Case Study on the Development of a Liquid Chromatography-High Resolution Mass Spectrometry Method for Non-Targeted Detection of β-Lactam Containing Antibiotics and their Transformation Products

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Introduction

Antibiotics are prolifically used to treat bacterial infections in both humans and animals; their cumulative toxic effects on aquatic organisms are not well understood and their widespread use is predicted to lead to the development of antibiotic resistance in bacteria. Therefore, they are understandably recognised as Chemicals of Emerging Concern (CECs). The β-lactam functionality is considered responsible for antibiotic/antibacterial activity hence the detection of β-lactam-containing chemicals and their transformation products (TPs) are of particular interest.

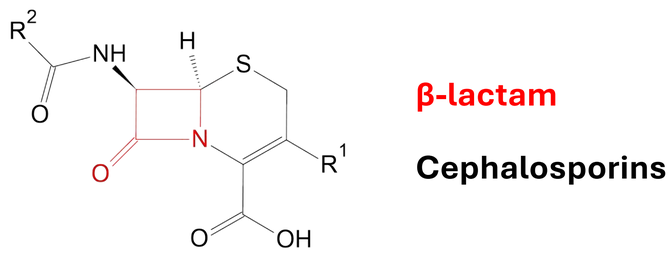

Figure 1 - Structure of β-lactam functionality (red); structure of cephalosporin (black).

Metabolite identification was required during a biodegradation study in activated sludge (OECD TG 314b) on a [^{14}C]-cephalosporin antibiotic (Figure 1). For any environmental fate study, the position of the radiolabel is required to be at stable location within the molecule, however this stable location is not always the region of interest in terms of activity or toxicity. Additionally, metabolite identification is only triggered by radiolabelled TPs, which could result in a misleading profile of non-toxic metabolites. Hence, a screening method was developed for the identification of TPs containing a β-lactam ring by liquid chromatography-high resolution mass spectrometry (LC-HRMS).

Methods

Analysis was performed on a LTQ Orbitrap mass spectrometer (Thermo Scientific, USA). Reference standards were purchased from Sigma-Aldrich, the target test substance was provided by Roche Ltd. For the development of the method, an abiotic and a biotic OECD TG 314b test vessel were dosed with non-radiolabelled material and left at study conditions for four days, along with analogous control samples. The HPLC methods used were performed on C18 HPLC columns, using a water/acetonitrile mobile phase with formic acid or ammonium acetate modifiers.

Method Development

A range of cephalosporin antibiotic reference standards were analysed for common fragments that contained or indicated the presence of the β-lactam ring. Structural assignments of all the MS^2 fragments were made, aided by Compound Discoverer software (Thermo Scientific, USA). Three fragments were selected that indicated the β-lactam functionality was present in the parent molecule (Figure 2).

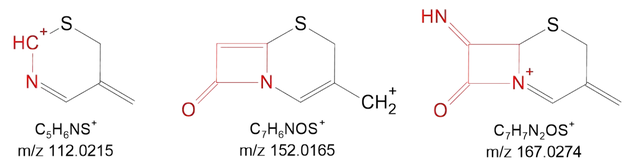

Figure 2 - Selected fragments that show or indicate the presence of a β-lactam ring.

Study samples were analysed with data dependent (dd) scanning and with source induced dissociation (SID), considered comparable to all ion fragmentation (AIF). The likely candidate fragments were chromatographically extracted in study samples. Not all the reference standards were triggered for fragmentation by ddMS^2, which was suspected due to the close retention times of the peaks. By further optimisation of dynamic exclusion parameters, it was possible to obtain at least one fragment scan for each of the reference standard peaks. Ultimately, a targeted mass list of the reference standard precursor ions had to be used to obtain reliable fragmentation data using ddMS^2. It was possible to identify all reference standards from the extracted ion chromatograms (EICs) of the three candidate fragment masses (Figure 3).

