Application Note: 320

Analysis of a Verapamil Microsomal Incubation using Metabolite ID and Mass Frontier™

Key Words

- Metabolism Study
- Structure Elucidation
- Metabolite ID
- Mass Frontier

Chromatography and Mass Spectrometry Application Note

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Introduction

The characterization of a drug's metabolites plays a crucial role throughout its development. In the early discovery phase, studies are designed to help the medicinal chemist quickly identify metabolic "hot spots" that might require chemical modification in order to improve pharmacokinetics. At this early stage, the emphasis is typically on analyses with fast turnaround speeds, due to the large number of compounds involved. As the development process moves forward and the number of drug candidates is reduced, the emphasis shifts from speed to completeness, especially when regulatory and toxicity issues are involved. By the time a drug candidate reaches the latter stages of development, a team of scientists might spend years exhaustively characterizing its metabolic products.

LC/MS has become the technique of choice in these metabolism studies due to its inherently high level of sensitivity and specificity. In a typical analysis requiring less than one hour to complete, data sufficient to identify and characterize dozens of metabolites spanning a wide range of concentrations can be acquired. However, the information content in these LC/MS experiments can be extremely high, necessitating the need for efficient software tools. These software tools should satisfy the needs of both 1) the medicinal chemist in early stage discovery who must quickly screen large numbers of compounds for major metabolites and, 2) the needs of the researcher in late-stage development who must completely characterize all metabolites, including those present at very low levels. The Metabolite ID and Mass Frontier software packages have been designed to satisfy these diverse requirements.

Overview

Metabolite ID and Mass Frontier have been designed to work in conjunction with each other to efficiently extract information from LC/MS data files obtained from in vivo and in vitro metabolism samples. In order to fully utilize the capabilities of these software packages, MS^2 as well as MS data should be acquired. Following a typical work flow, Metabolite ID is used to identify both expected and unexpected metabolites. Once the modifications in the sample have been determined, MS^2 spectra can then be exported from the Metabolite ID software package to the Mass Frontier software package for more in-depth characterization. For example, Metabolite ID might determine that a drug molecule has three distinct sites of hydroxylation. Mass Frontier would then be used to locate the specific sites in the molecule where the hydroxylation occurs.

In this application note, Metabolite ID and Mass Frontier are utilized for the analysis of microsomal incubations of Verapamil, a calcium channel blocker used to treat various cardiovascular conditions. The goal of this publication is to describe how Metabolite ID and Mass Frontier can be used to extract the maximum amount of information from the data.

Experimental

Verapamil hydrochloride (Sigma-Aldrich) at a concentration of 100 um was incubated for four hours with liver microsomes (In Vitro Technologies) and Uridine diphosphate glucuronic acid (UDPGA) before termination with an equal volume of acetonitrile. A high drug concentration and a long incubation time were used in order to generate the maximum number of metabolites. The UDPGA was added to generate phase two glucuronide conjugates. Microsomes from male Cynomolgus monkeys, male Wistar rats, and male Beagle dogs were used to probe interspecies variations in metabolism. The incubation mixtures were analyzed without any additional sample preparation or cleanup using a Finnigan™ LCQ™ Advantage ion trap mass spectrometer equipped with a Finnigan Surveyor® HPLC system. The mass spectrometer was operated in a data-dependent acquisition mode in which both MS and MS^2 spectra were acquired without the need to specify parent masses. In this mode, the acquisition software probed the MS spectra in real-time on a scan-by-scan basis to select the two most intense parents ions for MS^2 analysis. By analyzing both the first and the second most intense parent ions, information on co-eluting metabolites was obtained. Dynamic Exclusion™ was not employed in these analyses in order to avoid the possibility of missing modifications such as hydroxyla-

tions that can occur multiple times with closely spaced retention times. The ion source of the mass spectrometer was operated in the positive ion electrospray ionization mode. The HPLC separation was achieved using a 150×2.1 mm 5 µm BetaBasic® 18 column (Thermo Electron) and the following HPLC gradient conditions:

Time (min)	% A	% B	Flowrate (mL/min)
0	95	5	0.2
3	95	5	0.2
18	40	60	0.2
21	10	90	0.2
29	10	90	0.2
30	95	5	0.2
40	95	5	0.2

A=95% water : 5% acetonitrile : 0.1% formic acid and B=95% acetonitrile : 5% water : 0.1% formic acid. Injection volume was 10 µL.

