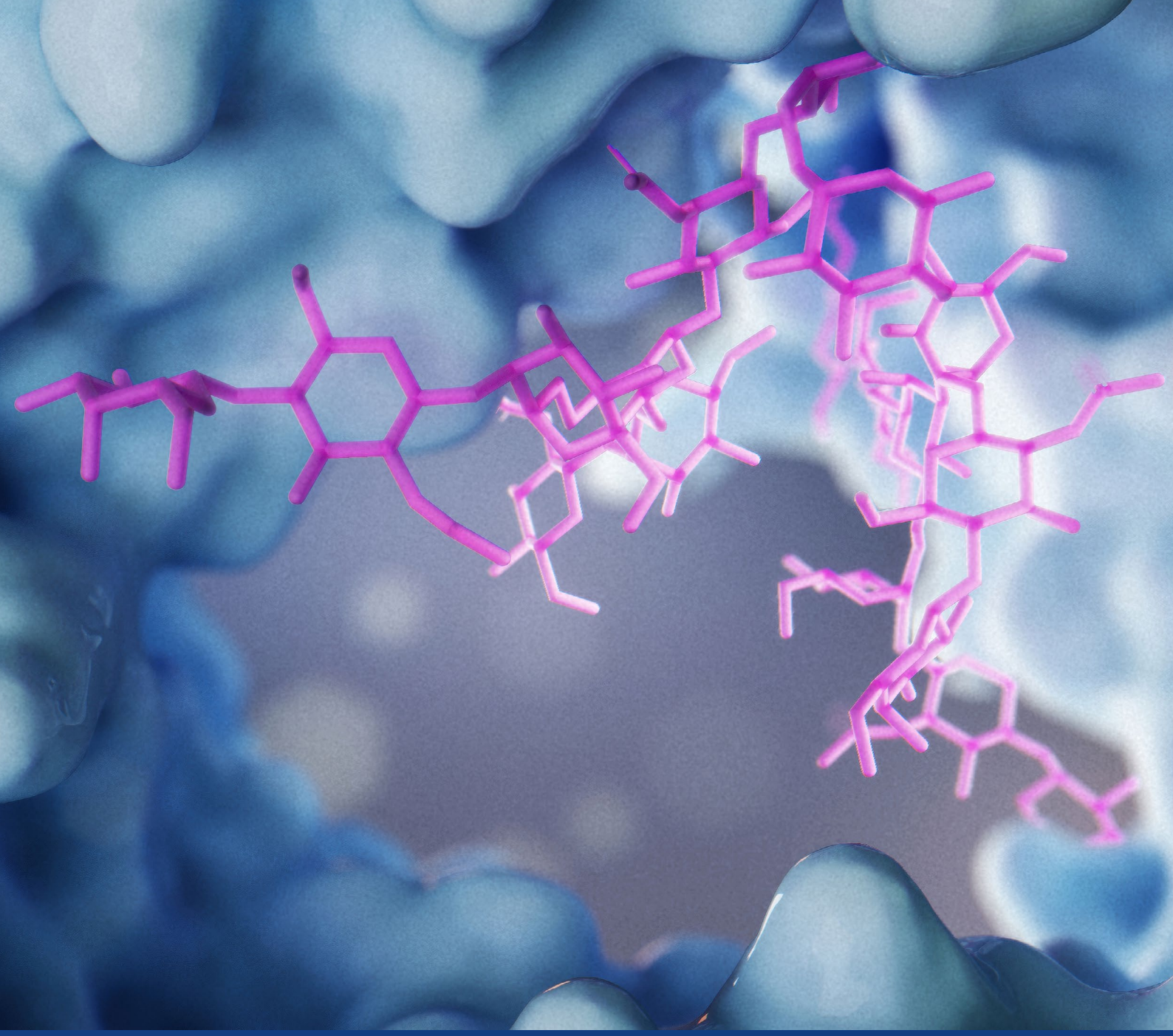

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Contents

Introduction

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 acetonitril[illegible]entrifuged, and supernatant was used for LC-MS/MS a[illegible]ysis.

