Application Note: 441

PIF: Precursor Ion Fingerprinting – Searching for a Structurally Diagnostic Fragment Using Combined Targeted and Data Dependent MS^n

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Key Words

- Active Pharmaceutical Ingredient
- DMPK
- Mass Frontier™ Software
- Metabolite Identification
- Precursor Ion Fingerprinting

Overview

Purpose
Demonstrate the validity of the PIF methodology for an API, maropitant, and its metabolites.

Methods
LC with semi-targeted and data dependent MS^n and in silico fragmentation.

Results
Tagged greater than ten and elucidated two metabolite structures using PIF information for the API and metabolites.

Introduction

There is an increasing desire to reduce development time and cost devoted to ill-fated lead candidates and therefore a growing need for complete characterization of each compound earlier in the discovery process. Discovery DMPK experiments are becoming routine resulting in a need for more rapid and automated methods of verification that a putative metabolite is related to the parent drug. Here we present a simplified Precursor Identifying Fragment (PIF) technique which readily lends itself to routine automation and that offers the advantage of metabolite identification with no *a priori* knowledge of the active pharmaceutical ingredient (API) or any biotransformation products.

Microsomal Incubation

Maropitant sample is prepared at 1 mg/mL in 50/50 MeOH/H_2O. Incubation is carried out for 60 min at 37 °C using BD Gentest™ Sprague Dawley rat microsomes 452521.

PIF Methodology

1. Construct spectral trees by Intelligent acquisition of PIF Data for Control/t=0 and in vitro incubations using preset ion trap methods.
2. Use Mass Frontier for Component Identification
3. Compare Control and Sample component spectral trees to Determine a Precursor Identifying Fragment for the API.
4. Construct the XIC at all levels of MS^n of the Precursor Identifying Fragment to map probable metabolites.
5. Use Difference Spectra to determine the likely metabolic transformation(s).
6. Use Mass Frontier to Determine the Metabolite Structure from the set of possible metabolites.

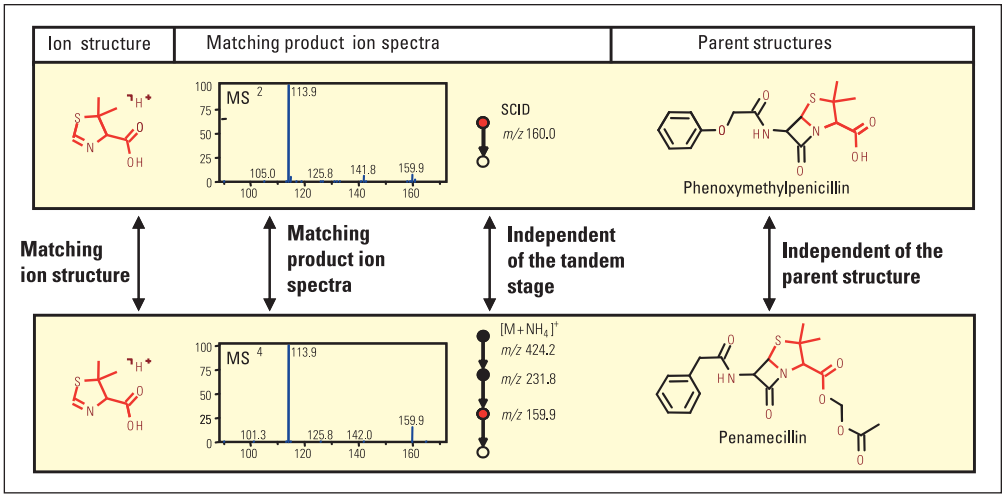
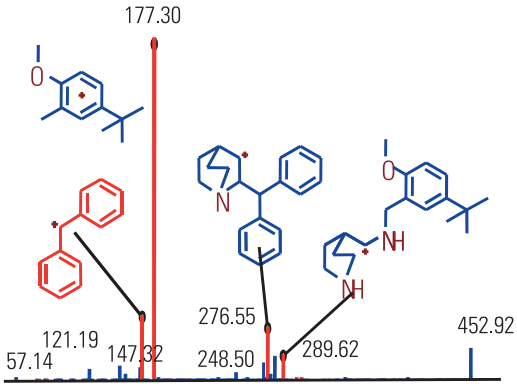


Figure 1: Demonstration of the Quasi-equilibrium Theory for Penamecillin and Phenoxymethylpenicillin

Results

Define the Precursor Identifying Fragment

Multiple experiments involving control only were carried out under various conditions of data dependent acquisition including: with/without parent list for MS2, variation of N from 2 to 5 in Top N MS3, and with/without an MS4 step. Optimized conditions – those conditions which gave sufficient information for elucidation of the API structure in the control – are listed in Figure 2.