Metabolite ID Analysis

Background subtraction is an optional step that was used for the verapamil example presented here. The ***background subtraction*** feature includes a scaling factor by which the control file's intensity can be multiplied (to ensure complete background subtraction) and a time offset (to correct for any retention time drift between the source and the control file). Metabolite ID also provides a manual background subtraction feature that allows the user to specify a specific mass range and time range to be subtracted.

After the optional background subtraction, the appropriate data file is opened and the ***parent drugs*** of interest are specified as illustrated in Figure 1. Users can select from previously created drug lists or create new entries by pressing the new Item tab. The Modifications button allows the user to select individual modifications of interest from the extensive list provided or define custom modifications. An alternative approach that can be employed when the user has no prior knowledge of their drug's metabolism is to use all of the modifications supplied. The results of this latter approach, applied to this verapamil example, are shown in Figure 2. These results were generated by clicking the Results button. The top portion of the display in Figure 2 shows extracted ion chromatograms corresponding to the masses of the parent drugs and modifications specified. From the three chromatograms displayed, one can conclude that verapamil undergoes demethylation and dehydrogenation but not decarboxylation.

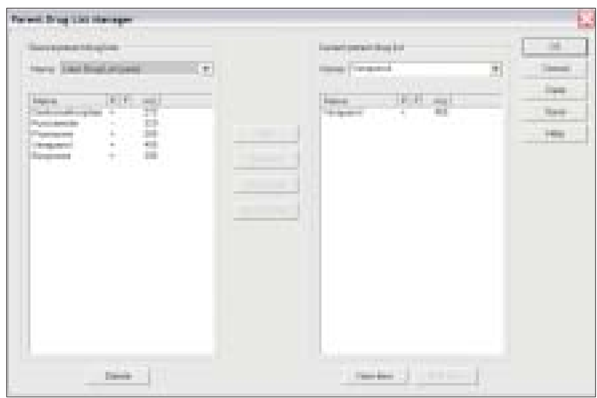

Figure 1. Archived lists of parent-drugs on the left. The currently active drug list is on the right.

The bottom portion of Figure 2 consists of two windows containing the MS and MS^2 spectra. Generated by clicking the cursor on the apex of the peak in the "Dehydrogenation" chromatogram the MS spectrum shows a parent mass of 453 and a mass of 277 in the MS^2 spectrum. This is a loss of 176 mass units which is characteristic of a glucuronide conjugate rather than a simple dehydrogenation product. This example demonstrates the utility of having quick and easy access to MS and MS^2 spectra. This metabolite is likely the result of a three step process where alkyl cleavage at the tertiary amine group of verapamil initially produces a previously documented metabolic product at mass 290 which then undergoes demethylation to produce a free hydroxyl group which is the site of glucuronidation in the final step (Figure 3). The results for the mass 453 metabolite should prompt the user to return to the Modification List Manager dialog box and create custom modifications corresponding to the proposed mass 291 metabolite and its demethylated analogue.

Figure 2. Initial search results using only the factory provided list of modifications

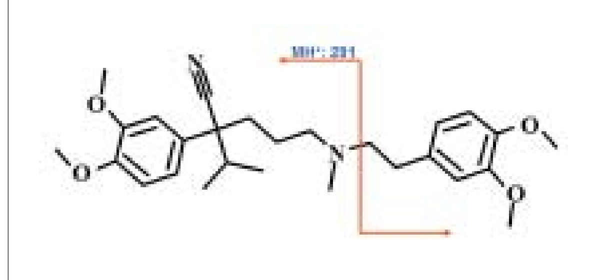

Figure 3. A fragmentation of verapamil.

Table 1 summarizes the results that were obtained by applying the list of single and multiple modifications provided with Metabolite ID to the verapamil incubation with monkey microsomes. None of these modifications were detected in the monkey microsome control data file.