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

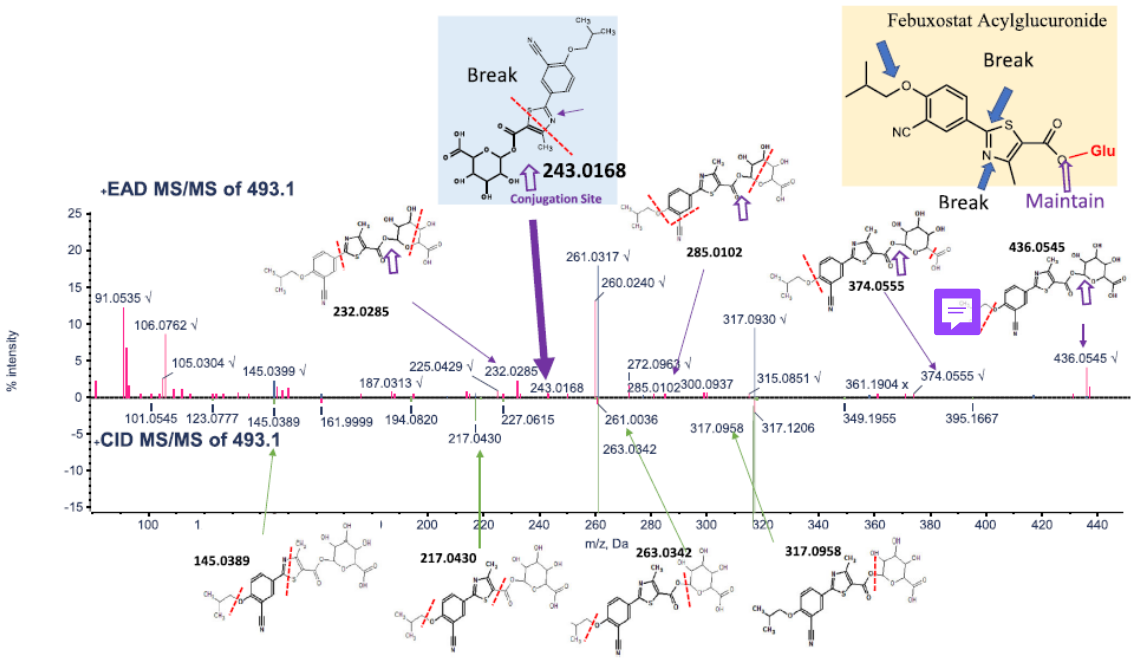
Results and Discussion

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



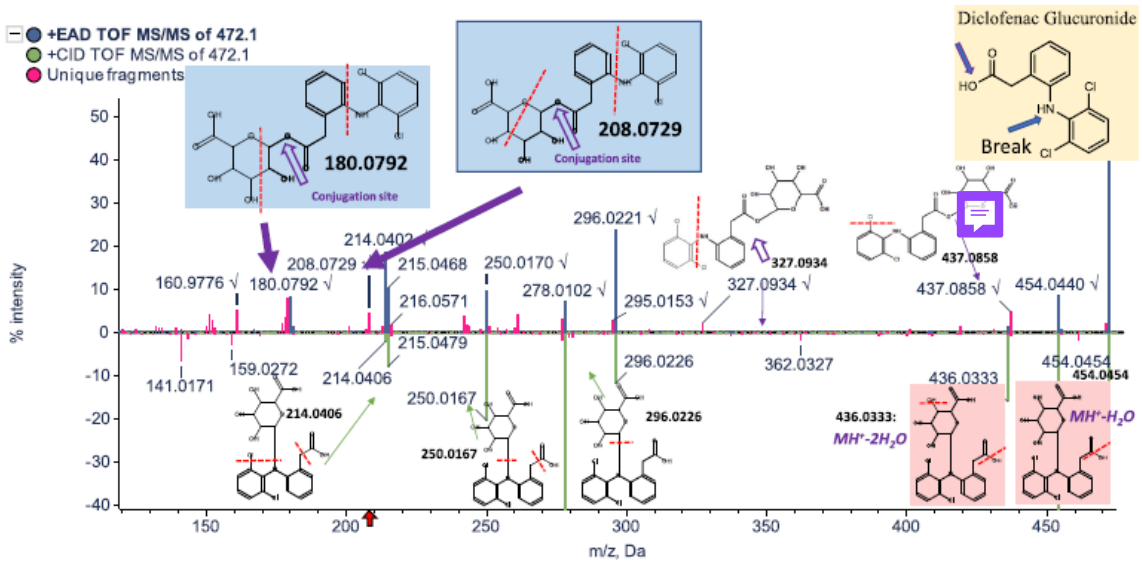
EAD vs. CID MS/MS fragmentation of Febuxostat glucuronide. CID (bottom) cleaves the weak glucuronidation bond early, making site identification challenging. EAD preserves the glucuronide partially or fully, producing distinct signature ions (magenta) that facilitate localization of the modification.

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.

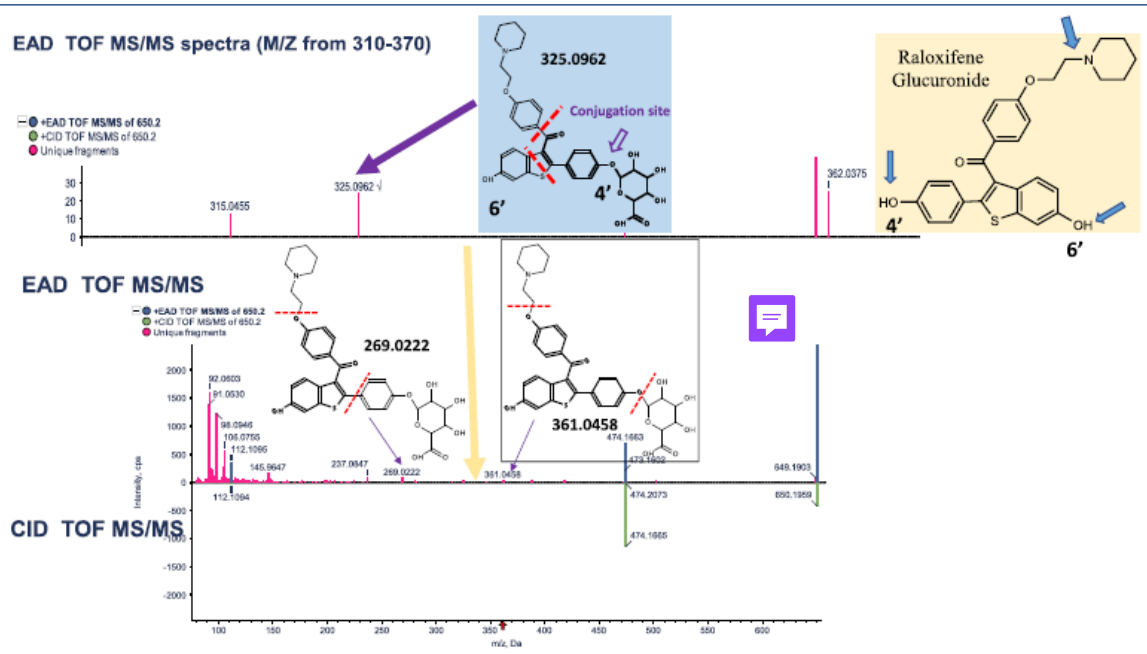
Raloxifene Glucuronidation and GSH Conjugation

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).

