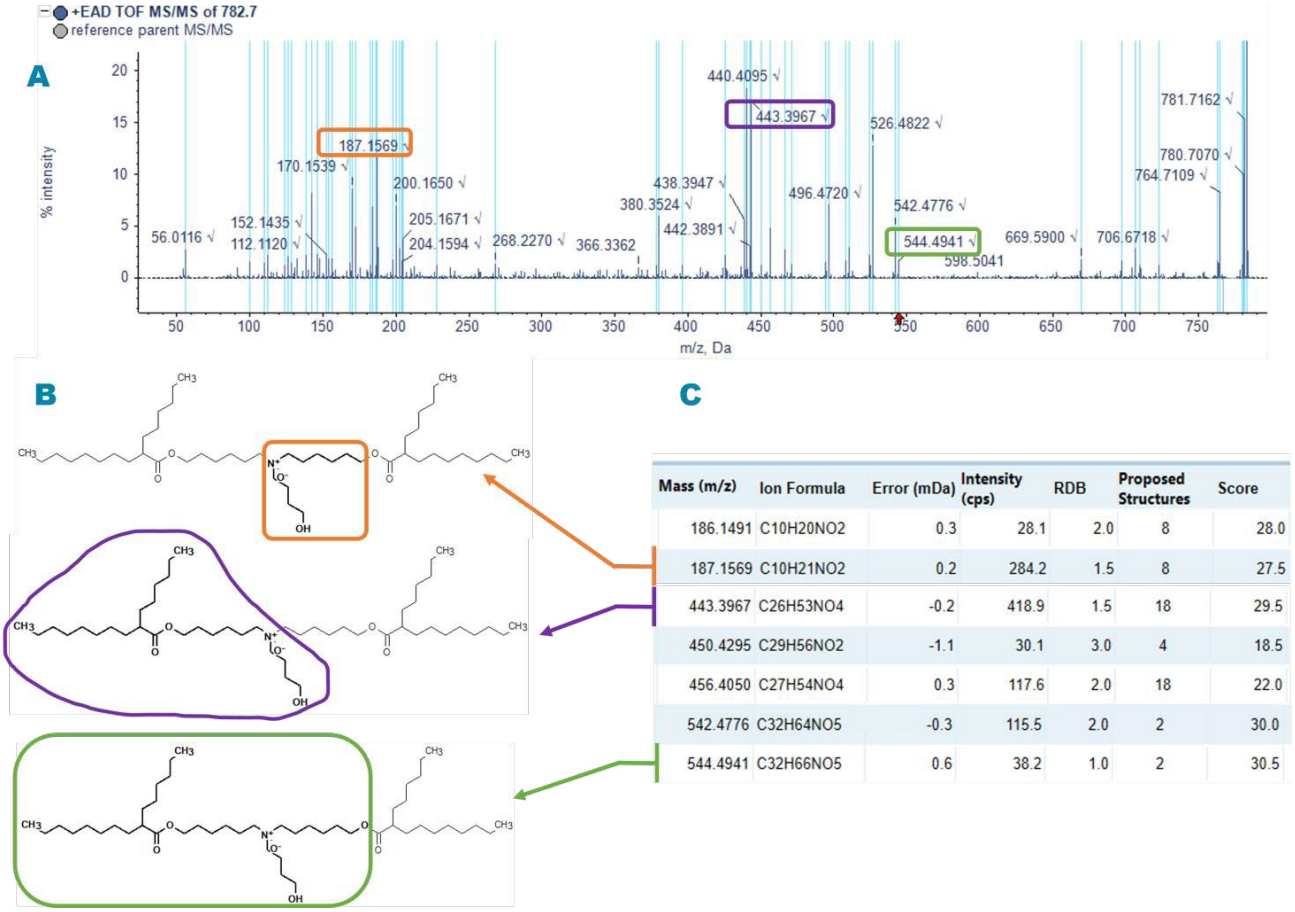
Name	Formula	m/z	RT (min)	Vendor 1		Vendor 2		Vendor 3	
				Rank	% Area	Rank	% Area	Rank	% Area
Gain of CH2	C49H97NO5	780.744	12.33	2	0.46	7	0.24	1	1.00
Gain of 2 CH2	C50H99NO5	794.759	12.99	1	0.52	2	1.16	3	0.63
Oxidation on N	C48H95NO6	782.722	11.04	3	0.26	5	0.45	4	0.30
Desaturation	C48H93NO5	764.714	12.06	4	0.23	6	0.42	6	0.16
Gain of CH2	C49H97NO5	780.744	12.54	5	0.17	1	1.37	7	0.07
Loss of C4H8O	C44H87NO4	694.670	10.60	6	0.12	11	0.10	-	-
Desaturation	C48H93NO5	764.712	11.60	7	0.03	9	0.20	8	0.16
Loss of C22H42O2	C26H53NO3	428.410	2.41	8	0.03	10	0.12	10	0.01
Loss of CH2	C47H93NO5	752.712	11.59	-	-	3	0.56	9	0.04
Loss of CH2	C47H93NO5	752.712	11.96	-	-	4	0.56	2	0.75
Loss of 4 CH2	C44H87NO5	710.665	9.97	-	-	-	-	5	0.30
Loss of CH2	C47H93NO5	752.713	12.21	-	-	8	0.22	11	0.01

Confirming the structure of selected ALC-0315 impurities

Confirming the structure of identified impurities relies on information attained by the MS/MS data. EAD can provide an in-depth characterization of singly charged ionizable lipids.[4,5] The Molecule Profiler software interpretation workspace capitalizes on the information-rich MS/MS spectrum to facilitate the automatic structural elucidation and localization of chemical alteration sites.