Mass of Modification	Mass of Metabolite	Proposed Assignment
-30	425	Loss of CH_2O or double demethylation/dehydrogenation
-28	427	double demethylation
-14	441	demethylation
-2	453	N-dealkylation/demethylation/glucuronidation
+2	457	oxidation/demethylation
+162	617	demethylation/glucuronidation
+192	647	oxidation/glucuronidation

Table 1

Metabolite ID provides two additional tools that assist in the identification of unknown or unusual modifications. The first is ***Chro Search*** and the second is ***Correlation Search***. Chro Search employs an algorithm which sorts the intensities of all the ions in all the MS spectra and reconstructs extracted ion chromatograms for the "n" most intense ions. The user can customize Chro Search by setting intensity, time range, and mass range thresholds for the search. The results of Chro Search applied to the background-subtracted verapamil monkey microsome incubation data file are shown in Figure 4.

The table along the left-hand side of Figure 4 shows the "n" most intense ions for which extracted ion chromatograms were constructed. The MS^2 spectrum of the parent drug is always displayed in the bottom right-hand corner. The results for m/z 330 (from row 11 in the table) are shown at the top. This represents a loss of 125 daltons from the parent drug. This is not a common metabolic mass loss and would have been difficult to predict otherwise. Inspection of the MS spectrum that corresponds to the apex of the largest peak in the extracted ion chromatogram of m/z 330 clearly indicates that m/z 330 represents the protonated molecular weight of an actual compound. Comparison of the MS^2 spectrum of this peak (bottom left) with that of the parent drug (bottom right) reveals a large common fragment ion at m/z 303. This supports the conclusion that m/z 330 represents a metabolite.

