

In-Vitro Metabolism Studies Using Data-Dependent™ LC/MSⁿ

Chromatography and Mass Spectrometry Application Note

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*The data presented here can be acquired using the Finnigan LCQ
series of ion trap mass spectrometers.*

Introduction

An integral part of the process by which a new drug candidate is evaluated and characterized involves the investigation of its rates and routes of metabolism. Due to their convenience, relative simplicity and reliability, *in-vitro* systems are used early in the drug discovery process to compare the biotransformation pathways across different species and to gain preliminary information on the metabolic routes to be expected in humans.

The current methodologies to characterize drug metabolites generally utilize LC/MS and LC/MS/MS, but frequently the data obtained is not sufficient to locate the site of metabolism on a candidate molecule. The Finnigan LCQ series, with their ability to perform multi-stage MS fragmentation, offer MS³ and MS⁴ routinely during an HPLC run. These second and third order product ion spectra afford data that allow metabolite identification with greater specificity. An additional strength of the Finnigan LCQ series is their ability to perform automated Data-Dependent experiments. This means that the mass spectrometer makes real-time decisions about which MS experiment to perform based on the spectrum just acquired.

This approach will be illustrated with the example of the analysis of metabolites derived from glyburide (glibenclamide), a potent sulfonylurea drug.^{1,2}

Goal

In this report, the application of benchtop ion trap API mass spectrometry to characterize *in-vitro* metabolites is discussed. The utility of Data Dependent MS/MS²/MS³ analyses, where the mass spectrometer makes “real-time” decisions about the experiment to be performed, are demonstrated using the characterization of glyburide metabolites as an example.

Experimental Conditions

Microsomal fractions were prepared from rat, dog, monkey, and human liver as described previously.³ Reaction mixtures (250 µL) with 5 or 50 M glyburide, 1 mg of microsomal protein/mL, 0.1 M potassium phosphate pH 7.25, 1 mM NADP, 10 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase/mL were incubated at 37 °C for 30 min. They were then quenched by addition of 250 µL of acetonitrile and the precipitate was removed by centrifugation. The supernatant was diluted with 500 µL of 10 mM ammonium acetate, pH 5.0, before analysis.

Metabolic products were separated using a Prodigy™ 5 mM C8 150×2 mm column with a 10×2 mm guard column. Solvent A was 10 mM ammonium acetate, pH 5.0 and solvent B was acetonitrile. The metabolites were eluted using the following linear gradient: 0 min, 30%B; 30 min, 30%B; 35 min, 60%B; 60 min, 100%B; flow 0.2 mL/min. The mass spectrometer used was a Finnigan LCQ. The entire 0.2 mL/min flow was directed into the source of the mass spectrometer without splitting, with the first 2.1 min diverted to waste using the built-in automated divert valve. The ion transfer tube was operated at 250 °C and sheath and auxiliary gases were set to 80 and 25, respectively. A relative collision energy of 25% was used for all MS experiments with an isolation width of 7.0 u to allow passage of ³⁵Cl and ³⁷Cl isotope peaks within a single scan.

Strategy

An effective strategy for metabolite characterization is to 1) obtain MSⁿ data on the unmetabolized drug (used as a reference for following experiments), 2) perform a Data-Dependent experiment to screen the metabolites, and 3) conduct selective multi-stage MSⁿ experiments to locate more specifically the site of metabolism. Since the samples utilized microsomal preparations fortified with NADPH, only oxidative metabolism occurred. This made the analysis slightly simpler since the most likely metabolic products were an unmodified parent, mono-oxygenated metabolites, and possibly di- or tri-oxygenated metabolites. A list of ions corresponding to the [M+H]⁺ for glyburide and its potential metabolites was entered in

Key Words

- Metabolite characterization
- Finnigan™ LCQ™ ion trap
- Sensitivity
- *In-vitro* metabolites

the method setup for the analysis in order to prevent the instrument from obtaining spectra on irrelevant, but potentially intense, ions in the samples.