Control method populates parent list with API *m/z*.

Sample method populates parent list with API and selected Phase I/Phase II metabolite *m/z*(s).

1. Semi-targeted data dependent **MS/MS** from list
2. Data-dependent **MS3 of Top 3** most intense ions from MS2
3. Data-dependent **MS4 of Most intense** ion from MS3

Figure 2: Intelligent Acquisition of Ion Trees for the API in Control/t=0 samples and API + Metabolites in t>0 samples

Results from the optimized acquisition for the API, Maropitant, are shown in Figure 3. The semi-targeted MS2 spectrum in the top panel shows 7 major fragments; the two dd-MS3 spectra in the bottom panel show subsequent fragmentation of the two most intense peaks in the MS2 spectrum: *m/z* 177 and *m/z* 276. The Precursor Identifying Fragment, *m/z* 167.3, is present in the MS2 spectrum (though not the most intense) and in the 470→276 MS3 spectrum.

Use PIF to Identify Metabolites of Maropitant

The optimized acquisition conditions were used for acquisition of PIF data for t=60 min. incubation. Metabolites are rapidly tagged in the chromatogram by the presence of the Precursor Identifying Fragment at any MS^n level in their spectral tree. This is shown in Figure 4 which displays the XIC for *m/z* 167 in the MS2 spectrum (purple trace), the MS3 spectrum (green trace) and the MS4 spectrum (red trace). The API, Maropitant, and ten of its metabolites (> 5% of the API) labeled. For reference, the complete base peak chromatogram is displayed in grey. This PIF approach is analogous to the highly selective precursor ion scanning – but is faster, can be done across all levels of MS^n, and requires no *a priori* knowledge about the API or its metabolites.

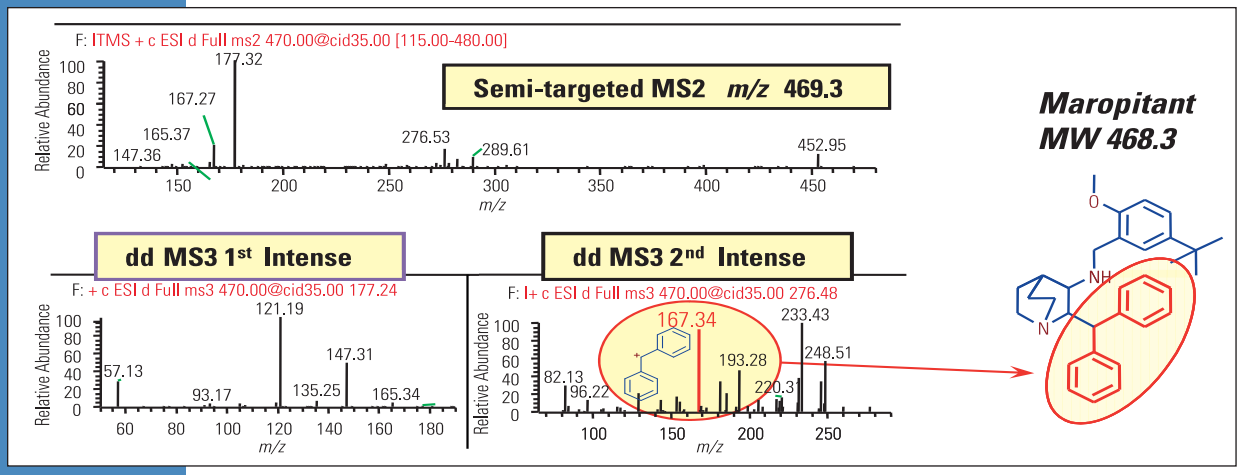


Figure 3: Define the Precursor Identifying Fragment for the API from the Control Spectral Tree Data