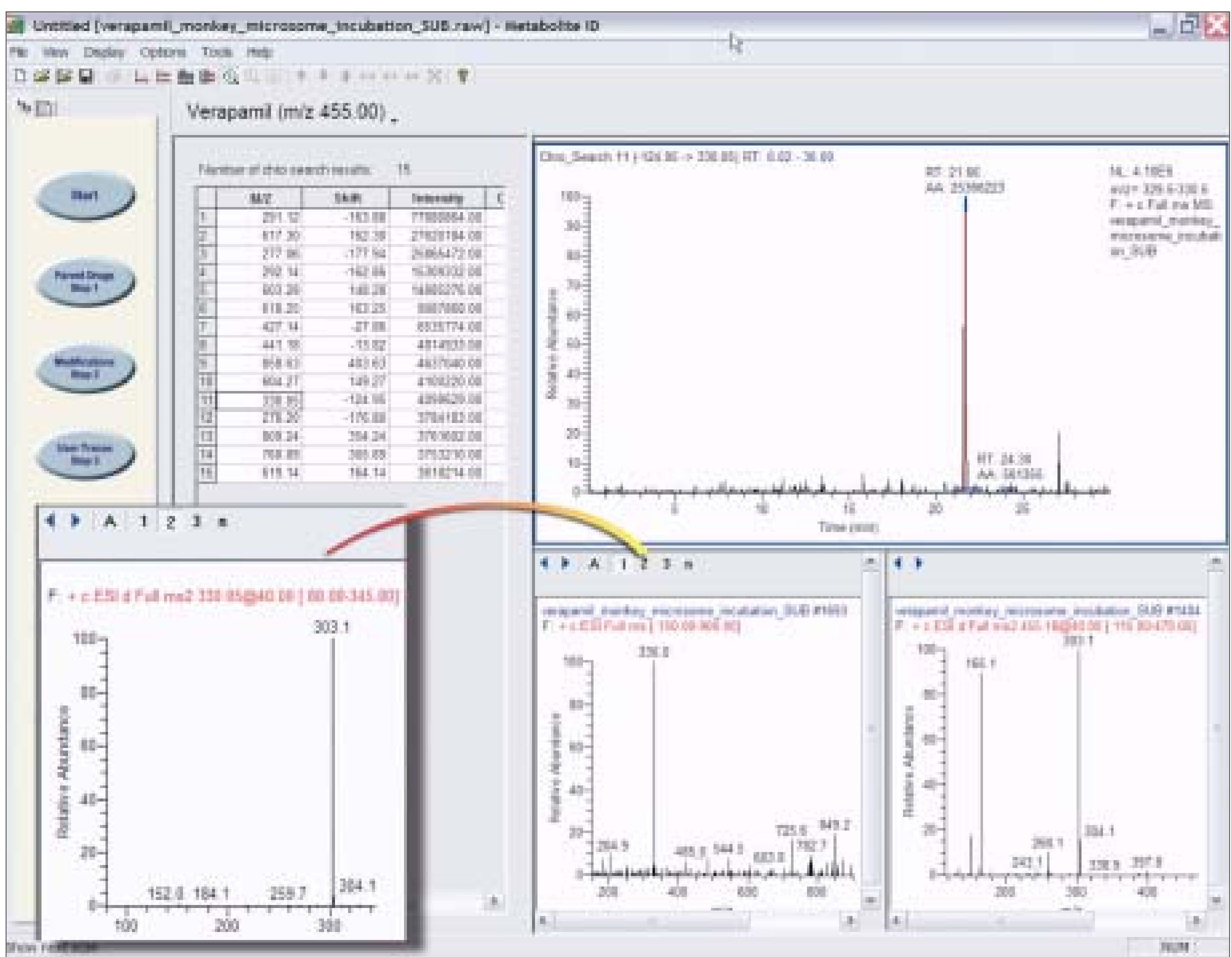

Figure 4. Results of Chro Search.

Table 2 summarizes the modifications that were found by the Chro Search algorithm for the verapamil monkey microsome incubation data file. The user could probe the data further by modifying the 'Threshold' or 'Most Intense' Chro Search parameters.

Mass of Modification	Mass of Metabolite	Proposed Assignment
-178	277	N-dealkylation/demethylation
-164	291	N-dealkylation
-125	330	?
-28	427	double demethylation
-14	441	demethylation
+148	603	double demethylation/glucuronidation
+162	617	demethylation/glucuronidation

Table 2

The other algorithm that Metabolite ID provides for searching for unexpected metabolites is the Correlation Search. This search compares all of the MS^2 spectra in a selected data file with the MS^2 spectrum of the parent drug specified in the Parent Drug List Manager dialog box. The MS^2 spectra are compared based on intensities and masses. The correlation score is a measure of the overlap between the MS^2 spectra of a potential metabolite and the reference scan.

The results of a correlation search of the verapamil monkey microsome incubation data file are shown in Figure 5. The table at the left gives the results in terms of "Parent M/Z, RT (min), and Correlation". The top chromatogram on the right-hand side is the "Modification Summary". It sums together the extracted ion chromatograms of all of the modifications specified in the Modification List Manager dialog box. The second chromatographic trace in Figure 5 is the "Correlation Summary". It sums together the correlation score of every MS^2 scan in the specified data file. The mass spectrum in the middle of the figure is the MS^2 spectrum of the reference scan of verapamil. The spectrum at the bottom of the display is the MS^2 spectrum of the scan selected by mouse-clicking in the data table.

The lengthy list of masses can be pared down using the following logic. Mass to charge values that appear at numerous retention times are most likely background ions and can be eliminated. The m/z values that appear too early or too late in the chromatogram relative to the parent drug are also candidates for elimination. Finally, those m/z values that do not exhibit at least 2-3 successive MS^2 scans with similar spectra are reasonable candidates for elimination. After applying these constraints to the original table of data, only those modifications at m/z of 330.1 and 441.2 are identified as possible metabolites.