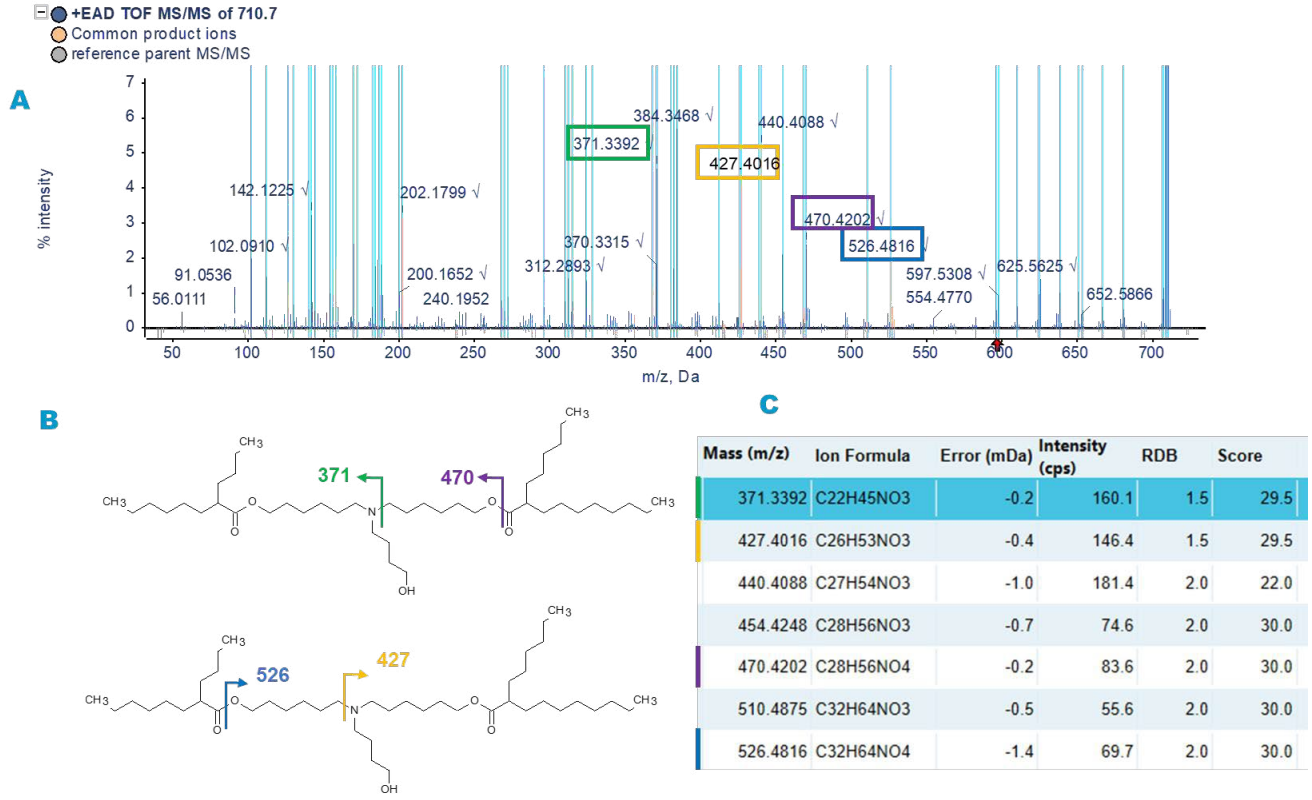
N-oxidation of ionizable lipids can lead to covalent modification of ribonucleotides and a loss of mRNA potency.[1] A peak observed in the samples from the 3 vendors at RT 11.0 min with an *m/z* value of 782.7 was identified by Molecule Profiler software as an oxidized version of the main peak. This peak was assigned a structure with oxidation on the N of the head group (N-oxide ion) based on the EAD data (Figure 3A). The presence of diagnostic ions at m/z 187.2, 443.4 and 544.5 support the oxygen incorporation into the N of the head group. Figures 3B and 3C show the representative diagnostic fragment ions and their corresponding structures.