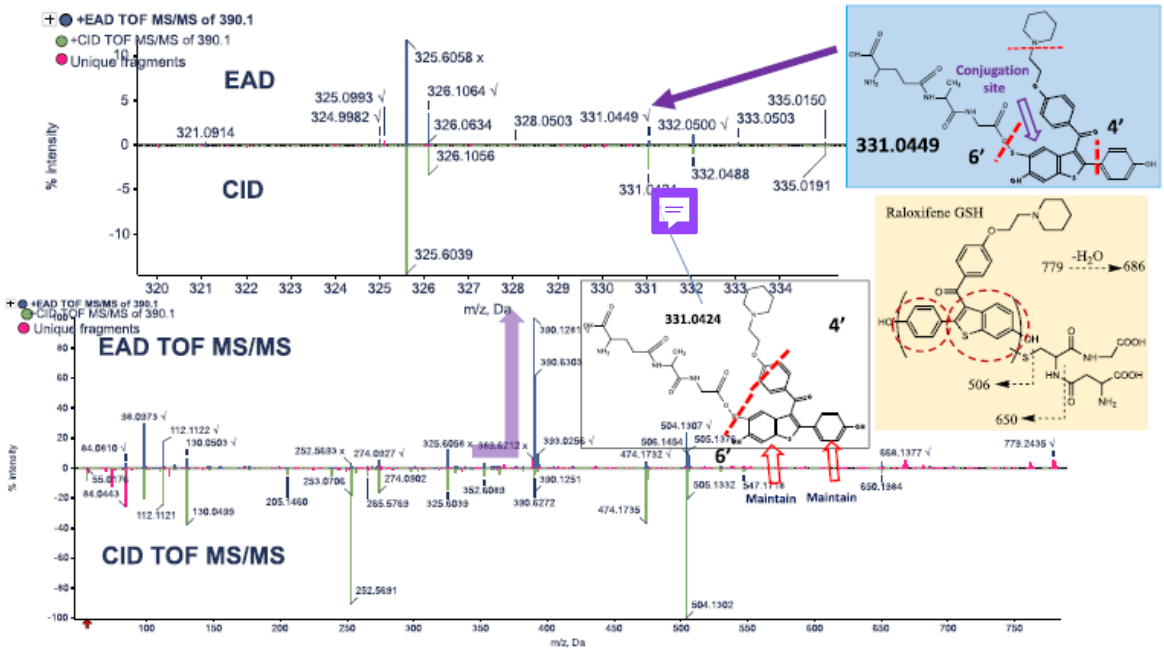
For the GSH conjugate (m/z 779.2), EAD analysis revealed a fragment (m/z 331.0449) that retained the benzo-thiophene ring and incorporated sulfur from GSH, indicating GSH conjugation on the benzo-thiophene ring, consistent with previously published NMR data (Figure 4).

Figure 3



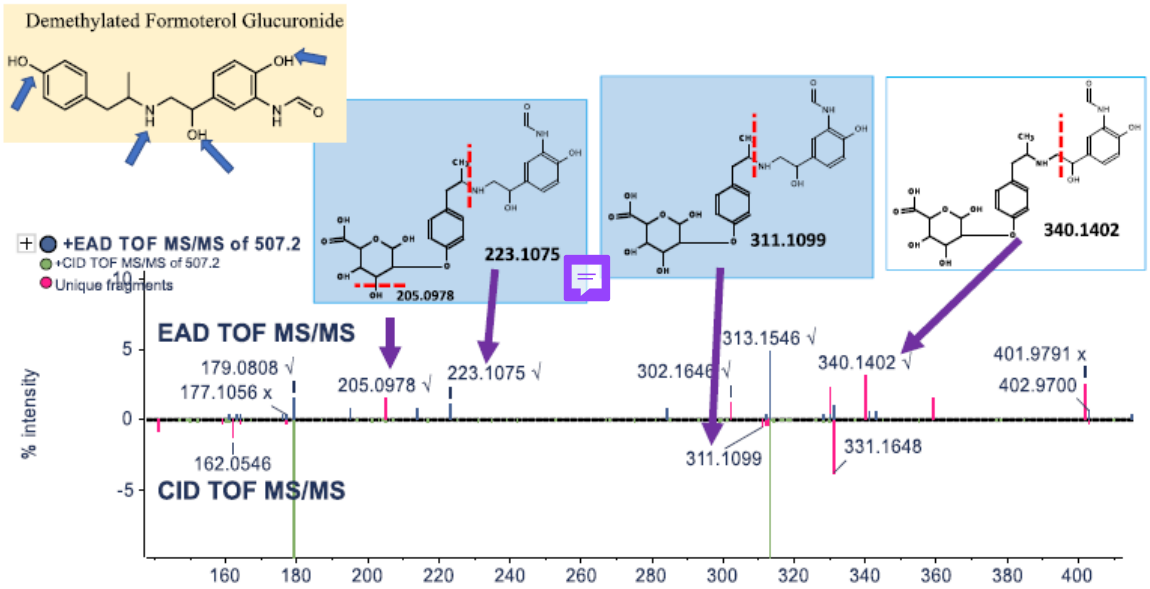
Structural characterization of Raloxifene-glucuronide via EAD and CID MS/MS. Two potential conjugation sites were identified, with a unique EAD fragment confirming glucuronidation at the 4'-OH of the phenol ring while preserving the glucuronide moiety.

