

# PIF: Precursor Ion Fingerprinting. Searching for a Structurally Diagnostic Fragment Using Combined Targeted and Data Dependent MS<sup>n</sup>

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## 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<sup>n</sup> and in silico fragmentation.

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

## Introduction

In the current market the cost of development of a single drug is well in excess of 1B\$. 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* 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<sub>2</sub>O. Incubation is carried out for 60 mins. 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<sup>n</sup>** 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.

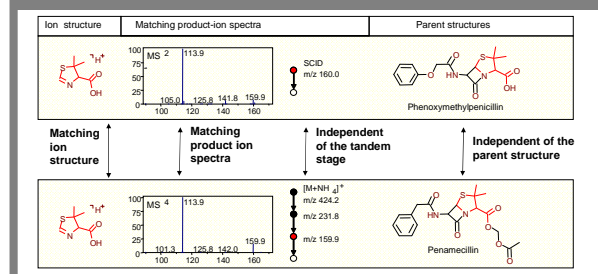
## Quasi-equilibrium theory:

The concept of the precursor ion fingerprint is derived from quasi-equilibrium theory (QET) originally formulated by Rosenstock *et al.* and discussed by Fred McLafferty in *Interpretation of Mass Spectra*<sup>1</sup>:

*"The probabilities of the various possible decomposition products of an ion depend upon its structure, internal energy, and the energy deposited during ion activation, but are independent of the ionization method, the structure of the precursor ion, and the formation mechanism of the ion undergoing decomposition."*

A demonstration of QET for two related structures is shown in Figure 1. We apply QET in an ion trap based method for rapidly classifying metabolites by the presence of the Precursor Identifying Fragment at any level of MS<sup>n</sup> in the spectral tree.

FIGURE 1. Demonstration of the Quasi-equilibrium Theory for Penicillin and Phenoxymethylpenicillin.

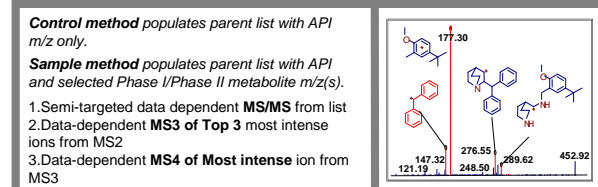


## Results

### Define the Precursor Identifying Fragment

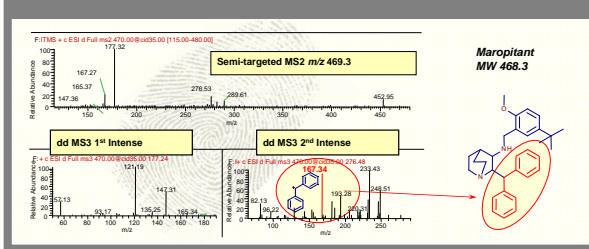
Multiple experiments involving control only were carried out under various conditions of data dependent acquisition including: with/without parent list for MS2, variation of N from 2 to 5 in Top N MS3, and with/without an MS4 step. Optimized conditions - those conditions which gave sufficient information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

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.

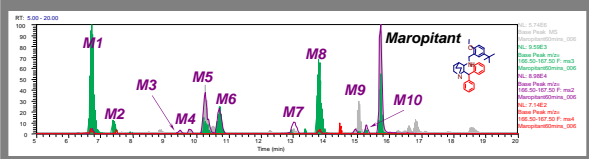
FIGURE 3. Define the Precursor Identifying Fragment for the API from the Control Spectral Tree Data



### Use PIF to Identify Metabolites of Maropitant

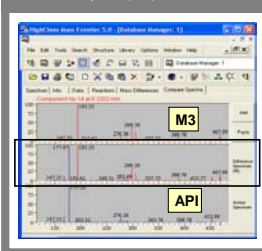
The optimized acquisition conditions were used for acquisition of PIF data for *t*=60 min. incubation. Metabolites are rapidly tagged in the chromatogram by the presence of the Precursor Identifying Fragment at any MS<sup>n</sup> level in their spectral tree. This is shown in Figure 4 which displays 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<sup>n</sup>, and requires no *a priori* knowledge about the API or its metabolites.**