A unique peak at RT ~10 min with m/z of 710.7 was observed only in the sample from Vendor 3. An information-rich EAD MS/MS spectrum was acquired on the ZenoTOF 7600 system, despite its low 0.3% abundance in the 10000-fold diluted sample. Molecule Profiler software assigned the structure of the impurity as the loss of 2 ethyl groups from the side chain of ALC-0315. Figure 4 shows the EAD-MS/MS spectrum and the structural elucidation of the impurity. Diagnostic fragment ions with m/z of 470.4 and 371.3 suggest the probable loss of 4 CH2 groups from one of the side chains, with 2 CH2 on each of the alkyl chain branches. Fragment ions with *m/z* values 427.4 and 526.5 suggest that the other side of the chain remained intact (Figure 4).



Mass (m/z)	Ion Formula	Error (mDa)	Intensity (cps)	RDB	Proposed Structures	Score
186.1491	C10H20NO2	0.3	28.1	2.0	8	28.0
187.1569	C10H21NO2	0.2	284.2	1.5	8	27.5
443.3967	C26H53NO4	-0.2	418.9	1.5	18	29.5
450.4295	C29H56NO2	-1.1	30.1	3.0	4	18.5
456.4050	C27H54NO4	0.3	117.6	2.0	18	22.0
542.4776	C32H64NO5	-0.3	115.5	2.0	2	30.0
544.4941	C32H66NO5	0.6	38.2	1.0	2	30.5

Figure 3. Structural elucidation of the oxidation site of ALC-0315 by Molecule Profiler software. A) The EAD-based MS/MS spectrum of the oxidized impurity. When a peak was assigned to a proposed structure or formula, the peak was highlighted in light blue. B) The diagnostic fragment ions pinpointing the oxidation to the N of the headgroup. C) Selected fragment ions corresponding to the assigned peaks in panel A.

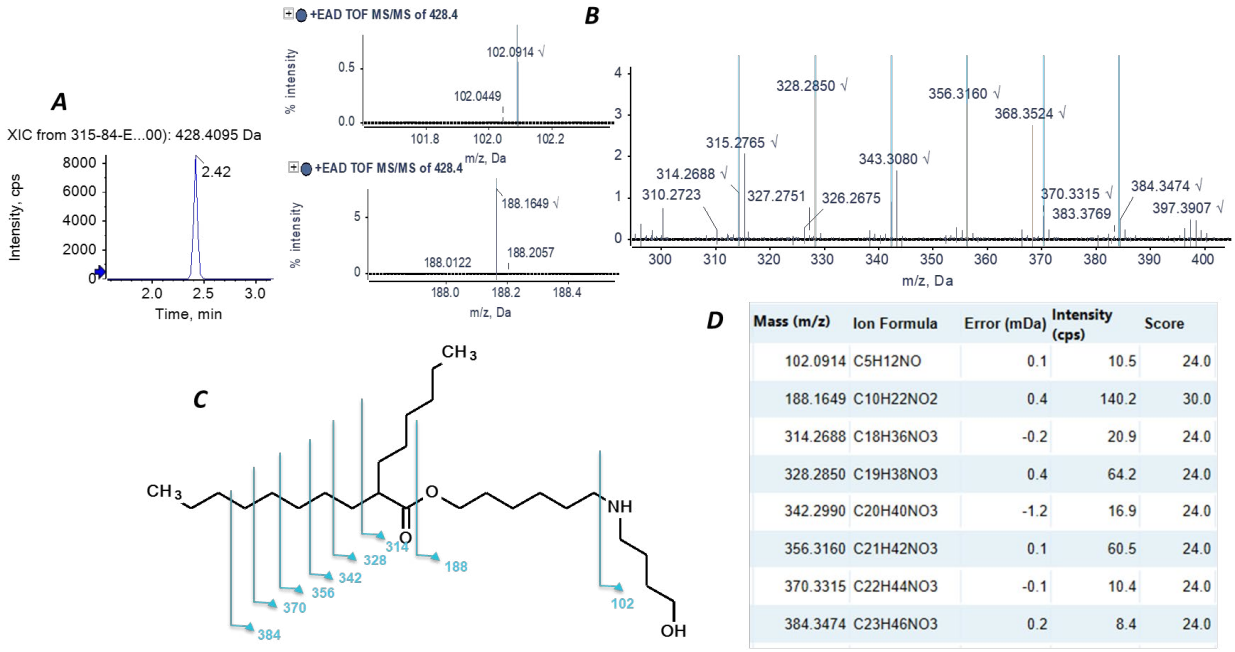


Mass (m/z)	Ion Formula	Error (mDa)	Intensity (cps)	RDB	Score
371.3392	C22H45NO3	-0.2	160.1	1.5	29.5
427.4016	C26H53NO3	-0.4	146.4	1.5	29.5
440.4088	C27H54NO3	-1.0	181.4	2.0	22.0
454.4248	C28H56NO3	-0.7	74.6	2.0	30.0
470.4202	C28H56NO4	-0.2	83.6	2.0	30.0
510.4875	C32H64NO3	-0.5	55.6	2.0	30.0
526.4816	C32H64NO4	-1.4	69.7	2.0	30.0

Figure 4. Structural elucidation by Molecule Profiler software of a distinct impurity with m/z of 710.6 found in the sample from Vendor 3. A) The EAD-based MS/MS spectrum of the impurity. B) The diagnostic fragment ions indicating the loss of 4 CH2 groups from one side of the chain. C) Selected fragment ions that correspond to the assigned peaks in panel A.