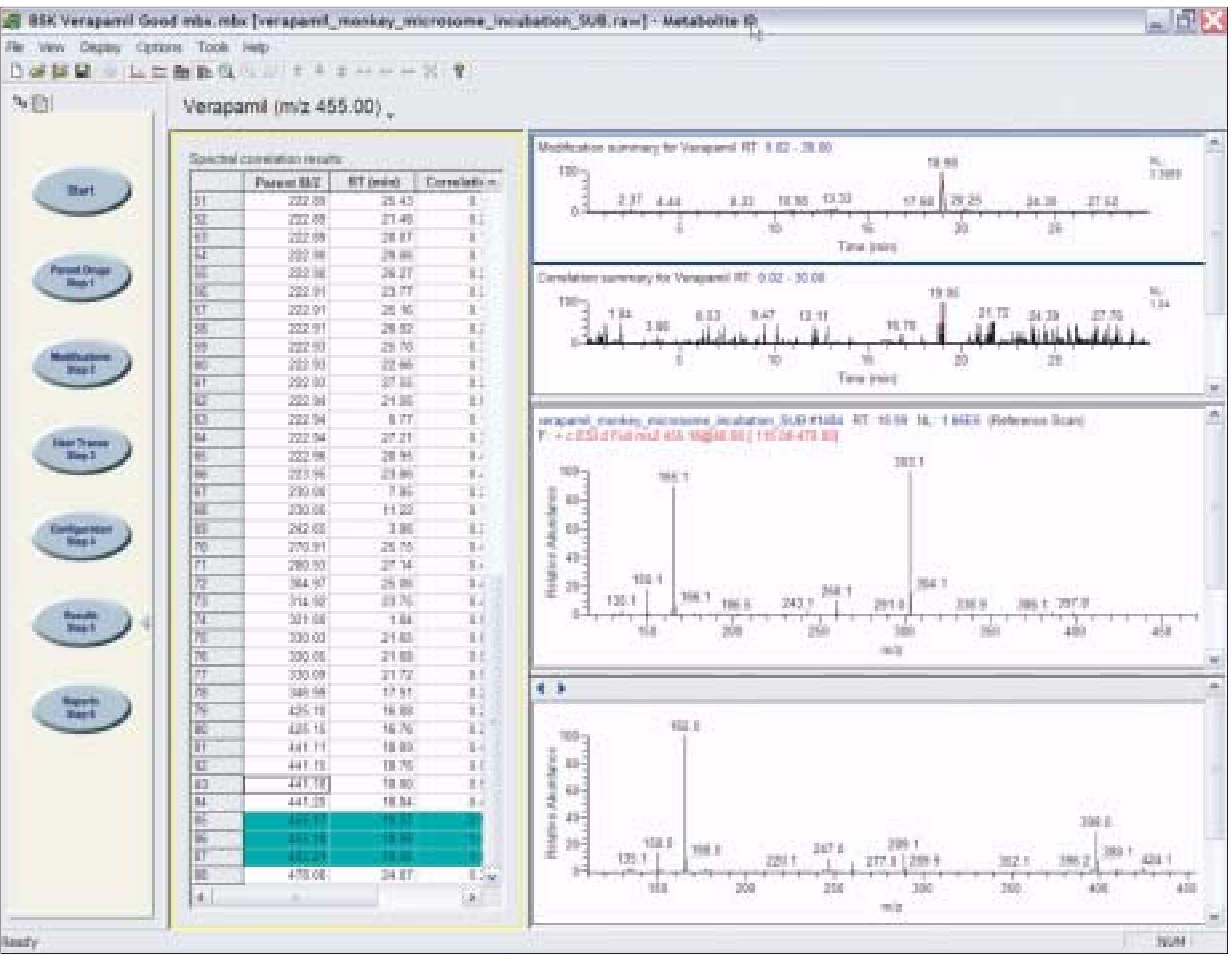

Figure 5. Results of Correlation Search.

Metabolite ID also provides the user with the capability to change the reference scan. In the case of verapamil, it makes sense to perform an additional correlation search using the MS^2 spectrum of the N-dealkylation metabolite (MH^+: 291) described earlier. This makes sense because any metabolite resulting from the same alkyl cleavage would most likely correlate better with this reference than with the parent drug. Using the logic described for the previous correlation search, the m/z values of 277, 307, and 425 can be flagged as likely metabolites.

Table 3 summarizes the results of these two correlation searches. Further correlation searches using additional reference scans could be performed.

Reference Scan (parent mass)	Mass of Modification	Mass of Metabolite	Proposed Assignment
455	-125	330	?
	-14	441	demethylation
291	-178	277	N-dealkylation/demethylation
	-148	307	N-dealkylation/oxidation
	-30	425	Loss of CH_2O or double demethylation/dehydrogenation

Table 3

The final step in characterizing the metabolites of verapamil is to evaluate all of the possible combinations of the modifications discovered to this point. The complete list of all possible modifications is listed in Table 4.