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

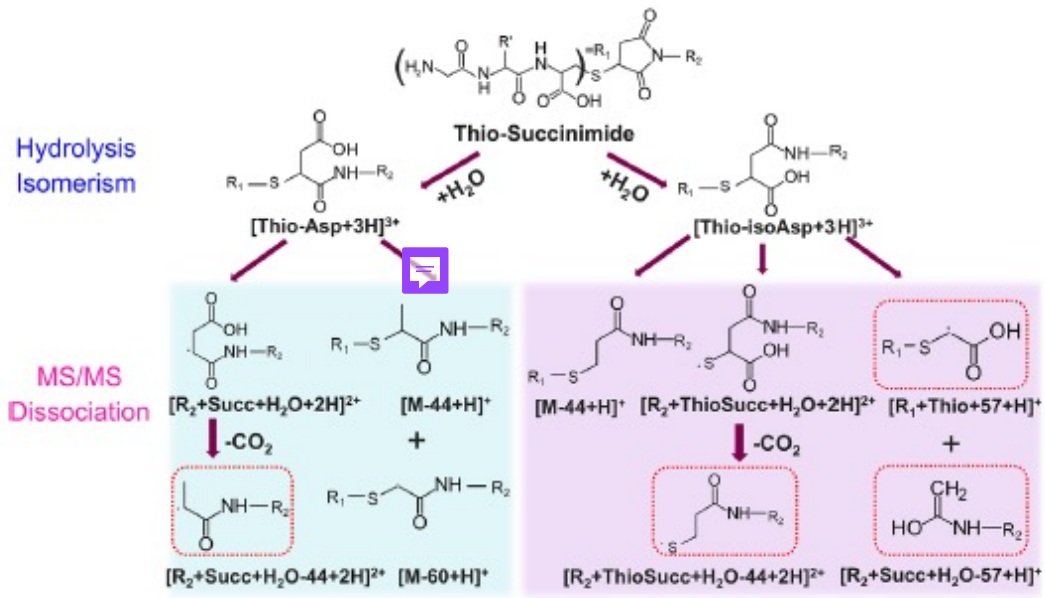
Tryptic digested peptides were separated on a C_{18} UPLC column. The ZenoTOF 7600 operated in full-scan MS mode (m/z 100-1500) with collision energy set at 10 V. For MS/MS, collision-induced dissociation (CID) used a collision energy of 40 V, while EAD employed a kinetic energy of 11 eV.

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

Figure 1

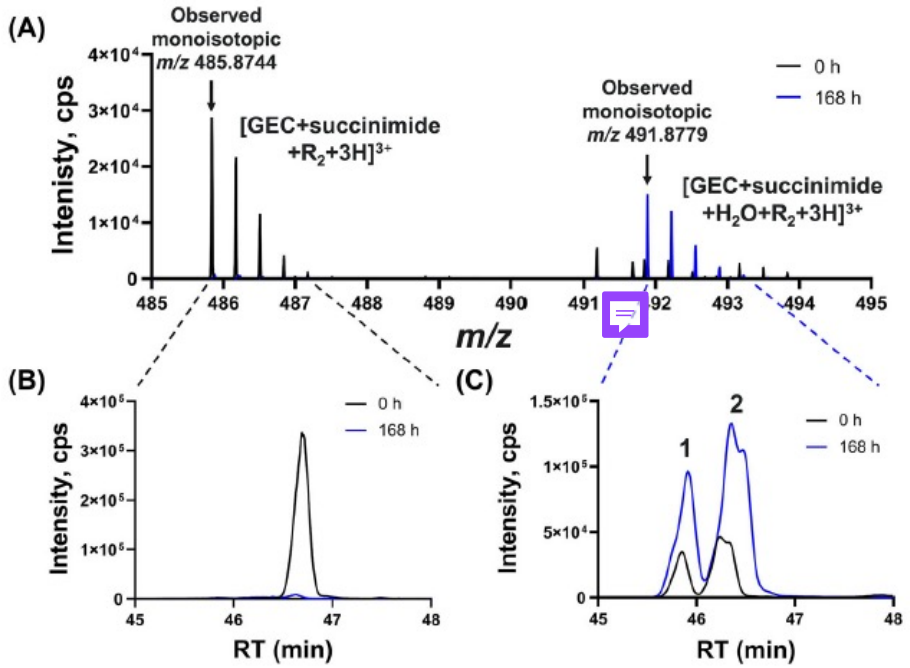


Scheme of thiosuccinimide hydrolysis mechanism and the proposed structures of product ions after electron-activated dissociation (EAD). Product ions in the red boxes are diagnostic ions with predominant charge state indicated. R_1 is a tripeptide GEC, and R_2 is linker-payload. The linker payload is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

(R_2) to demonstrate the hydrolysis process (Figure 1). 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):