Molecule Profiler software elucidated the structure of low-abundance impurities with high confidence, leveraging the sensitivity gained by the Zeno trap and the information-rich EAD MS/MS spectra. Figure 5 shows the structural elucidation of an impurity (m/z = 428.4) with only 0.01% abundance in the sample from Vendor 3. In addition to the signature ions m/z values of 102.1 and 188.2 for definitive structural elucidation, EAD induced a series of C-C bond breaking, indicating the thorough structural analysis of singly charged ionizable lipids.

The workflow presented here offers a streamlined solution to characterize ionizable lipids and analyze lipid impurities. This workflow capitalizes on the detailed information obtained by EAD, in addition to the speed, sensitivity and wide dynamic range of the ZenoTOF 7600 system accompanied by the automatic structural elucidation capabilities of Molecule Profiler software. The workflow can be implemented for the routine analysis of ionizable lipids, quality control raw material testing and stability studies of LNP formulations. The data generated from this workflow can guide the design of safer, more efficient and more stable novel ionizable lipids to further unlock the potential of LNP-based therapeutics.



Mass (m/z)	Ion Formula	Error (mDa)	Intensity (cps)	Score
102.0914	C5H12NO	0.1	10.5	24.0
188.1649	C10H22NO2	0.4	140.2	30.0
314.2688	C18H36NO3	-0.2	20.9	24.0
328.2850	C19H38NO3	0.4	64.2	24.0
342.2990	C20H40NO3	-1.2	16.9	24.0
356.3160	C21H42NO3	0.1	60.5	24.0
370.3315	C22H44NO3	-0.1	10.4	24.0
384.3474	C23H46NO3	0.2	8.4	24.0

Figure 5. Structural elucidation of the low-abundance impurity with m/z 428.4 in the sample from Vendor 3. A) The XIC at m/z = 428.4095. The impurity that eluted at 2.41 min was analyzed in detail. B) The EAD-based MS/MS spectrum of the impurity. When a peak was assigned to a proposed structure or formula, the peak was highlighted in light blue. C) The structure of the impurity. D) Selected fragment ions corresponding to the assigned peaks in panel B

Conclusions

- Sensitive detection and confident identification of impurities in ionizable lipid ALC-0315 from 3 different suppliers were achieved using information-rich MS/MS spectra generated by EAD on the ZenoTOF 7600 system
- Molecule Profiler software assigned the TOF MS/MS spectral peaks produced by the ZenoTOF 7600 system, identifying diagnostic product ions and elucidating the structure of ALC-0315 and related impurities
- The ZenoTOF 7600 system and Molecule Profiler software increased confidence in LNP product effectiveness and safety by profiling lipid raw materials and monitoring unwanted impurities such as N-oxide derivatives

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Efficient transfer of the high-speed CE-SDS method from the PA 800 Plus system to the BioPhase 8800 system

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This technical note introduces a new capillary electrophoresis sodium dodecyl sulfate (CE-SDS) method on the BioPhase 8800 system, equivalent to the gold standard high-speed (HS) method on the PA 800 Plus system. The new method enables a seamless method transfer from the PA 800 Plus system to the BioPhase 8800 system, offering higher throughput with 8 parallel capillaries(Figure 1).

The introduction of the PA 800 Plus system and the IgG Heterogeneity and Purity Assay has made the CE-SDS method an essential analytical tool for characterizing therapeutic biomolecules. In the fast-paced environment of process development and support, where thousands of samples are screened annually, there is a demand for quick, reliable sample analysis method that can be efficiently transferred throughout the pipeline. To support this demand for even higher throughput, the IgG HS method is easily transferrable from the PA 800 Plus system to the 8-capillary BioPhase 8800 system.

Key features of the HS CE-SDS method

- **Seamless transition from the PA 800 plus system to the BioPhase 8800 system:** By taking advantage of the flexibility of the hardware settings on BioPhase software, an equivalent IgG HS CE-SDS method was developed
- **Same outstanding performance:** Consistency between runs and cartridges demonstrates the method's reproducibility. Inter-capillary reproducibility over 96 samples is <5% for light chain (LC), glycosylated (HC) and non-glycosylated heavy chains (ng-HC).
- **Similar migration time as original PA 800 Plus method**: Migration time of the 10 kDa marker on the BioPhase 8800 system high-speed method is within 1 minute (min) window compared to the migration time on the PA 800 Plus system.
- **The MW estimation** for lysozyme and carbonic andrydrase was better than 16%.