Mass of Modification	Mass of Metabolite	Proposed Assignment
-178	277	N-dealkylation/demethylation
-164	291	N-dealkylation
-162	293	N-dealkylation/demethylation/oxidation
-148	307	N-dealkylation/oxidation
-125	330	?
-42	413	triple demethylation
-30	425	loss of CH_2O or double demethylation/dehydrogenation
-28	427	double demethylation
-14	441	demethylation
-12	443	double demethylation/oxidation
-2	453	N-dealkylation/demethylation/glucuronidation
+2	457	demethylation/oxidation
+148	603	double demethylation/glucuronidation
+162	617	demethylation/glucuronidation
+164	619	double demethylation/oxidation/glucuronidation
+178	633	demethylation/oxidation/glucuronidation
+192	647	Oxidation/glucuronidation

Table 4

Once the metabolite analysis has been completed, Metabolite ID provides a number of different ways to display the final results. The ***Modification Peak Summary Table***, displayed in Figure 6 demonstrates the broad dynamic range over which Metabolite ID can identify metabolites. For the verapamil monkey microsome incubation, metabolites ranging in amounts from 0.2% to 40.4% of the total peak area were characterized.

Mass Frontier Analysis

Mass Frontier provides a collection of modules that assist the user in managing, interpreting, and classifying mass spectra. In this publication, Mass Frontier will be used to locate the specific sites of metabolic modifications identified previously with Metabolite ID. The general approach will be as follows:

- From Metabolite ID, export the MS^2 spectra of the metabolite of interest and a reference compound
- Draw the structure of the reference compound using the ***Structure Editor*** module in Mass Frontier.
- Associate the structure just drawn to the corresponding MS^2 spectrum of the reference compound previously exported to the ***Spectra Manager*** module.
- Use the ***Fragments & Mechanisms*** module to assign peaks in the experimental MS^2 spectrum of the reference compound with specific portions of its structure.
- Compare the peaks in the MS^2 spectrum of the metabolite of interest with the assigned peaks in the MS^2 spectrum of the reference compound to determine the sites of modification in the metabolite.

The assumption in this type of analysis is that the modification does not alter the MS^2 fragmentation pathways of the metabolite. Comparison of the MS^2 spectrum of the metabolite with the reference compound can indicate where the modification has occurred.

The approach just described will be applied to the demethylated metabolite (MH^+: 441) at 18.80 minutes.

The MS^2 spectrum of the demethylated metabolite eluting at 18.80 minutes and the unmodified verapamil are exported from Metabolite ID to the Spectra Manager module of Mass Frontier. The structure of verapamil is drawn using the Structure Editor module and is associated and stored with the experimental MS^2 spectrum of verapamil. The Fragments & Mechanisms module then interprets the experimental MS^2 spectrum of verapamil.

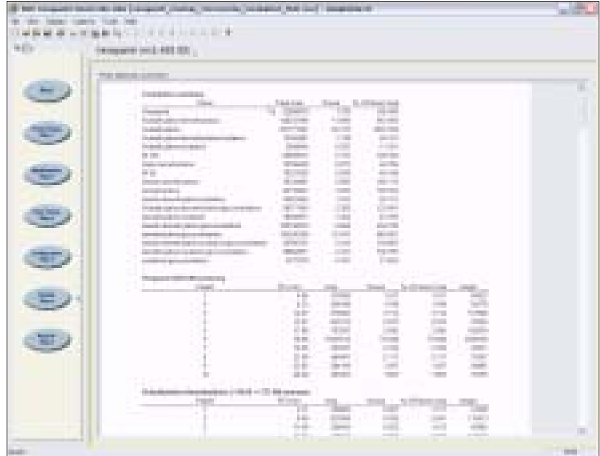

Figure 6. Modification Peak Summary report.

The fragmentation pathways and structures (Figure 7) can be subjected to further fragmentation simulations. This module of Mass Frontier makes its structural assignments using a known list of unimolecular rearrangement and fragmentation reactions. The user is able to control which of these reactions will actually be considered.

The Spectra Manager and Fragments & Mechanisms modules work together to automatically highlight those peaks in the experimental MS^2 spectrum explained by the predicted fragmentation. In the case of verapamil, Mass Frontier was able to make structural assignments for the MS^2 peaks at m/z's 165, 260, and 303.