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

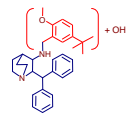


### Elucidate the Structures of M3 and M7

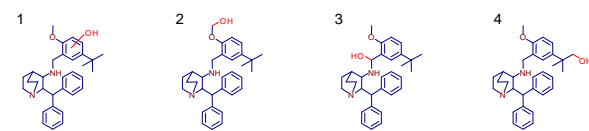
FIGURE 5. Difference spectrum for M3 *m/z* 486 and Maropitant (API)



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 a shift of the fragment at *m/z* 177.5 to *m/z* 193.3 with the same nominal mass difference  $\Delta m=16$  Th observed for the shifted parent. The phase I metabolite **M3** is most likely hydroxylation of the O-methylated substructure:



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<sup>n</sup> 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 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.

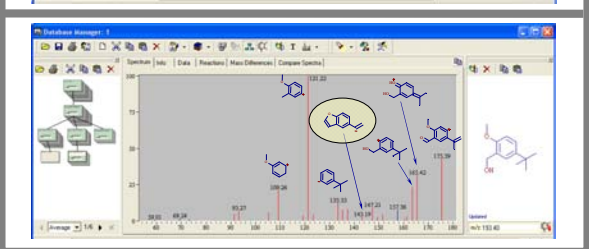
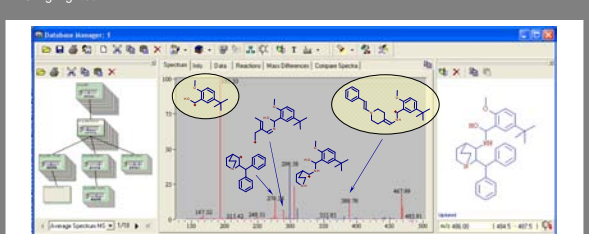
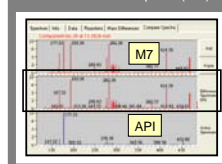


FIGURE 7. Difference spectrum for M7 *m/z* 486 and Maropitant (API)



The MS2 difference spectrum for **M7** and the API is shown in the middle panel of Figure 7. Careful examination of its many components reveals the shift of the fragment at *m/z* 177.5 to *m/z* 193.3 explaining the difference of 16 Th. Thus, the phase I metabolite **M7** is most likely hydroxylation of 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.

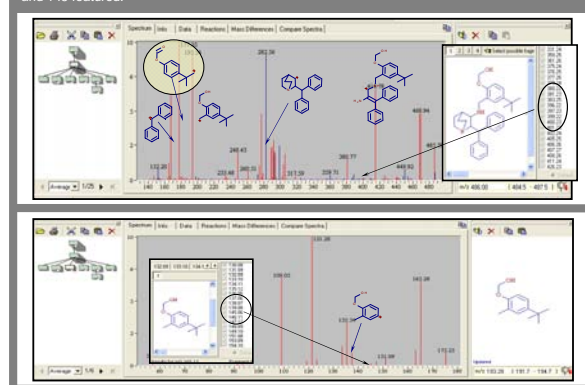
In order to determine the structure of the second hydroxylated metabolite we make further use of the Mass Frontier difference spectrum at various stages of MS<sup>n</sup>, comparing **M3** (structure assigned) with **M7** (structure unassigned).

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.

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.

FIGURE 9. a) Annotated MS2 spectrum for hydroxylated metabolite M7 with key fragment at *m/z* 177 highlighted. b) Annotated 486→193 MS3 spectrum for M7 with key region between *m/z* 140 and 145 featured.



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:

1. Availability of MS<sup>n</sup> spectral tree data which provides sequential fragmentation pattern information.
2. Ability to assign structures in the observed MS<sup>n</sup> spectra based on *in silico* fragmentation done in Mass Frontier.

## Conclusions

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

Traditional precursor ion scanning techniques, although highly selective, are typically less sensitive as a result of low 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.

(1) F. McLafferty, F. Turecek, p. 106, *Interpretation of Mass Spectra*, 3<sup>rd</sup> Edition, 1993, University Science Books.