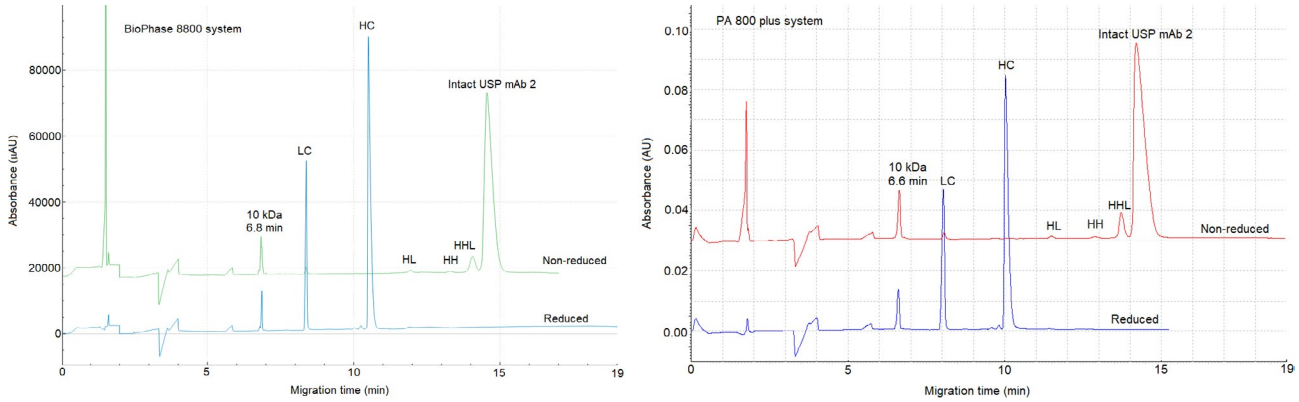

Figure 1: Separation of USP mAb 002 under reducing and non-reducing conditions on the BioPhase 8000 system (left) and on PA 800 Plus (right) using the HS CE-SDS method. Separation conditions are in the methods section. Migration time of 10 kDa marker on the BioPhase 8800 system using the HS CE-SDS method is within 1 min window compared to the migration time on the PA 800 Plus system.

Introduction

CE-SDS is widely used in biotherapeutic analyses for lot release, stability testing, formulation-buffer screening, process development, cell line development and product characterization. There are 2 versions of the CE-SDS separation method available on the PA 800 Plus system: the IgG high-resolution (IgG HR) and IgG HS. The IgG HR method was created for the high resolution between the ng-HC and the HC. Both methods have been adopted, validated, transferred and used in analytical development and quality control for numerous commercial molecules. However, only the IgG HR separation method was previously available on the BioPhase 8800 system. The IgG HS separation method on the PA 800 Plus system was created to reduce the total analysis time by 15 minutes, without compromising the data quality compared to the IgG HR method. The effective separation length is the fundamental difference between the IgG HR and HS methods. Effective length is the distance the molecule travels from introduction to detection. In the IgG HS method, the effective length is 10 cm. This distance is much shorter than the traditional IgG HR, where the effective length is 20 cm with a 30 – 40 min separation time. Consequently, the separation is considerably quicker.

This study proposes a new CE-SDS-based method for the BioPhase 8800 system that matches the performance of the IgG HS CE-SDS method on the PA 800 Plus system.

Methods

Samples: USP mAb 002, Monoclonal IgG1 - United States Pharmacopeia (USP) Reference Standard (P/N: 1445547-2MG) was from USP (Rockville, MD). NISTmAb, Humanized IgG1k Monoclonal Antibody (P/N: 8671) was from NIST (Gaithersburg, MD). IgG control standard (P/N: 391734) was from SCIEX (Framingham, MA).

Reagents: The BioPhase CE-SDS Protein Analysis Kit (P/N: C30085) was from SCIEX. The β-mercaptoethanol (β -ME) (P/N: M3148-25ML) and iodoacetamide (IAM) (P/N: I6125) were from Sigma-Aldrich (St. Louis, MO).

Capillary cartridges: For the BioPhase 8800 system, the CE-SDS analysis was performed using a BFS capillary cartridge – 8 x 30 cm (P/N: 5080121) from SCIEX. For the PA 800 Plus system, the CE-SDS analysis was performed using a BFS pre-assembled capillary cartridge (P/N: A55625) from SCIEX.

Capillary electrophoresis instrument and consumables: The BioPhase 8800 system (P/N: 5083590F) and a starter kit of 4 sample plates, 4 reagent plates and 8 outlet plates (P/N: 5080311) were from SCIEX. CE-SDS analysis was performed using UV detection at 220 nm.

The PA 800 Plus system (P/N: A51963AE) was equipped with a PDA detector. The detection wavelength was 220 nm.

Instrument methods: On the PA 800 Plus system, the capillary conditioning method comprises 0.1M NaOH rinse at 20 psi for 10 min, 0.1M HCl rinse at 20 psi for 5 min, ddi H2O rinse at 20 psi for 2 min,SDS-MW gel buffer rinse for 10 min at 70 psi and a voltage separation at 15 kV for 10 min. The separation method consists of 0.1M NaOH rinse at 70 psi for 3 min, 0.1M HCl rinse at 70 psi for 1 min, ddi H2O rinse at 70 psi for 1 min and SDS-MW gel buffer rinse for 10 min at 70 psi, 2 water dips, an injection at 5 kV for 20 seconds, and the separation at 15 kV for 20 min

The separation method used on the BioPhase 8800 system is shown in Figure 2.