The Finnigan LCQ was set up to perform the following Data-Dependent experiment: When one of the ions from the list was detected in MS (and above a user-defined threshold), the mass spectrometer automatically acquired a product ion mass spectrum (MS^2) for this ion. Next, a second order product ion (MS^3) mass spectrum was collected for the base peak from the MS^2 spectrum. This $MS/MS^2/MS^3$ sequence was repeated throughout the duration of the chromatographic peak. At the end of the peak, the mass spectrometer returned to MS mode until another ion from the mass list was detected and the cycle was repeated for this new ion.

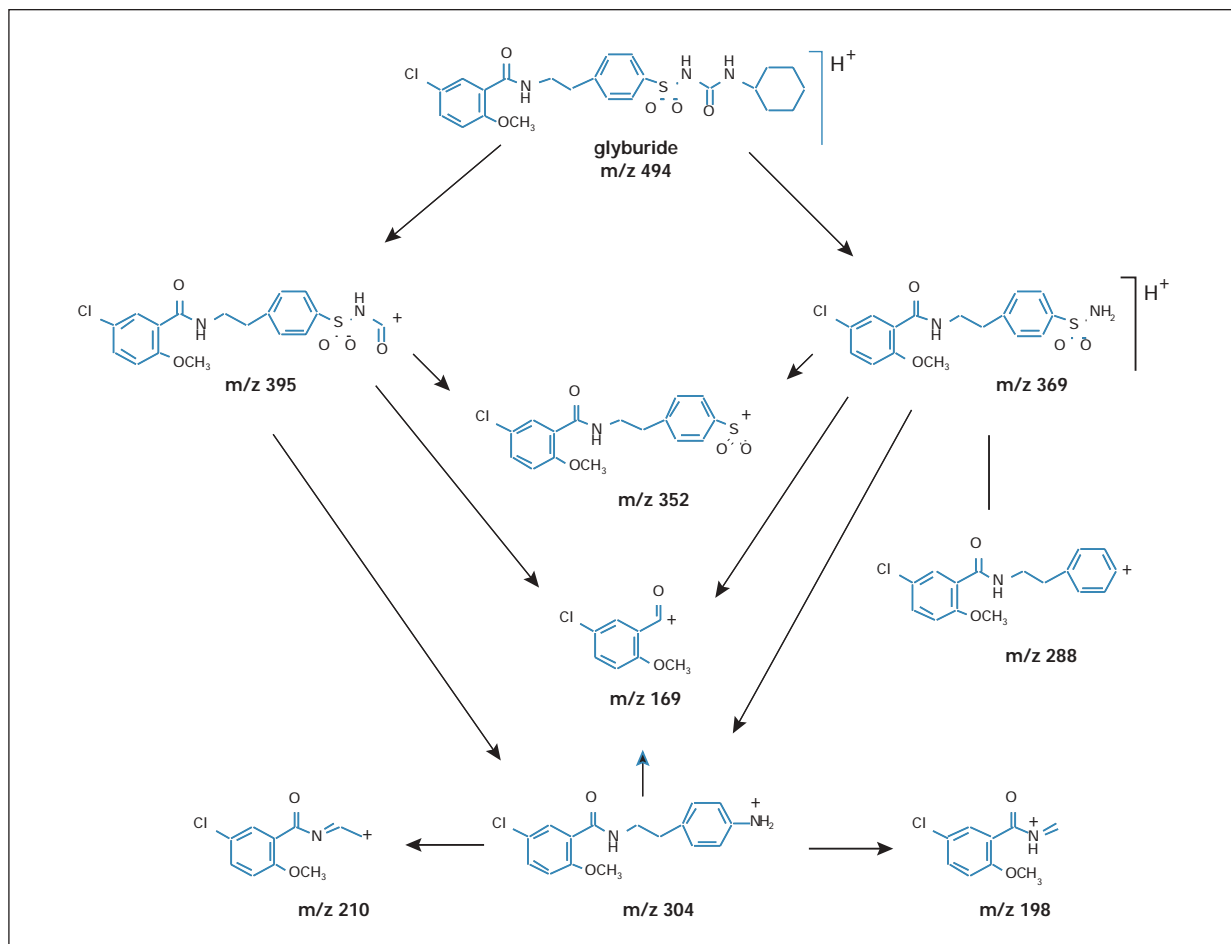
Having obtained MS, MS^2 and MS^3 data, the retention time, molecular weight and significant structural information were obtained in this one analysis. Additional structural data were collected from subsequent LC/MS^n analyses designed to collect MS^n data for specific ions of interest. Although the Data Dependent analyses provide a tremendous amount of data from a single chromato-