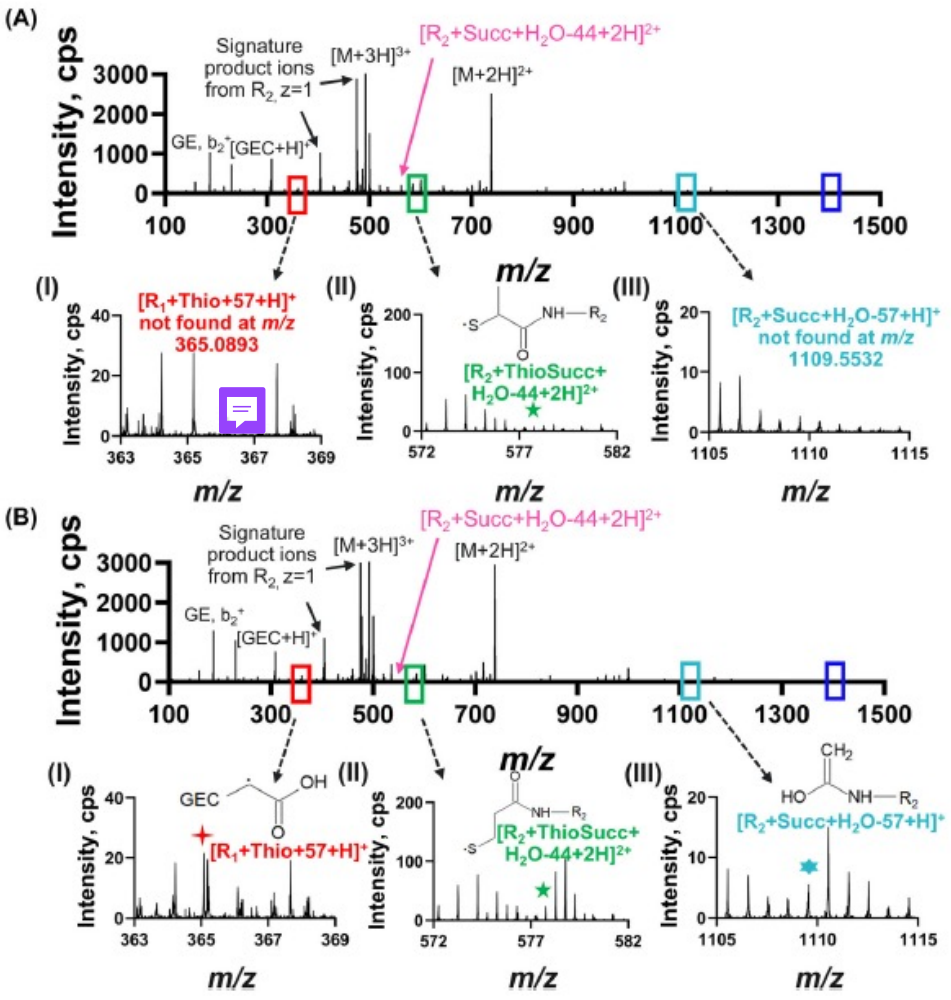
- A singly charged product ion with m/z 365.0839 was observed in Peak 2 but not in Peak 1, corresponding to $[R_1 + Thio + 57 + H]^+$ with a -15 ppm mass difference from theoretical.
- A singly charged product ion with m/z 1109.5403 was observed in Peak 2 but not in Peak 1, corresponding to $[R_2 + Succ + H_2O - 57 + H]^+$ 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 labelled 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

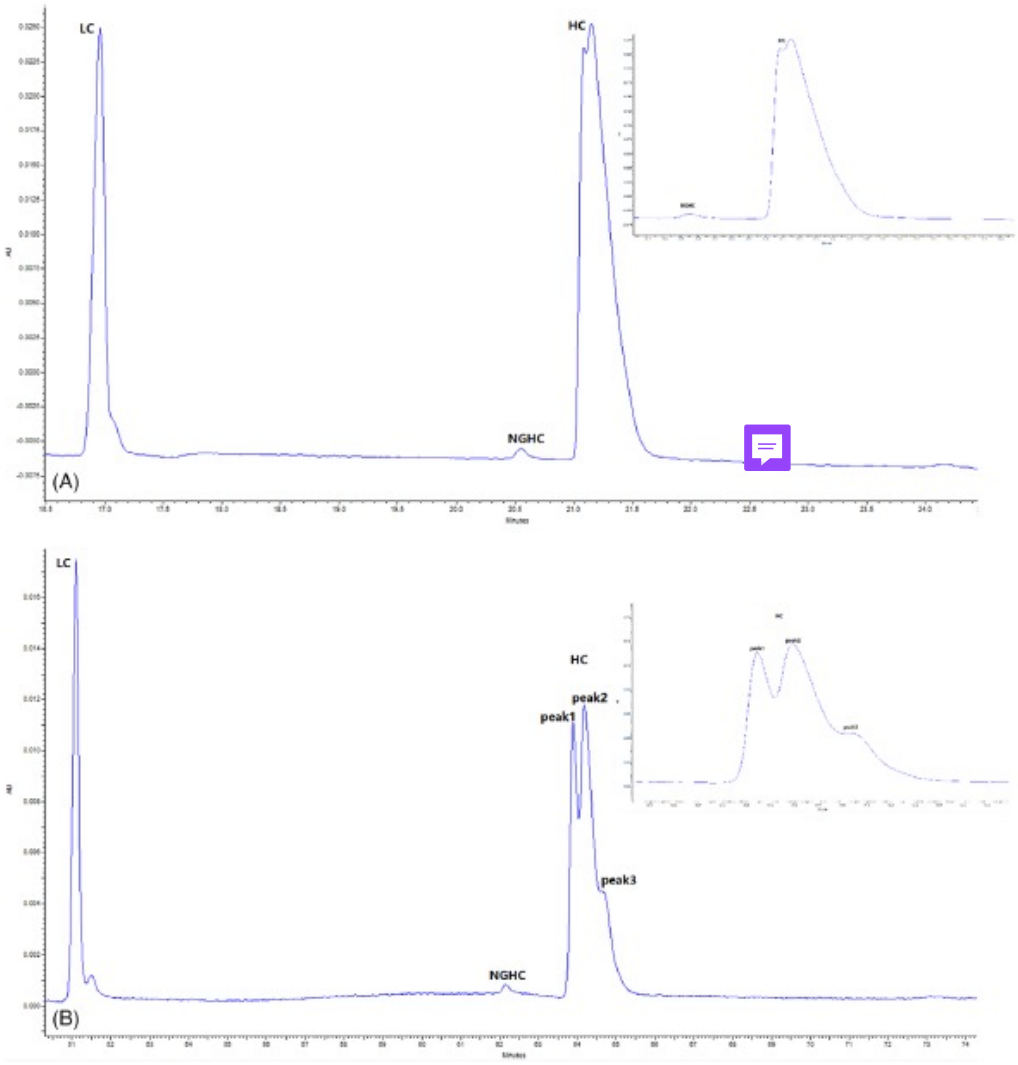
The glycosylated heavy chain contained three glycoforms (G0F, G1F, and G2F) as determined by MS analysis. With standard CE-SDS conditions (20-cm effective capillary length), the glycosylated heavy chain formed only two peaks (Figure 1). When the method was optimized using a 40-cm effective length capillary, 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