Software: BioPhase software, version 1.2 e-license, was used to create methods and sequences for data acquisition and data analysis on the BioPhase 8800 system. The 32 Karat (version 10.1) software package was used for data acquisition and processing.

Method Duration: 34.3 min. Number of Actions: 10

Settings	Capillary Cartridge: 18.0 °C, Wait Capillary Length: 30.0 cm Capillary Type: Bare Fused Silica Current Limit: 600 µA , Enabled		Sample Storage: 25.0 °C, Wait Detector Type: UV, 220 nm, Wait Peak Width: 8 sec. Data Rate: 2 Hz
Rinse	Duration: 2.0 min. 80.0 psi		Inlet: Basic Wash Outlet: Waste
Rinse	Duration: 2.0 min. 50.0 psi		Inlet: Acid Wash Outlet: Waste
Rinse	Duration: 1.0 min. 50.0 psi		Inlet: Water Rinse Outlet: Waste
Rinse	Duration: 4.0 min. 80.0 psi		Inlet: CE-SDS Gel Buffer Rinse Outlet: Waste
Wait	Duration: 0.0 min.		Inlet: Water Dip 1 Outlet: Water Dip
Wait	Duration: 0.0 min.		Inlet: Water Dip 2 Outlet: Water Dip
Inject	Duration: 20 sec. -5.0 kV	Plate: Sample	Outlet: CE-SDS Gel Buffer Inj
Wait	Duration: 0.0 min.		Inlet: Water Dip 3 Outlet: Water Dip
Separate	Duration: 25.0 min. -30.0 kV, 20.0 psi, Both Ramp Time: 0.5 min. Autozero: 2.0 min.		Inlet: CE-SDS Gel Buffer Sep Outlet: CE-SDS Gel Buffer Sep
Wait	Duration: 0.0 min.		Inlet: Water Dip 1 Outlet: Water Dip

Figure 2: Proposed HS CE-SDS separation method for the BioPhase 8800 system.

Sample preparation for CE-SDS analysis under reducing and non-reducing conditions: The USP mA 2 IgG sample was used without any previous treatment. 10 µL of the USP mAb 2 IgG sample was mixed with 2 µL of 10 kDa marker, 83 µL of SDS sample buffer pH 9 and 5 µL of β -ME or 250mM IAM for reduced and non-reduced conditions, respectively. Then, the mixture was heat-denatured at 70°C for 10 min before CE analysis.

Results and discussion

Selecting 10 kDa as the internal mobility marker and defining a migration time window for 10 kDa

In CE, using an internal mobility marker is crucial. This is due to the consistent relative position between the marker and the sample peaks, which guarantees reproducibility for different runs, capillaries, chemistry lots, instruments and users.

The first step in developing the method was to set a migration time requirement. Although, any peak could be used as a marker, the migration time for a mAb molecule peak under reduced or non-reduced conditions can vary due to differences in the antibody hydrodynamic size and primary sequence. These differences can affect the hydrophobicity of the mAb. Consequently, using a mAb peak as a reference can impact assay accuracy and reproducibility, as it may change from molecule to molecule, making the method non-viable as a platform method.[1] Figure 3 illustrates the difference between

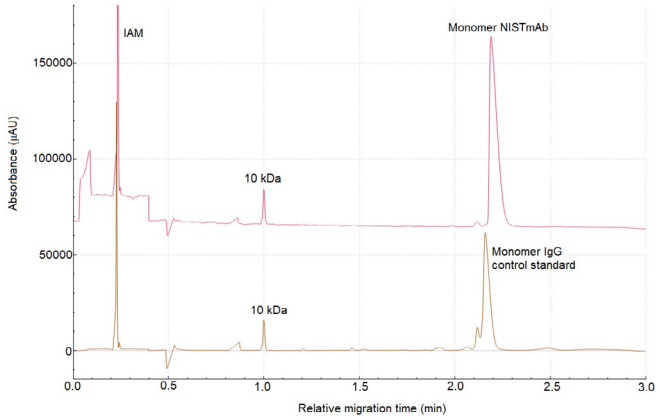

Figure 3: Migration time difference of 2 different mAbs under non-reducing conditions. The top trace is from NISTmAb, and the bottom trace is from the IgG control standard under non-reducing conditions using the IgG standard method on the BioPhase 8800 system (-15 kV/25°C).

the migration time of the monomer peak for NISTmAb and SCIEX's IgG control standard mAbs.

Defining a migration time window for the 10 kDa internal marker is more reliable than using a mAb peak, as this molecule exhibits a consistent migration pattern regardless of the antibody sample and the sample preparation conditions (reducing and non-reducing). Figure 1 shows that the migration time of the 10 kDa internal marker using the IgG HS method was around 6.6 minutes on the PA 800 Plus system under reducing and non-reducing conditions. Therefore, the 10 kDa molecule was used as a marker to investigate other instrument settings in this study. Arbitrarily, a requirement for the migration time of the 10 kDa on the BioPhase 8800 system was set at 6.6? +/- 1 min. Migration time of 10 kDa marker on the BioPhase 8800 system using the HS CE-SDS method was within 1 min window compared to the migration time on the PA 800 Plus system, demonstrating excellent data consistency between the two systems.