graphic analysis, a simple LC/MS analysis is still frequently valuable, especially when there are closely eluting analytes with the same molecular weight. The data from an LC/MS analysis will have more data points (since it is not interrupted with MS^n scans) to describe the chromatographic peaks better and reveal shoulders or minor peaks more clearly.

Results and Discussion

Glyburide was characterized using MS^2 , MS^3 , and MS^4 experiments in positive ion mode during an infusion of a 1 g/mL stock solution. A 7 amu isolation width was used to collect both ^{35}Cl - and ^{37}Cl -containing ions. Thus, the product ions included the Cl isotope pattern. These data are summarized in Scheme 1. These spectra were used as references to aid in interpretation of the spectra of metabolites. Shifts in masses observed in spectra for metabolites relative to spectra for glyburide, as well as differing fragmentation patterns facilitated characterization of the metabolite structures.

An LC/MS^n experiment was performed on a microsomal sample. The Data-Dependent analysis afforded



Scheme 1

MS, MS² and MS³ data—providing retention time, molecular weight and structural information. The reconstructed ion chromatogram (RIC) reproduced in Figure 1 indicated that there were seven metabolites that resulted from the incorporation of a single oxygen molecule (at 8.72, 9.92, 10.65, 11.89, 13.01, 16.79 and 23.87 min).

The MS spectra of all these metabolites were identical (see Figure 2) affording an [M+H]⁺ ion at *m/z* 510. The MS² spectra for the first six metabolites (see Figure 3) afforded ions at *m/z* 369, 395, 492, 352 and 169. With the exception of the ion at *m/z* 492 (elimination of H₂O) these ions were identical to those observed in the MS²