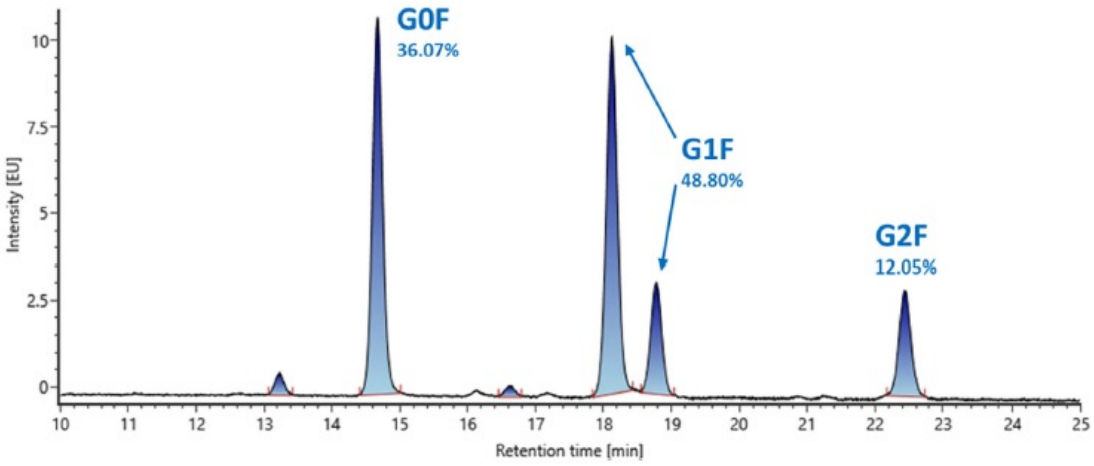
To prove that peaks 1, 2, and 3 were associated with glycosylation modifications, N-glycan cleavage was performed using PNGase F, and both peaks of the glycosylated heavy chain were completely transformed into a non-glycosylated heavy chain (Figure 3). Next, β1-4-galactosidase cleavage followed by reduced 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



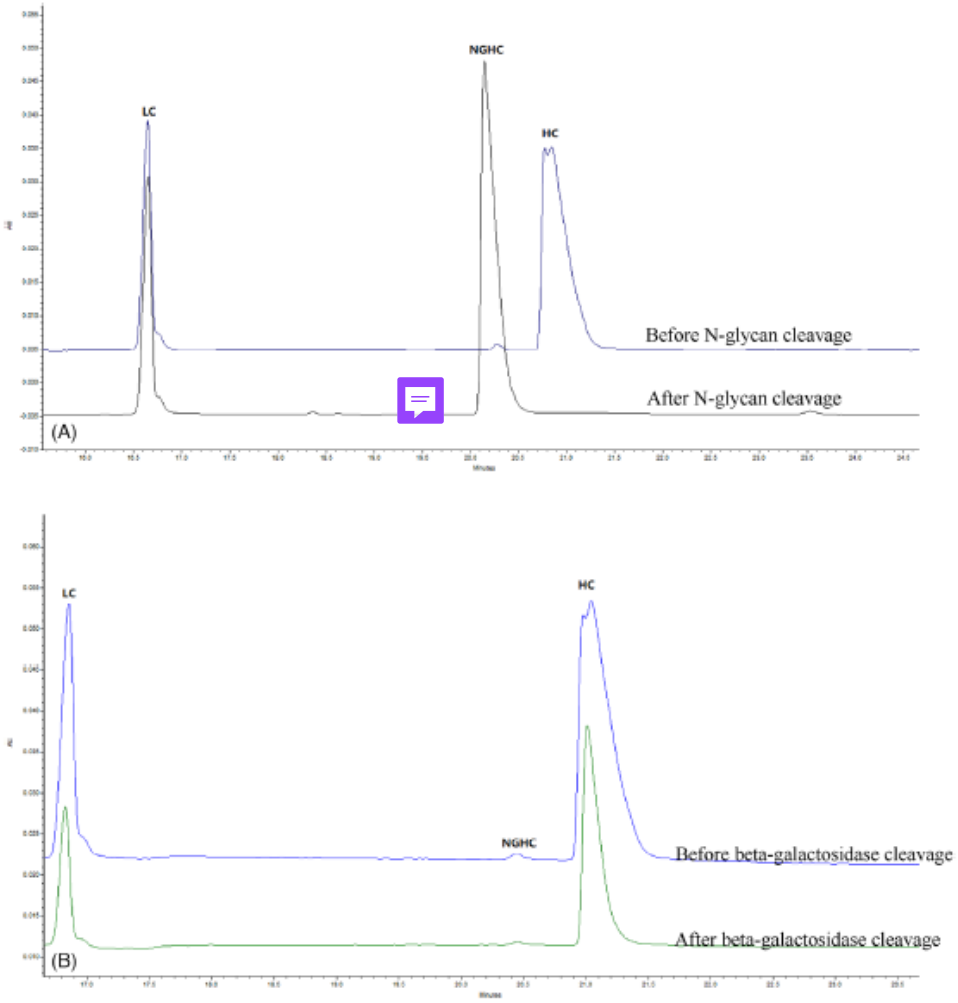
Reduced CE-SDS electropherograms of the anti-VEGFR-2 mAb. (A) Reduced CE-SDS separation was performed using a capillary with an effective length of 20 cm, and glycosylated heavy chains formed two peaks, with an enlarged view of glycosylated heavy chains shown in the upper right corner. (B) Reduced CE-SDS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