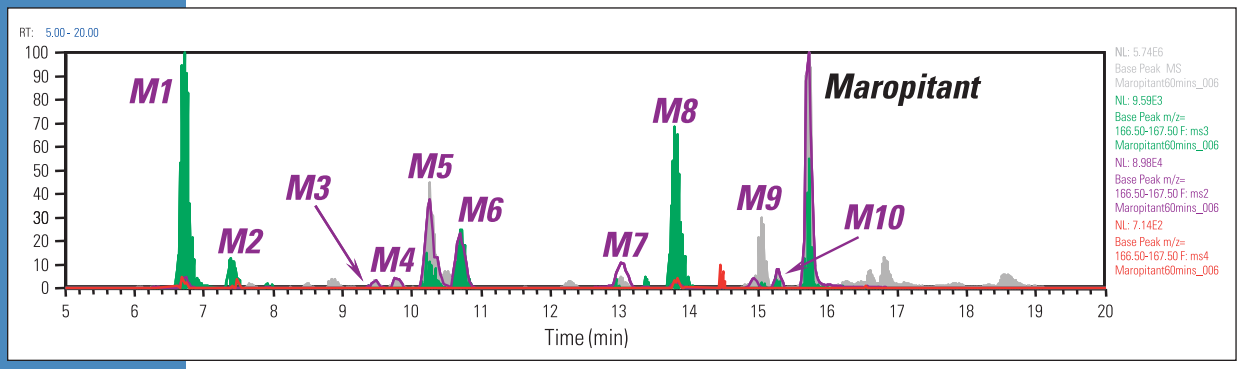
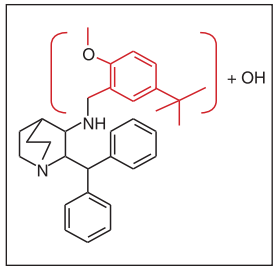


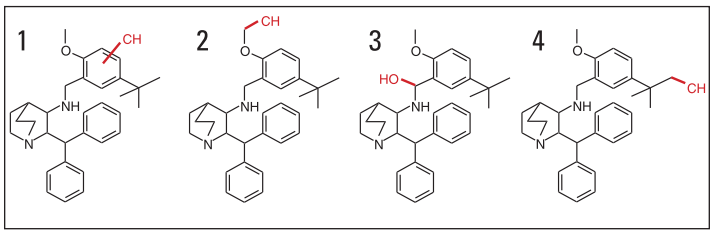
Figure 4: Track the Precursor Identifying Fragment: Extracted Ion Chromatogram for *m/z* 167

Elucidate the Structures of *M3* and *M7*

The difference spectrum for *M3* *m/z* 485.3 and the API *m/z* 469.3 is shown in the middle panel of Fig. 5. Examining its components we note the shift of the fragment at *m/z* 177.5 to *m/z* 193.3 with the same nominal mass difference Dm=16 Th observed for the shifted parent. Thus, the phase I metabolite *M3* is most likely hydroxylation on the O-methylated sub structure.



Based on the putative transformation derived from the difference spectrum shown in Figure 5, the following set of four possible metabolite structures can be derived:



Each of the above structures is fragmented in silico using Mass Frontier 5.0; the results are used to annotate the observed MS/MS spectrum. Spectral correlation is done by inspection with particular attention paid to those fragments which predominate in the difference spectrum shown in Figure 5. Following careful analysis of the MS2 spectrum and confirmation using MS3 and MS4 from the spectral tree, the most probable structure of *M3* based on highest MS^n spectral correlation is hydroxylation on the amide. The annotated MS2 and MS4 spectra are shown in Figure 6a and 6b. Key fragments at *m/z* 388.8 in the 486 MS2 spectrum and *m/z* 143.2 in the 298→193 MS4 spectrum are highlighted. These fragments are specific to hydroxylation at the amide (number 3 above).