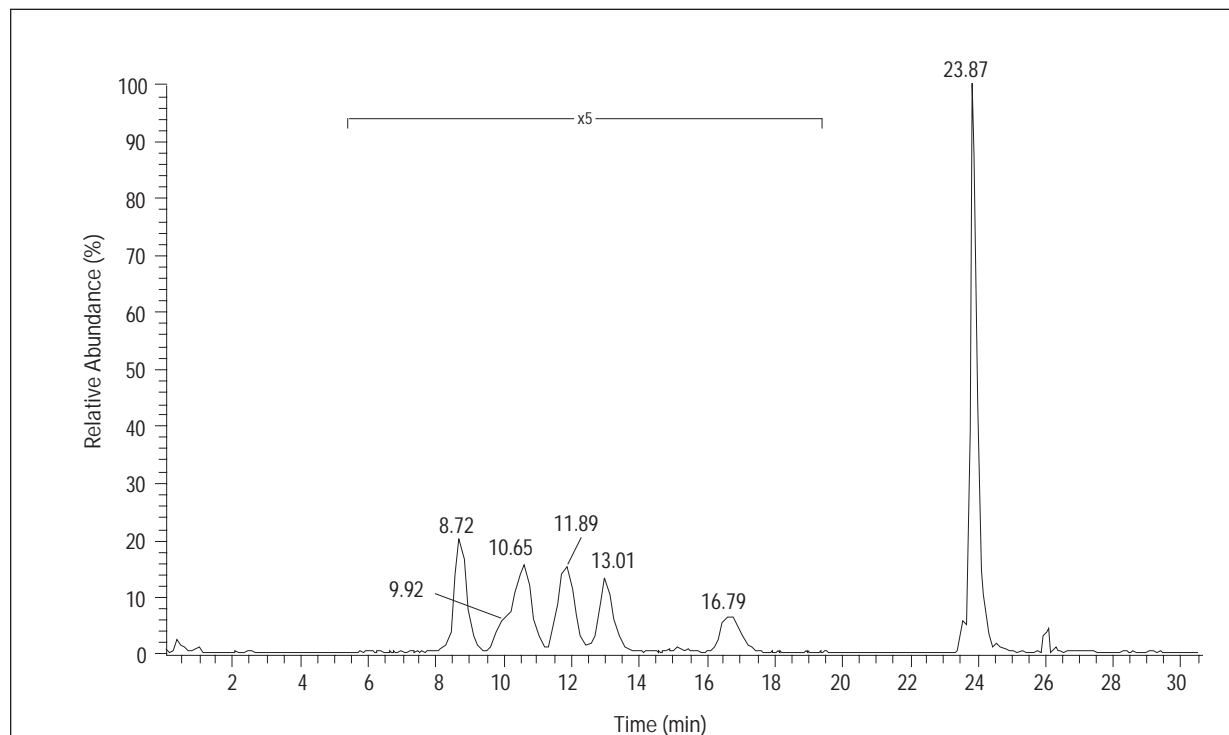


Figure 1. RIC *m/z* 510

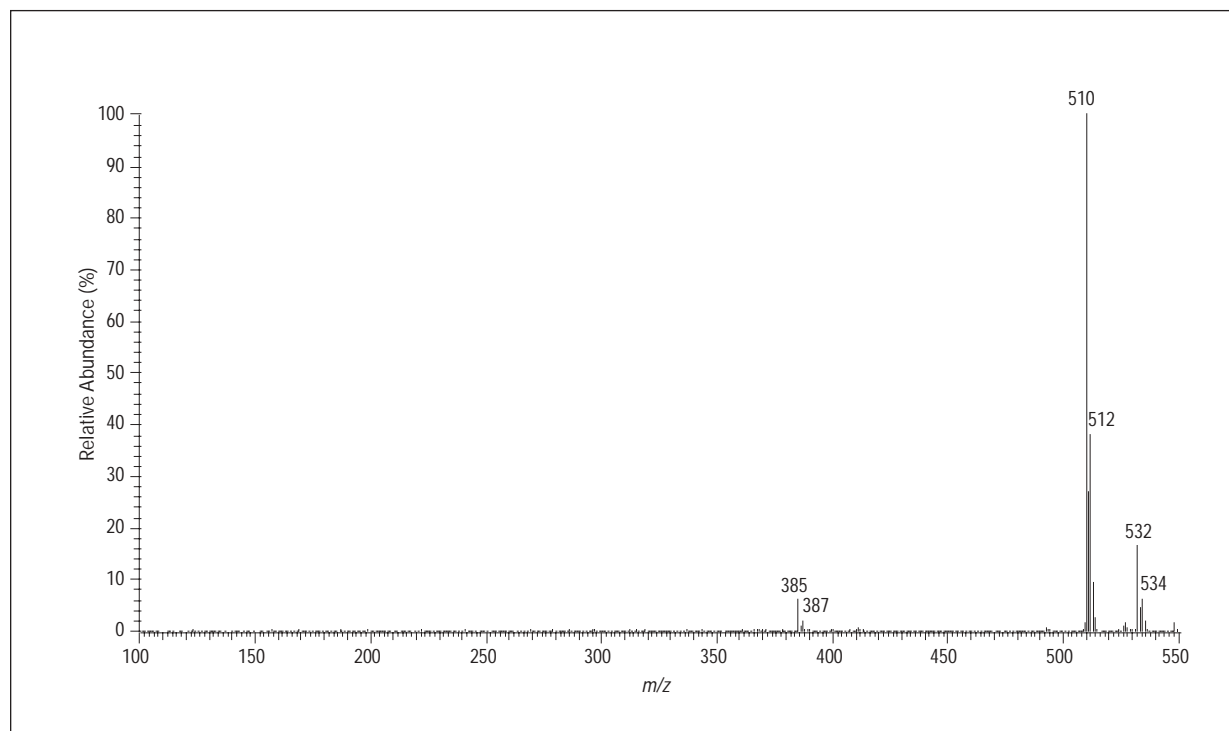


Figure 2. MS spectra

spectra of glyburide. This indicates that the site of metabolism was the cyclohexyl ring, since the loss of the cyclohexyl moiety resulted in an identical spectrum. Additionally the MS³ spectra for these metabolites were identical to the MS³ spectrum derived from glyburide.