Investigating the separation temperature and electric field strength

It is well known that 3 main factors can impact the separation time: capillary length, electric field (voltage), and separation temperature.[2]

On the BioPhase 8800 system, the total capillary length is fixed at 30 cm with 20 cm of effective length. Thus, the electric field and separation temperature are the only variables that can be adjusted in a method.

Figure 4 shows the separation of reduced USP mAb 002 under various voltages and temperature conditions.

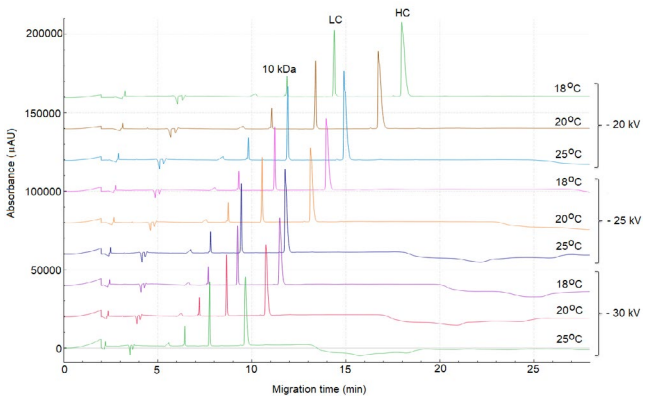

Figure 4: Overlay of the CE-SDS separation traces of USP mAb 002 at various separation voltages and temperatures.

As expected, the higher the electric field and temperature, the faster the migration time. However, as the peaks of interest migrated faster, the baseline fluctuation from the separation

gel buffer became more significant and could potentially interfere with peak integration. The background signal from the separation gel buffer was influenced by temperature and electric field. Figure 4 shows that the baseline noise was less significant at lower temperatures, i.e., 18°C, even at higher electric fields.

Even though the baseline interference was mitigated at 18°C, the migration time of the 10 kDa marker at the highest electric field (-30 kV) was 7.69 min, which falls outside the target range of 6.5 +/- 1 min. Therefore, further method optimization was carried out as described below.

Optimizing separation ramp time

Another method setting that is often overlooked is the ramp time. Ramp time is when the instrument's high voltage power supply goes from 0 kV to the set separation voltage. The ramp time setting is crucial because it directly impacts the reproducibility of the separation. When the voltage is applied, the electrode generates a sudden heat wave that propagates to the surrounding areas, affecting the capillary.[3-4] The temperature gradient may induce dispersion and reduce separation efficiency. However, theoretical models of the thermal gradient, as explained by Guiochon et al.,[5] indicate that this effect is negligible for capillaries with an inner diameter of 100 µm. The inner diameter of the capillary used in this application was only 50 µm.

Typically, in CE-SDS-based separation, the BioPhase 8800 and PA 800 Plus systems have a pre-set ramp time of 2 minutes. Figure 5 shows the influence of the ramp time on the separation of USP mAb 002 under reducing conditions. In the lower trace with a ramp time of 2 min, the 10 kDa marker migrated at 7.6 min. The upper trace shows a separation using a ramp time of 0.5 min, where the 10 kDa marker migrated at 6.8 min, which falls within the required migration time window. Therefore, 0.5 min was selected as the optimal ramp time. The final optimized HS CE-SDS method comprised an 18°C separation temperature and a-30 kV separation voltage with 0.5 min ramp time.

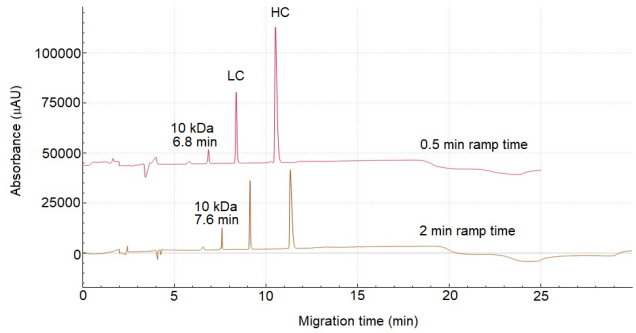

Figure 5: Overlay of electropherograms obtained with NISTmAb and USP mAb002 under non-reducing conditions using the IgG standard method from PA 800 Plus system (-30 kV/18°C).

Reproducibility of the method

To ensure the decrease in ramp time did not compromise the method's reproducibility, a discrete precision study was conducted using 2 cartridges. Figure 6 shows the average migration time values for the 10 kDa marker obtained from 2 cartridges. The error bar is the standard deviation from 96 separations per cartridge. The RSD for the 10 kDa migration time with both cartridges was better than 1.6%, demonstrating excellent assay reproducibility.