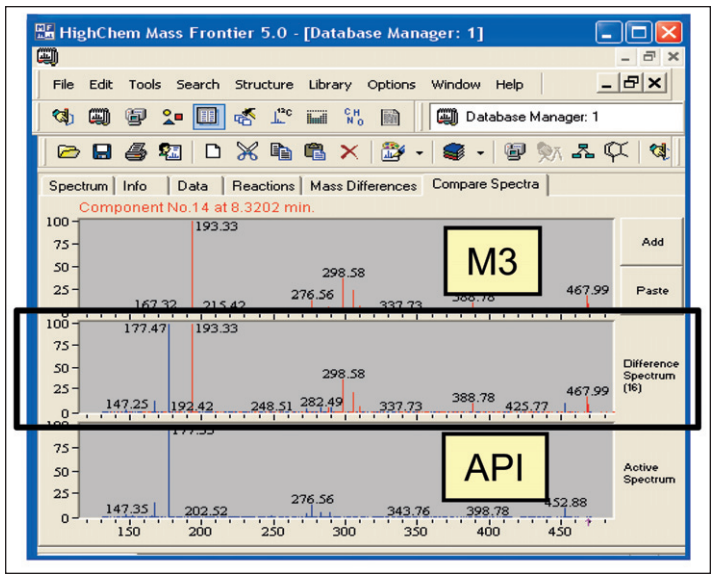


Figure 5: Difference spectrum for M3 *m/z* 486 and Maropitant (API)

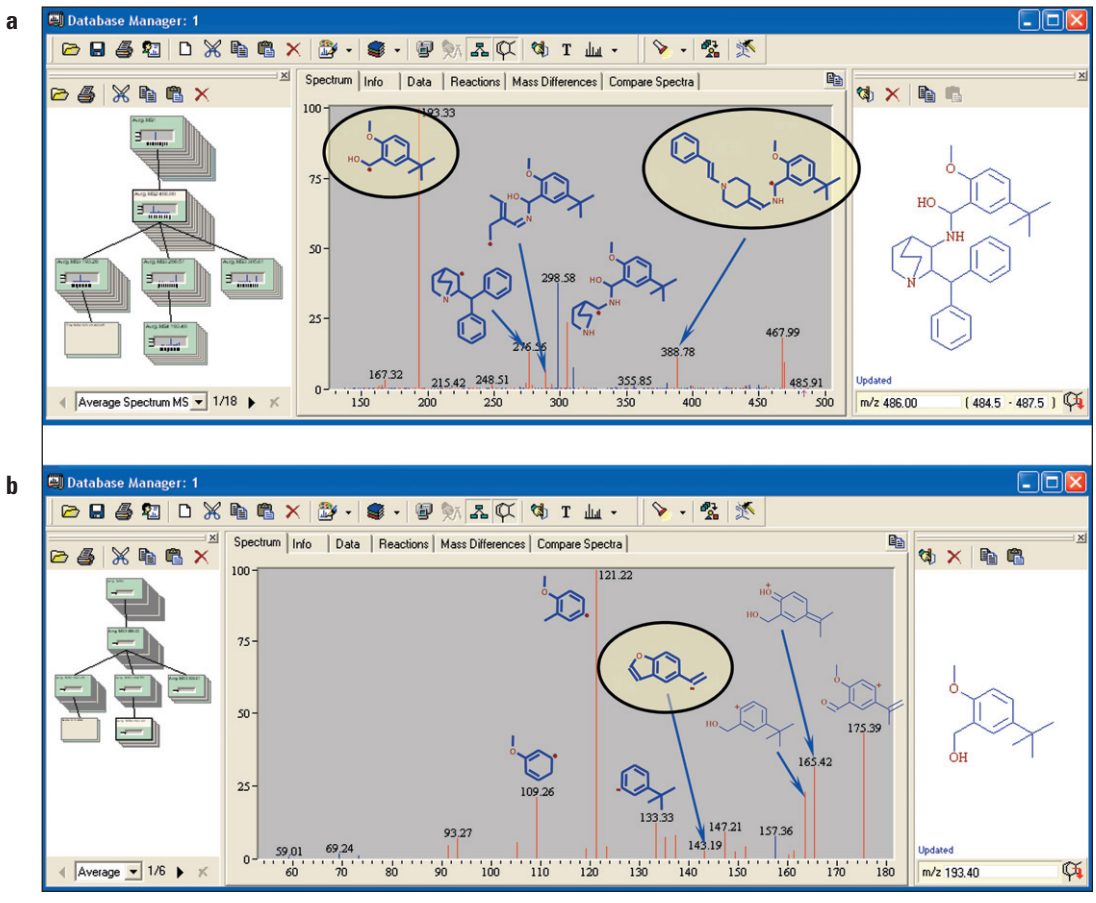


Figure 6: a) Annotated MS2 spectrum for hydroxylated metabolite *M3* with two key fragments at *m/z* 193 and 388 highlighted. b) Annotated MS4 spectrum for *m/z* 193 with key fragment at *m/z* 143 highlighted.