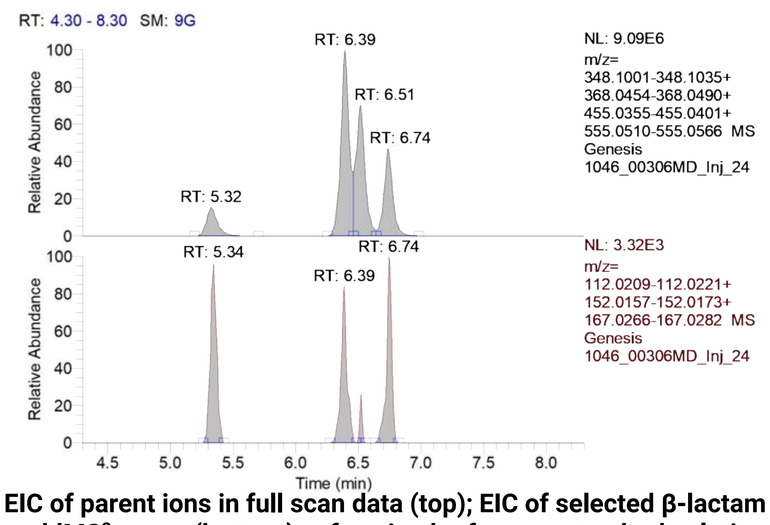

Figure 3 - EIC of parent ions in full scan data (top); EIC of selected β-lactam fragments in ddMS^2 scans (bottom), of a mixed reference standard solution.

SID was selected for the analysis of the OECD TG 314b study samples due to its ability to collect reliable fragmentation data, irrespective of co-eluting interference or the intensity of the precursor ion, without missing any target TPs.

Results

The lability of the targeted functional group lead to low detection levels, however, it was possible to detect a single β-lactam containing TP in the OECD TG 314b samples and it was considered that all β-lactam-containing TPs were found. By comparison to the same type of search on a stable moiety (also the selected location for the radiolabel) the effectiveness of the approach can be further demonstrated. Extraction of this stable fragment showed a chromatographic trace comparable to the radio-chem trace observed in the study (Figure 4).