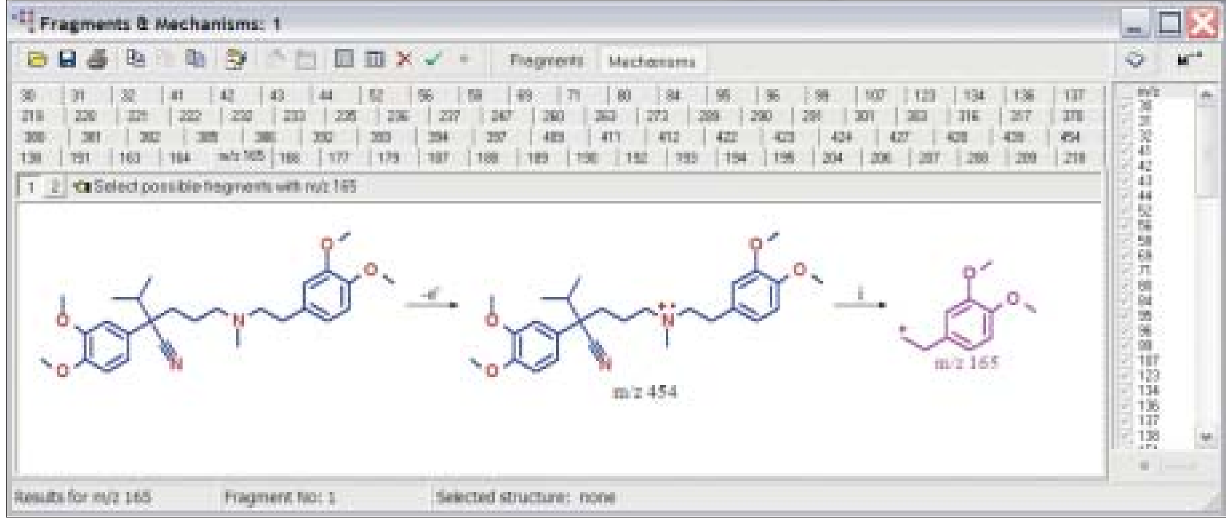

Figure 7. Fragmentation pathway shown for fragment m/z 165.