The MS² spectrum (see Figure 4) obtained from the metabolite at 23.87 min afforded ions at *m/z* 385, 367, 411, 492 and 169, indicating that the site of hydroxylation was not the cyclohexyl moiety. The data from the MS² spectrum in conjunction with the data from the MS³ spectrum (see Figure 5) allowed the fragmentation pathway to be delineated (see Scheme 2).

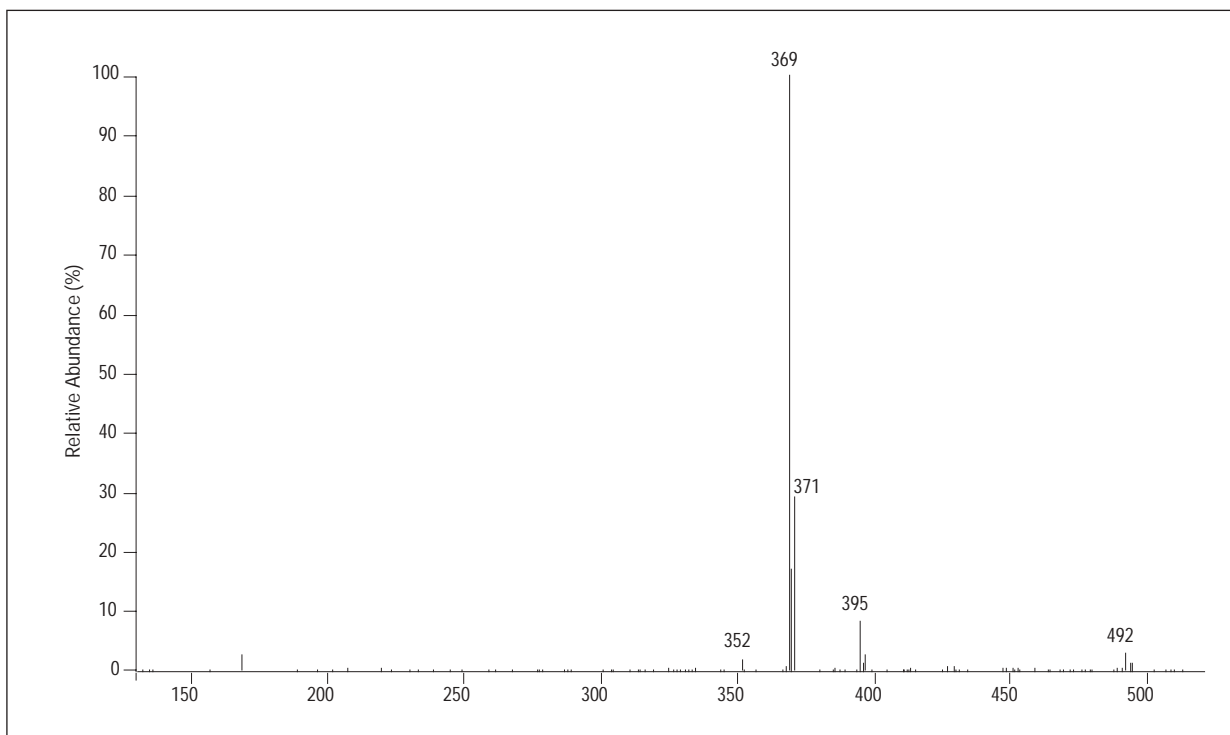


Figure 3. MS² spectra of early eluting metabolites

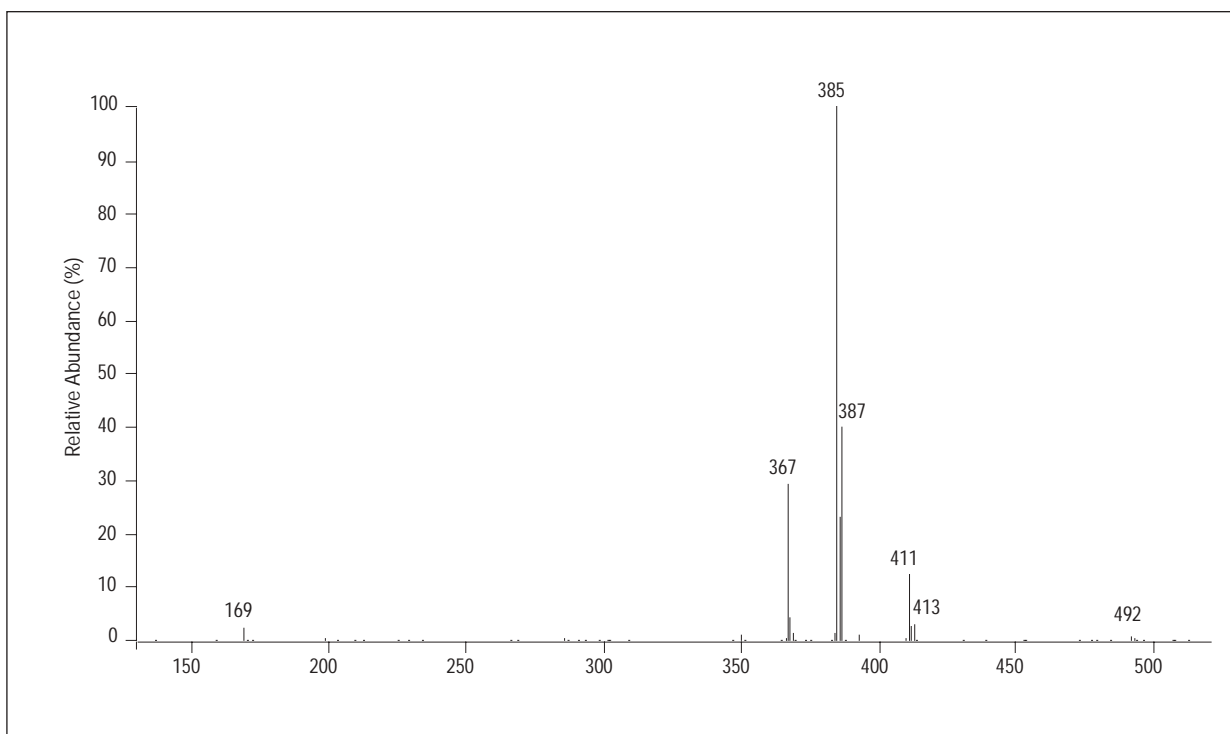


Figure 4. MS² spectrum of metabolite at 23.87 min

The data obtained thus far were compatible with three different metabolite structures (see Figure 6). To enable a more specific structural assignment, a further LC/MSⁿ experiment was performed using negative ion mode. These data are summarized in Scheme 3. The ions observed at *m/z* 323, 198 and 134 indicate that structure C in Figure 6 was incorrect.

Thus only two LC/MSⁿ experiments enabled the novel metabolite at 23.87 min to be identified as hydroxylation of the ethyl chain at either the benzylic position, or alpha to the amide nitrogen (structures A and B in Figure 6).