The MS2 difference spectrum for *M7* and the API is shown in the middle panel of Figure. 7. On careful examination of its many components we note again the shift of the fragment at *m/z* 177.5 to *m/z* 193.3 accounting for the difference of 16 Th. Thus, the phase I metabolite *M7* is most likely hydroxylation on the O-methylated substructure – the same conclusion drawn for *M3*. Even with the aid of accurate fragment masses, the structures of *M3* and *M7*, and hence the location of the biotransformation, cannot be unambiguously assigned based solely on the fragment masses in the MS2 spectral data.

In order to determine the structure of the second hydroxylated metabolite we make further use of the Mass Frontier difference spectrum at various stages of MS^n, comparing *M3* (structure assigned) with *M7* (structure unassigned).

The MS2 difference spectrum for *M3* and *M7* shown in Figure 8a contains multiple elements including the *M3* fragment at *m/z* 388.8 (not seen in the *M7* MS2 spectrum), and many *M7* fragments such as those at *m/z* 177.5, 282.4, and 414.6 (not seen in the *M3* MS2 spectrum).

As anticipated the MS2 difference spectrum for *M3* and *M7* does not contain the fragment at 193.3 as *M3* and *M7* have this (nominal mass) fragment in common. The 486→193 MS3 spectra for *M3* and *M7* (top and bottom panels of Figure 8b) confirm the hypothesis that these fragments have the same chemical formula and similar structures since they share substructures at *m/z* 165.3, 135.3, 121.3, 109.3 and 93.3. The circled region in the difference spectrum (middle panel of Figure 8b) provides information about the nature of the structural difference between *M3* and *M7*; *M7* lacks the fragment at *m/z* 143.

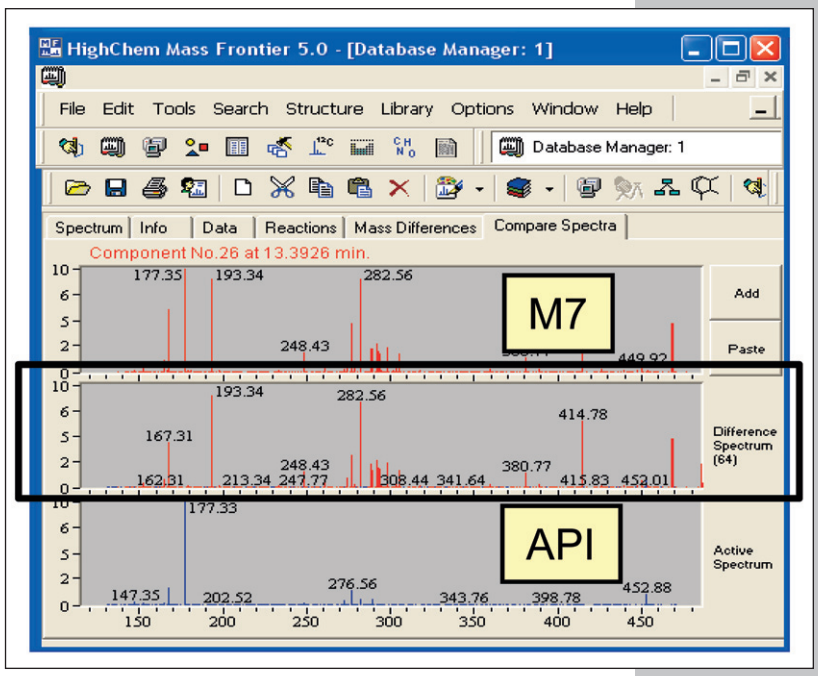


Figure 7: Difference spectrum for *M7 m/z* 486 and Maropitant (API)