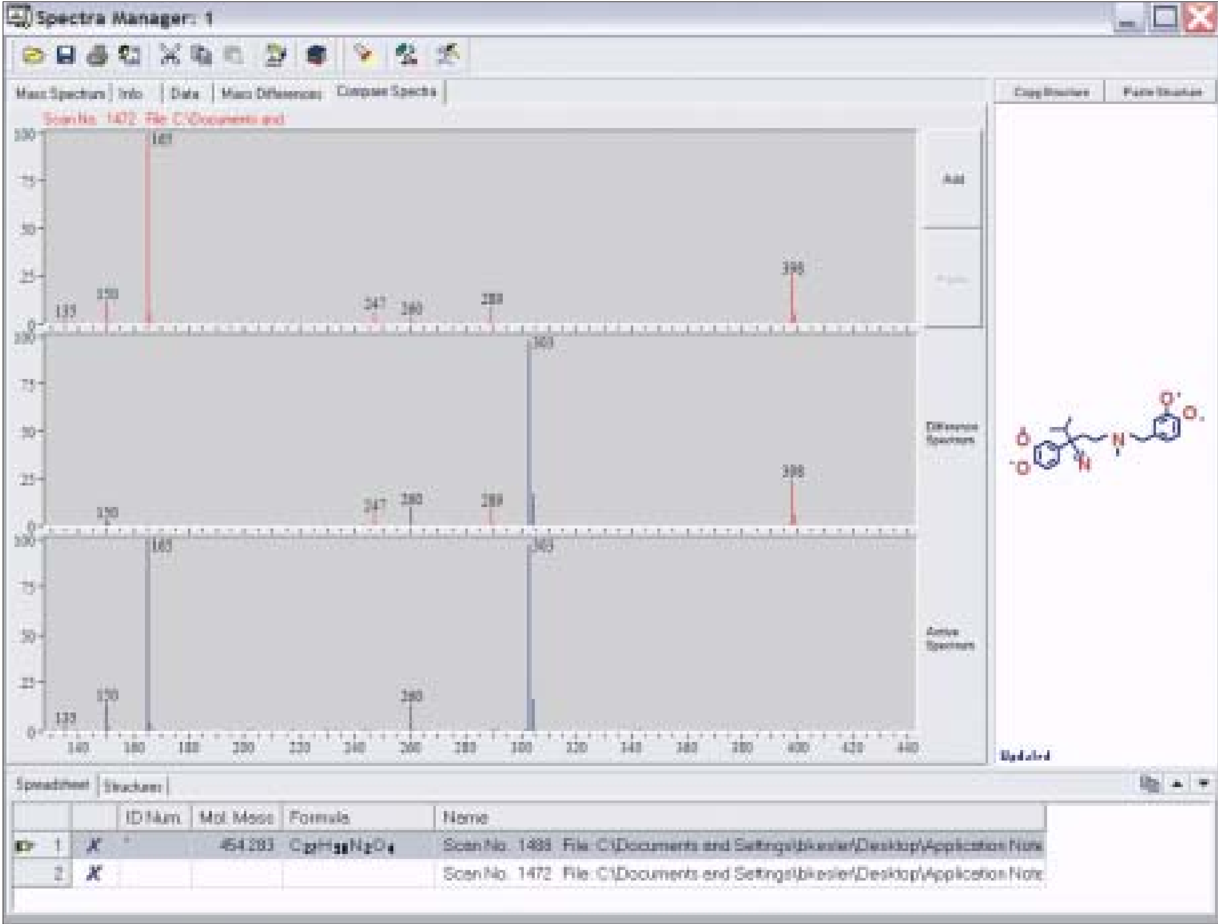

Figure 8. Comparison of verapamil MS^2 (bottom) with the demethylated metabolite (top).

The next step is to look for the three corresponding fragments in the experimental MS^2 spectrum of the demethylated metabolite. The MS^2 spectra of verapamil and its demethylated metabolite can be simultaneously viewed in the Spectra Manager window by selecting the "Compare Spectra" option (See Figure 8). An inspection of the spectra in this window indicates that the mass 165 and 260 peaks in the MS^2 spectrum of verapamil are unshifted in the MS^2 spectrum of the demethylated metabolite while the mass 303 peak has been shifted downwards by 14 daltons to mass 289.

Table 5 lists the three fragments along with the structures predicted by Mass Frontier. The structure of verapamil with its five possible sites of demethylation is shown in Figure 9. Sites 4 and 5 can be eliminated as possible sites of demethylated by the observation that the mass 165 MS^2 fragment of verapamil is unshifted in the demethylated metabolite. The same logic applied to the mass 260 MS^2 fragment eliminates sites 1 and 2. This leaves site 3 as the only possible site of demethylation and is confirmed by the mass 303 MS^2 fragment which is shifted lower by 14 daltons to mass 289. This N-demethylated metabolite is called norverapamil and has been previously described in the literature as the major demethylation metabolite of verapamil.

Using the same approach as above, the sites of demethylation in the two N-dealkylated/demethylated metabolites at 14.79 and 15.82 (both with MH^+:277) minutes can also be determined. However, in these examples the reference compound for comparison will be the N-dealkylated metabolite of verapamil rather than verapamil itself.

verapamil MS^2 fragment	Mass Frontier assignment	mass shift in demethylated metabolite	fragment contains modification?
165		0	no
260		0	no
303		-14	yes

Table 5

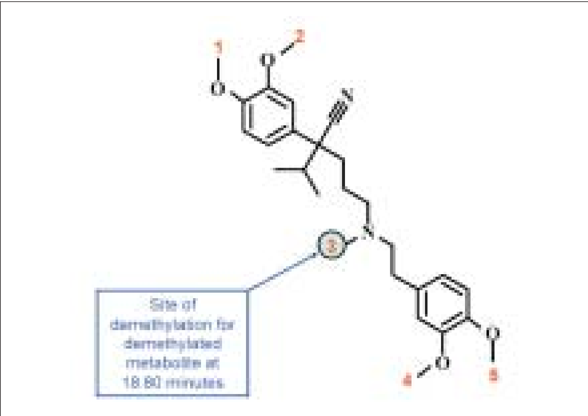

Figure 9. Structure of verapamil showing the site of demethylation for a metabolite.

Conclusion

The utility of the Metabolite ID and Mass Frontier software packages for identifying metabolites and elucidating their structures was demonstrated using a microsomal incubation of the drug, verapamil. Specifically, Metabolite ID quickly identified 17 different distinct types of modifications. It was shown that with Metabolite ID, a variety of different search tools can be employed to more completely characterize the metabolites in a sample. Mass Frontier was shown to be capable of assigning the specific sites of modification of metabolites using their MS^2 spectra.

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