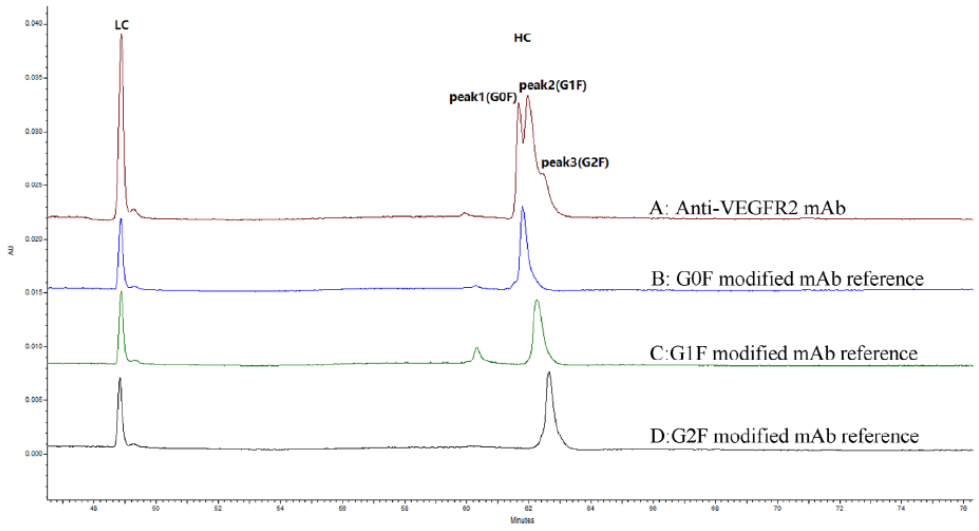
- Peak 1 was identified as G0F-modified heavy chain
- Peak 2 was identified as G1F-modified heavy chain
- Peak 3 was identified as G2F-modified heavy chain

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:

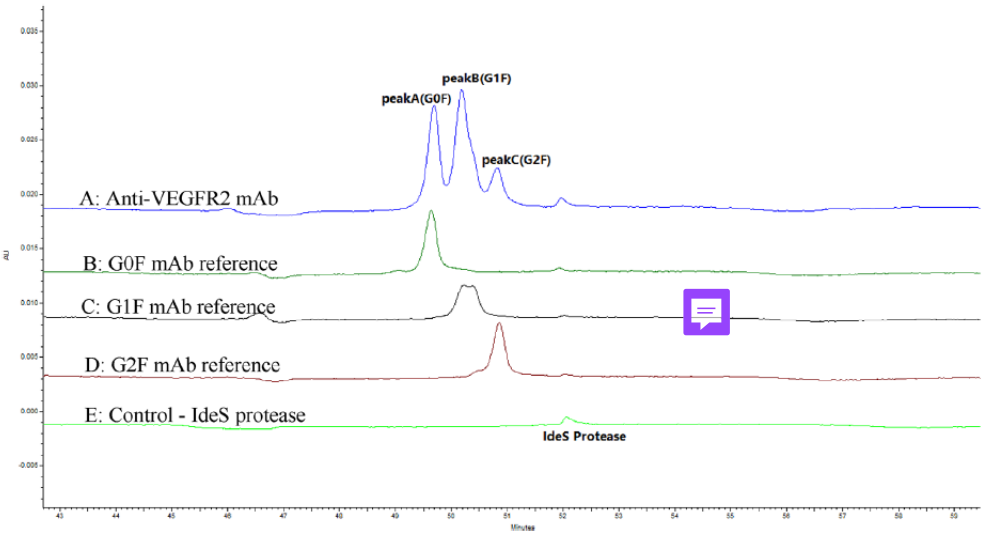
- G0F scFc: 25,231 Da (theoretical: 25,232 Da)
- G1F scFc: 25,393 Da (theoretical: 25,394 Da)
- G2F scFc: 25,555 Da (theoretical: 25,556 Da)

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.