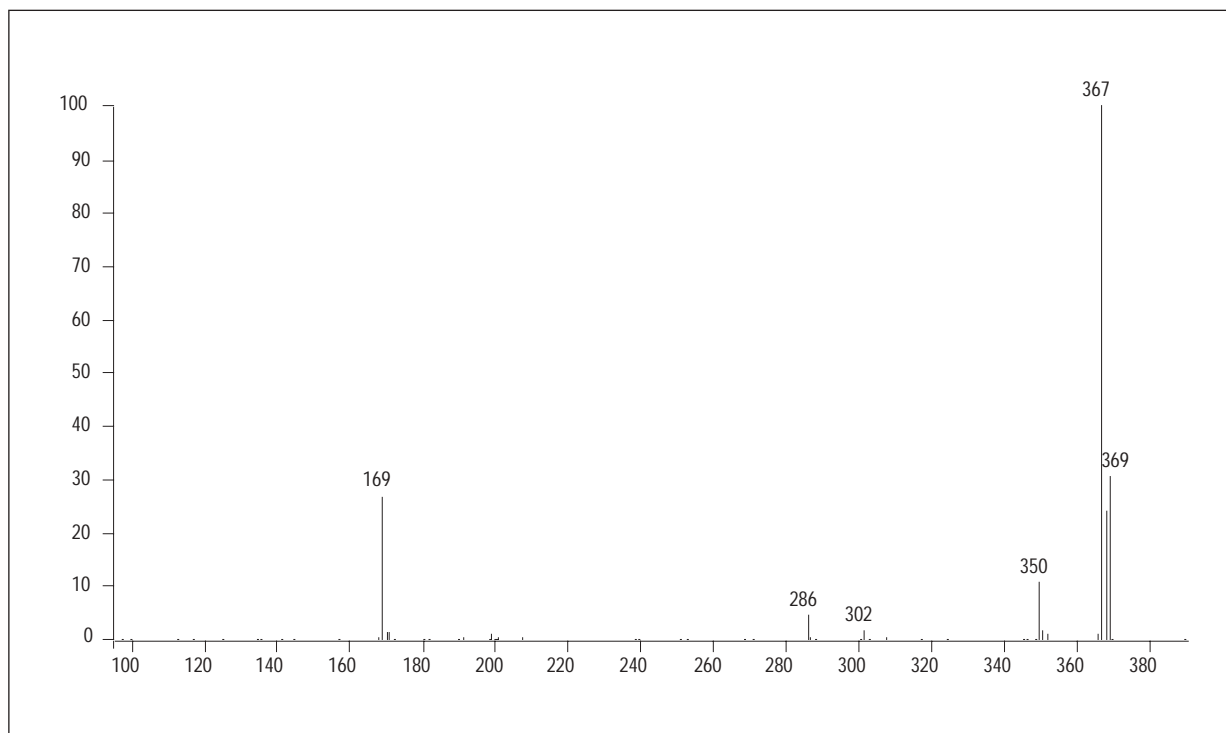


Figure 5. MS³ spectrum of metabolites at 23.87 min