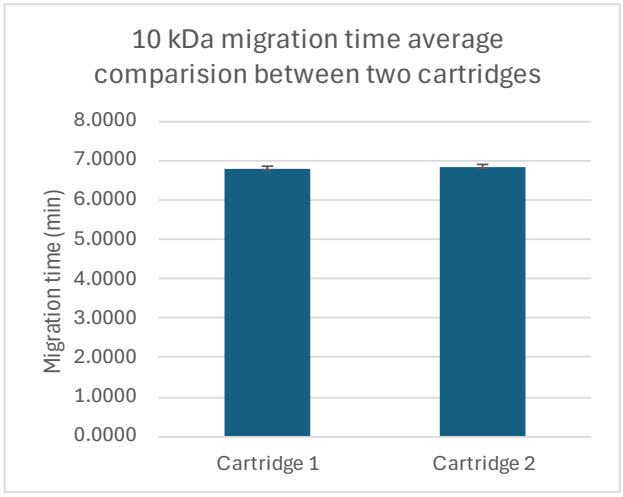

Figure 6: Average migration time values for the 10 kDa marker obtained from 2 cartridges with 96 samples per cartridge.

This method was also tested with the intact USP mAb under non-reducing conditions, and the CPA %RSD was 4.3%, indicating high assay reproducibility.

Molecular weight estimation

A simple experiment was conducted to estimate the molecular weight of carbonic anhydrase and lysozyme, aiming to determine whether the change in separation temperature adversely affected the estimation. Figure 7 shows an overlay of the molecular weight size standards (bottom, blue trace), lysozyme (middle, green trace) and carbonic anhydrase (top, red trace). The X-axis represents the molecular weight, while the Y-axis shows absorbance units.Table 1 presents the

corrected peak area average and the percentage of relative standard deviation for the LC, HC and ng-HC of the USP mAb 002 reference standard under reducing conditions. The consistency of these values, with a %RSD of less than 5% for both cartridges demonstrates the excellent reproducibility of the method.

Table 1. Average values and reproducibility of USP mAb 002 under reducing conditions

cartridge	Average values for corrected peak area		
	LC	ng-HC	HC
#1	7746.29	120.73	19107.87
#2	7105.25	110.04	17447.68
cartridge	%RSD for corrected peak area		
	LC	ng-HC	HC
#1	3.80	4.82	3.76
#2	3.41	4.76	3.26

Using the BioPhase software, the estimated molecular weight of lysozyme and carbonic anhydrase were determined to be 14.3 and 30.7 kDa, respectively, based on the separation of the molecular weight size ladder and point-to-point curve fitting. These values are in excellent agreement with the values listed in the UniProt database[6], with an accuracy value of 12% and 16% for lysozyme and carbonic anhydrase, respectively.

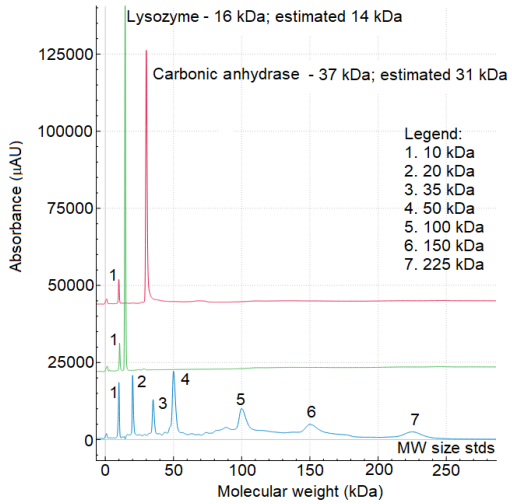

Figure 7: Overlay of electropherograms obtained with the molecular weight size standards (stds), lysozyme and carbonic anhydrase.

Conclusions

- Seamless transfer of the HS CE SDS method from the PA 800 Plus system to the BioPhase 8800 system with a higher throughput was accomplished
- Accurate molecular weight estimation with accuracy better than 16% was achieved
- Excellent assay reproducibility was demonstrated with %RSD of CPA < 5%
- A successful method transfer was achieved using 18 °C for separation temperature and 30 kV for separation voltage with a 0.5 min for ramp time

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Pharma MS

- Innovative drug metabolite identification and characterization using electron-activated dissociation
- Confident identification of phase 1 metabolites using electron-activated dissociation (EAD)
- Confident characterization and identification of glucuronide metabolites using diagnostic fragments from electron-activated dissociation (EAD)
- Comprehensive structural analysis of small molecules with heterocyclic rings
- Comprehensive characterization and preclinical assessment of an imidazopyridine-based anticancer lead molecule

Biopharma MS

- Detection and relative quantitation of host cell proteins in lentivirus preparations
- Approach for routine detection and quantitation of host cell proteins in NIST mAb using Zeno SWATH DIA
- Confident sequence analysis of a trispecific antibody using an electron-activated dissociation (EAD)-based middle-down workflow

Biopharma CE

- Multi-capillary gel electrophoresis to increase throughput for the purity analysis of biotherapeutic proteins
- Monitoring and determining the cause of antibody discoloration using capillary isoelectric focusing (cIEF) and electron-activated dissociation (EAD)
- Analytical characterization of the antibody drug conjugate Enhertu using multi-capillary electrophoresis

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