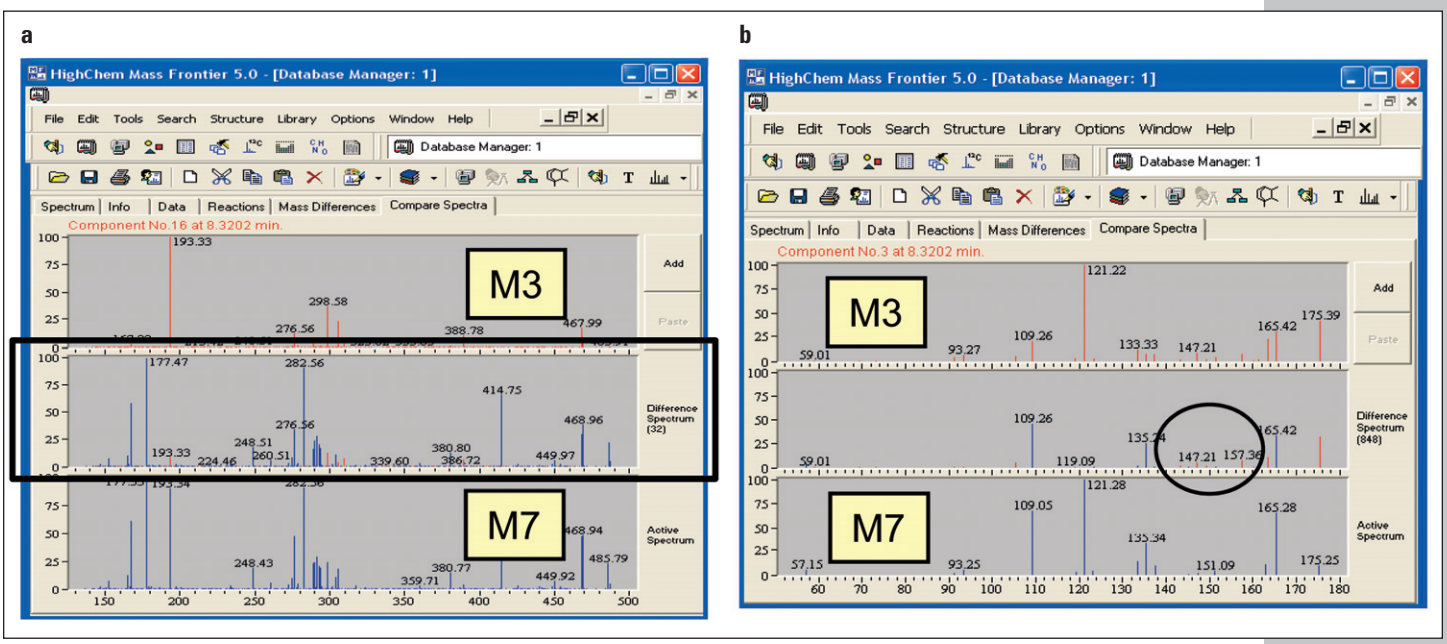


Figure 8: a) MS2 *m/z* 486 Difference spectrum for *M3* and *M7* and b) 486→193.3 MS3 Difference spectrum for *M3* and *M7*

Again using Mass Frontier 5.0 for in silico fragmentation of the remaining three possible structures for *M7* and estimating spectral correlation we propose *M7* to be hydroxylation of the O-methyl group. The annotated MS2 spectrum for *M7* is shown in Figure 9a.

The annotated 486→193 MS3 spectrum for *M7* is shown in Figure 9b. The insets in each figure display a structure and a partial list of its in silico fragments. In the MS2 spectrum (Figure 9a), one key fragment at *m/z* 177 is annotated and one key fragment from the *M3* MS2 spectrum (*m/z* 388) is noted absent. In the 486→193 MS3 spectrum for *M7* we note also the absence of the *M3* key fragment at *m/z* 143.

Confident assignment of the structures of *M3* and *M7* as hydroxylation of the O-methyl group was achieved for two primary reasons:

- Availability of MS^n spectral tree data which provides sequential fragmentation pattern information.
- Ability to assign structures in the observed MS^n spectra based on in silico fragmentation done in Mass Frontier.

At this time analysis of PIF data (described in the first panel) is almost entirely manual; however, improvements are being made in data processing methods which will allow automated processing and reporting of related compounds.