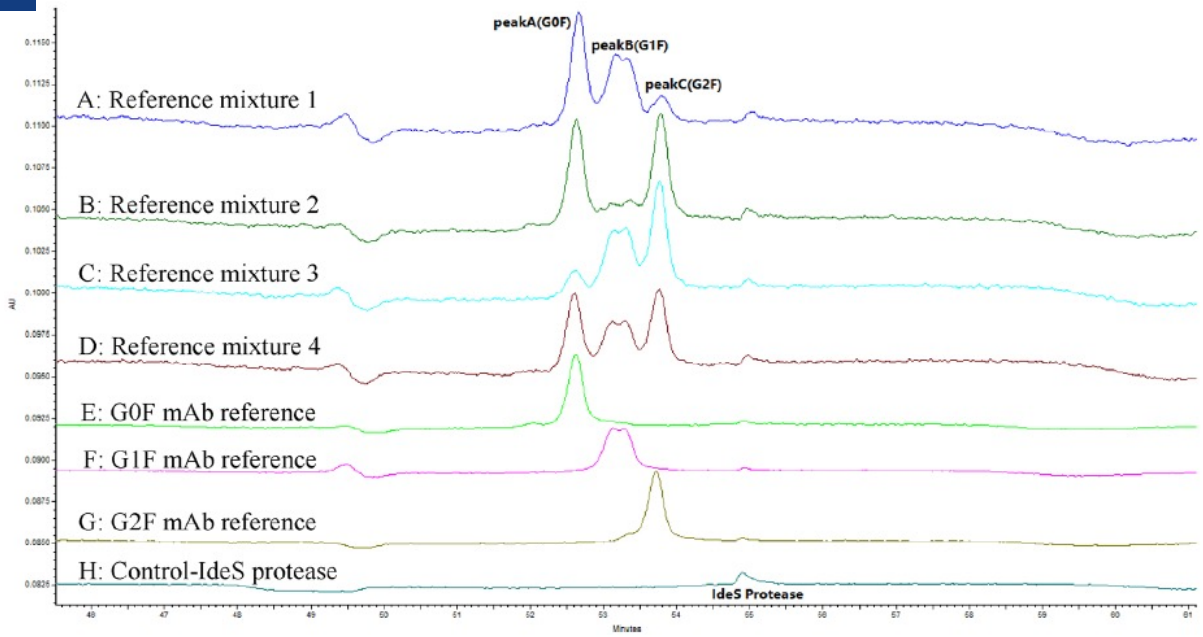
The separation of scFc fragments with different glycosylations showed much better resolution than heavy chains (resolution values for scFc fragments: 1.10 between peaks A-B and 1.35 between peaks B-C, compared to 0.51 and 0.18 for heavy chains). This allowed accurate identification (Figure 5):

- 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 application 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 of Strategic Marketing for Protein Therapeutics and Pharma at SCIEX, shares her extensive expertise on the integration of Mass Spectrometry (MS) and Capillary Electrophoresis (CE) in biopharmaceutical analysis. With over 15 years of research experience, Zoe discusses the complementary roles of MS and CE, the impact of recent technological advancements, and future trends shaping the industry. She delves into the challenges and benefits of these techniques, providing valuable insights from her work at SCIEX. Zoe highlights how MS and CE complement each other in biopharmaceutical development, offering high resolution and sensitivity for detecting biomolecules and detailed characterization of complex biomolecules. She also shares examples of integrated workflows, recent technological advancements, and future trends in the application of MS and CE. 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 when the company decided to expand its presence in the biopharma industry. 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 customers in the biopharma industry and building strong relationships that are essential for groundbreaking advancements. This experience has been highly rewarding.

In addition to close customer engagement, my team actively develops innovative workflows to ensure we stay at the forefront of cutting-edge methodologies. Each year, we deliver at least 15 high-quality technical notes, 3-4 publications with customers, and more than 20 conference presentations and posters in the biopharma field. We are also engaged in supporting next-generation instrumentation, software for instruments, and software launches.

How do MS and CE complement each other in biopharmaceutical analysis?

In biopharmaceutical analysis, MS and CE are complementary technologies and are both essential to the biopharmaceutical development process. CE offers high resolution and sensitivity for detecting biomolecules, while MS provides identification capabilities, allowing for the detailed characterization of complex biomolecules. CE is widely used throughout the entire biopharma pipeline, including in compliant environments for lot release assays, such as CE-SDS and ciEF. Although high-resolution mass spectrometry (HRMS) is more commonly used in research, discovery and development for biomolecule characterization, there is a noticeable trend of HRMS being adopted in quality control (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?

It happens frequently that the relative abundance of variant peaks in icIEF, the gold standard for charge variance analysis in biopharma, 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 because multiple rounds of fractionation are needed to generate sufficient samples for LCMS assays. After fractionation, verification is required to ensure that the collected sample accurately represents the peak of interest. During this process, undesired modifications can occur.

Recently, SCIEX launched a new icIEF-UV/MS platform that addresses this challenge. By maintaining the same icIEF separation, variant peaks can be directly detected and identified through HRMS system. This approach provides quick answers to questions, 30-minute separation, 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). However, I anticipate an increased integration of MS and MS, 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.

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, especially since CE-SDS can be automated. Additionally, compared 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. Moreover, CE-SDS is a much faster analysis and offers higher throughput.

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

In biopharmaceutical analysis using CE, scientists commonly face challenges such as improving throughput and accurately identifying different peaks. The upstream bioprocessing environment is fast-paced, with thousands of samples screened annually, leading to a high demand for 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.

To address this challenge, my 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.

Historically, charge variants are separated using icIEF, followed by offline fraction collection for 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 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 whitepaper 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 Strategic Marketing for Protein Therapeutics and Pharma 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. Since joining SCIEX in 2017, Zoe has led the biopharma protein therapeutics and pharma group.

In her role, she guides her team in fostering collaboration with key customers and building strong relationships that are essential for groundbreaking advancements. In addition to close customer 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.

Further Reading and Resources

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

- 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
- 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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