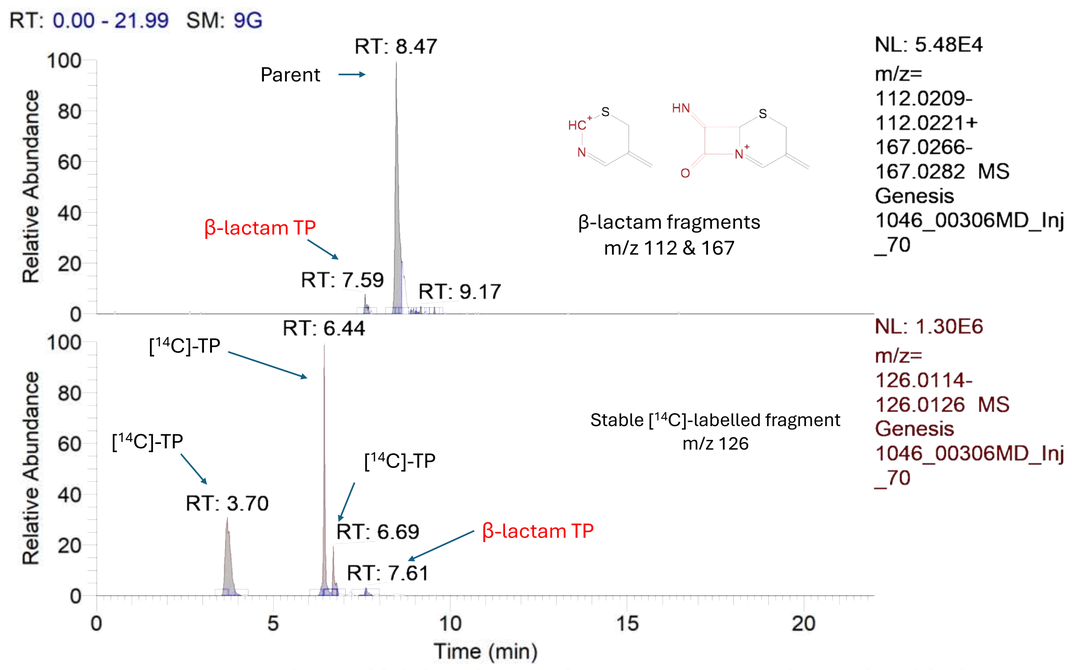

Figure 4 - Comparison of EIC of labile β-lactam fragments (top) and EIC of stable fragment containing the radiolabel (bottom), using SID in OECD TG 314b study sample.

Using SID, parent ions were not immediately apparent; parent ions needed to be determined by comparison with full scan data. Blank control samples were also vital to confirm the source of candidate peaks and avoid false positives.

For structural elucidation of the found metabolite, ddMS^2 data was collected using a targeted mass list containing the precursor ion found by SID. The MS^2 fragmentation data supported the suggestion that the structure of the metabolite contained a β-lactam ring.

Discussion

Identification of a single β-lactam metabolite was possible using SID. The difficulties associated with common fragment ion detection on a labile moiety are also ascribed to the lack of TPs found that exhibited this functional group. The increased probability of fragmentation and biodegradation at the site of the β-lactam ring improves the likelihood that all β-lactam containing metabolites were found in the study samples.

Recent advances in HRMS have improved the range of Orbitraps so that low mass fragments can now be found in the MS^2 data of high mass precursor ions. Improvements in acquisition times, the ability to overlay collision energies, and control over dynamic exclusion mean that ddMS^2 data can now be more confidently used to search for TPs. However, it is still possible that ddMS^2 will miss products of low intensity or those suffering from co-eluting interference.

Compound Discoverer (Thermo Scientific, USA) is a powerful processing tool that is limited to using ddMS^2 over SID/AIF. It can create a fragment ions search (FISh) trace that doesn't just target an isolated fragment but includes all detectable parent fragments in its creation. However, comparing the FISh trace to an isotope pattern trace of the same sample demonstrates the limitations of ddMS^2 for untargeted screening (Figure 5).

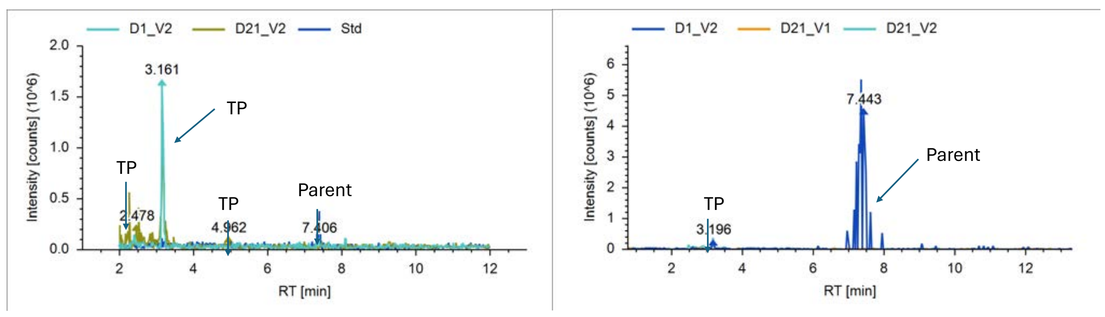

Figure 5 - Compound Discoverer (Thermo Scientific, USA) isotope pattern trace (left) and FISh trace (right)

Conclusions

The use of SID, comparable to AIF, allowed confident detection of all target metabolites with fast scan times and no loss in the quality of the chromatography. However, parent ions were not immediately apparent and blank controls were vital to avoid false positive results. Recent advances in HRMS and processing software greatly improve the quality and the power of ddMS^2 data, but manual review of the data is still considered vital to avoid missing target metabolites. Using a combination of AIF and ddMS^2 is the suggested approach for future studies.

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