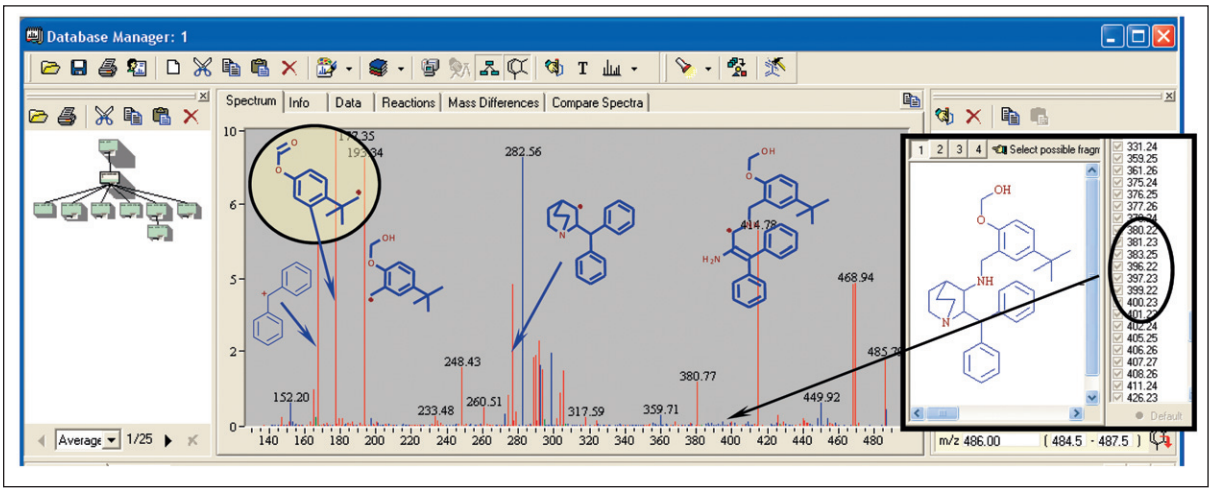


Figure 9a: Annotated MS2 spectrum for hydroxylated metabolite *M7* with key fragment at *m/z* 177 highlighted

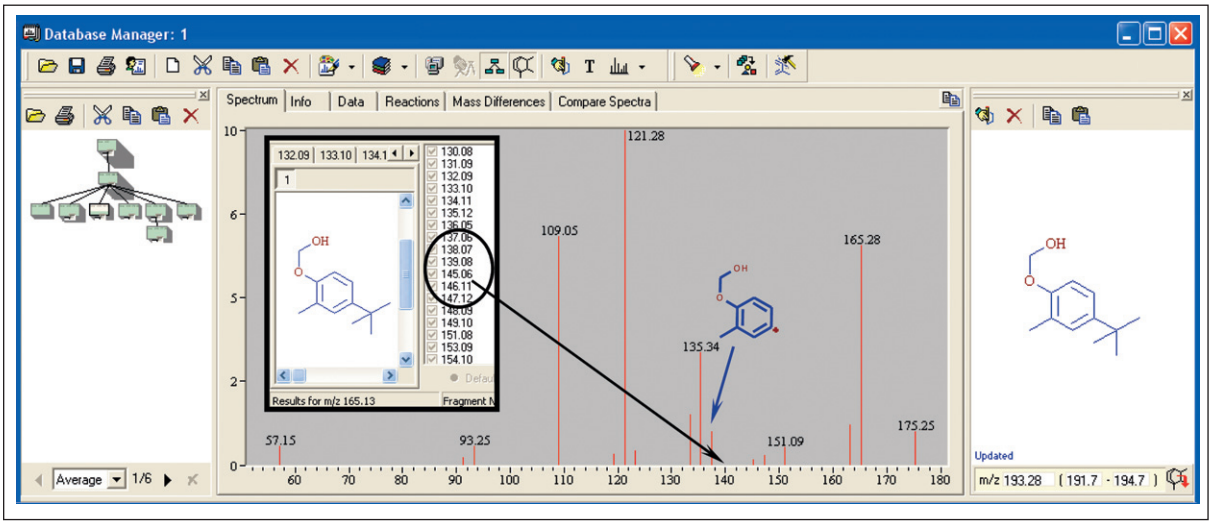


Figure 9b: Annotated 486→193 MS3 spectrum for *M7* with key region between *m/z* 140 and 145 featured

Conclusions

We have defined a Precursor Identifying Fragment (PIF) for Maropitant and used it to tag its metabolites from the precursor ion fingerprint information.

Traditional Precursor ion scanning techniques, although highly selective, are typically less sensitive as they require higher duty cycle in this mode of operation.

In contrast, the ion trap based PIF method we present is both rapid and sensitive due to fundamental duty cycle advantages. In addition, no *a priori* knowledge of the API or its metabolites is needed, making it amenable to routine automation with significant ease-of-use implications.

References

F. McLafferty, F. Turecek, p. 106, Interpretation of Mass Spectra, 3rd Edition, 1993, University Science Books.

Acknowledgements

We would like to thank Patrick Jeanville, strategic marketing manager for metabolism, for supplying the incubation samples.

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

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