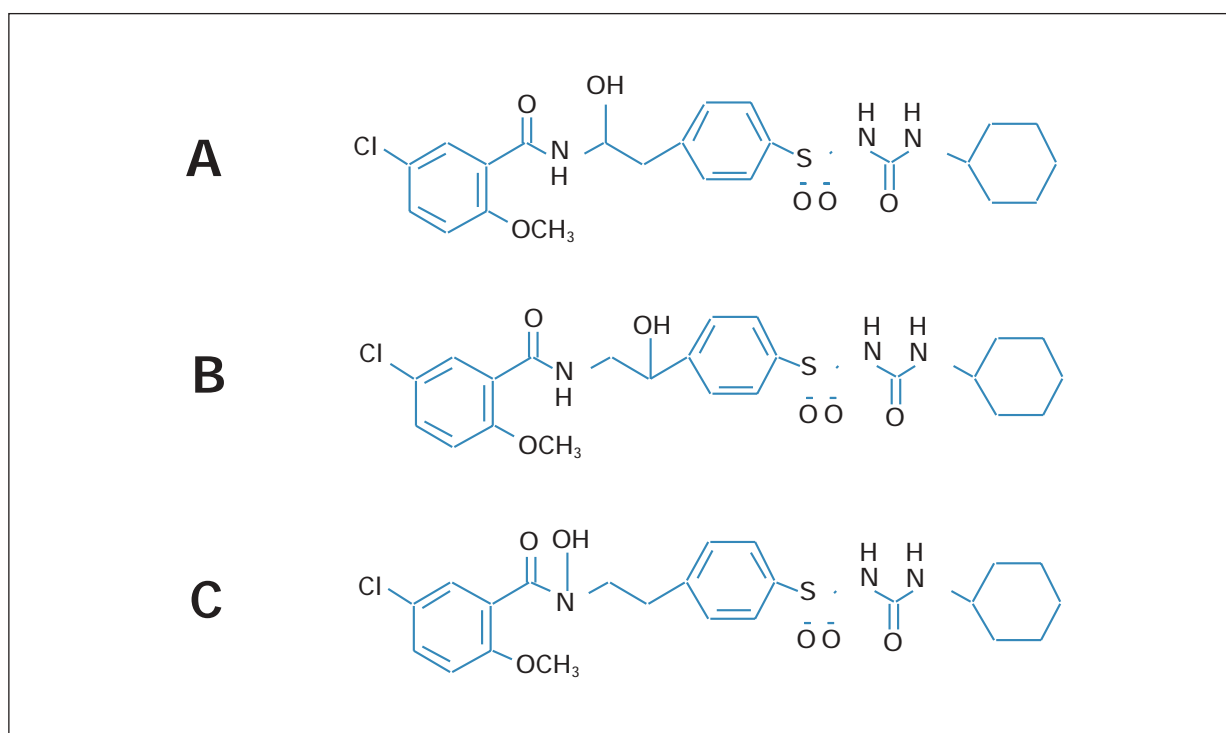
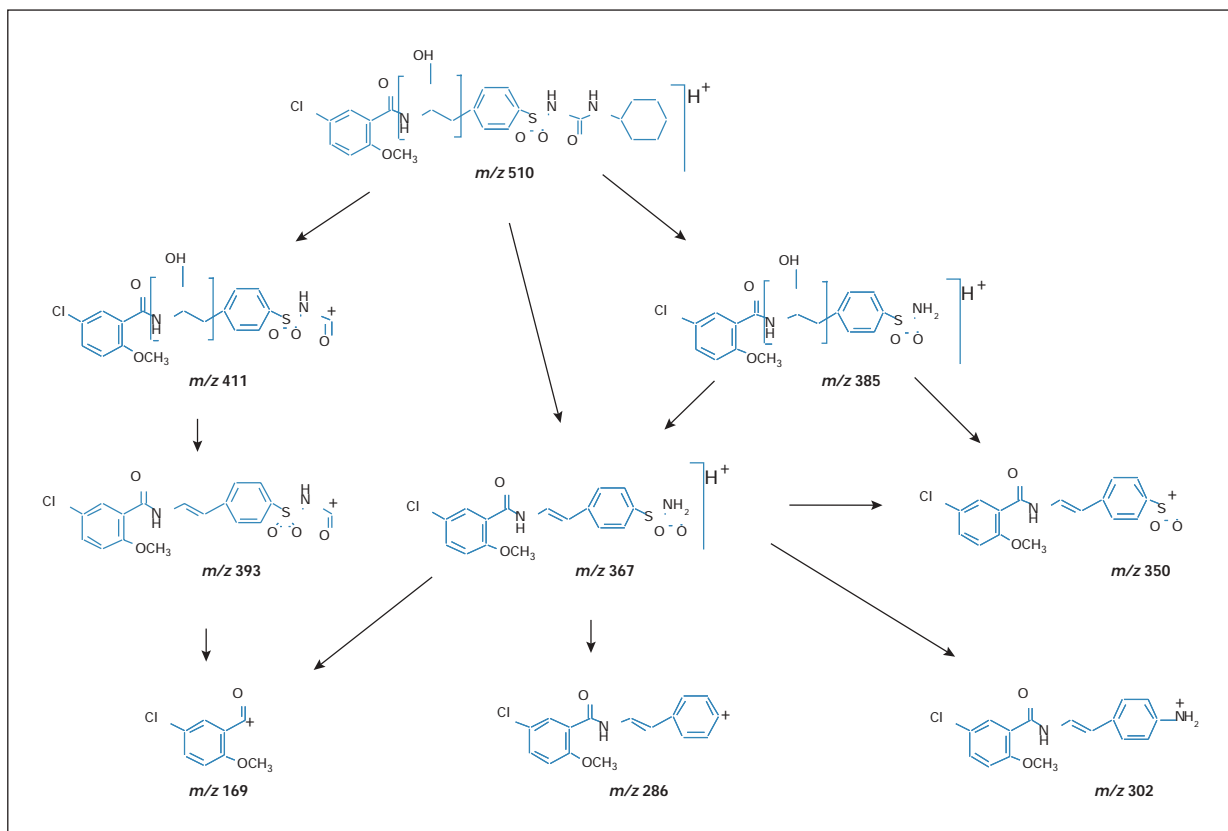
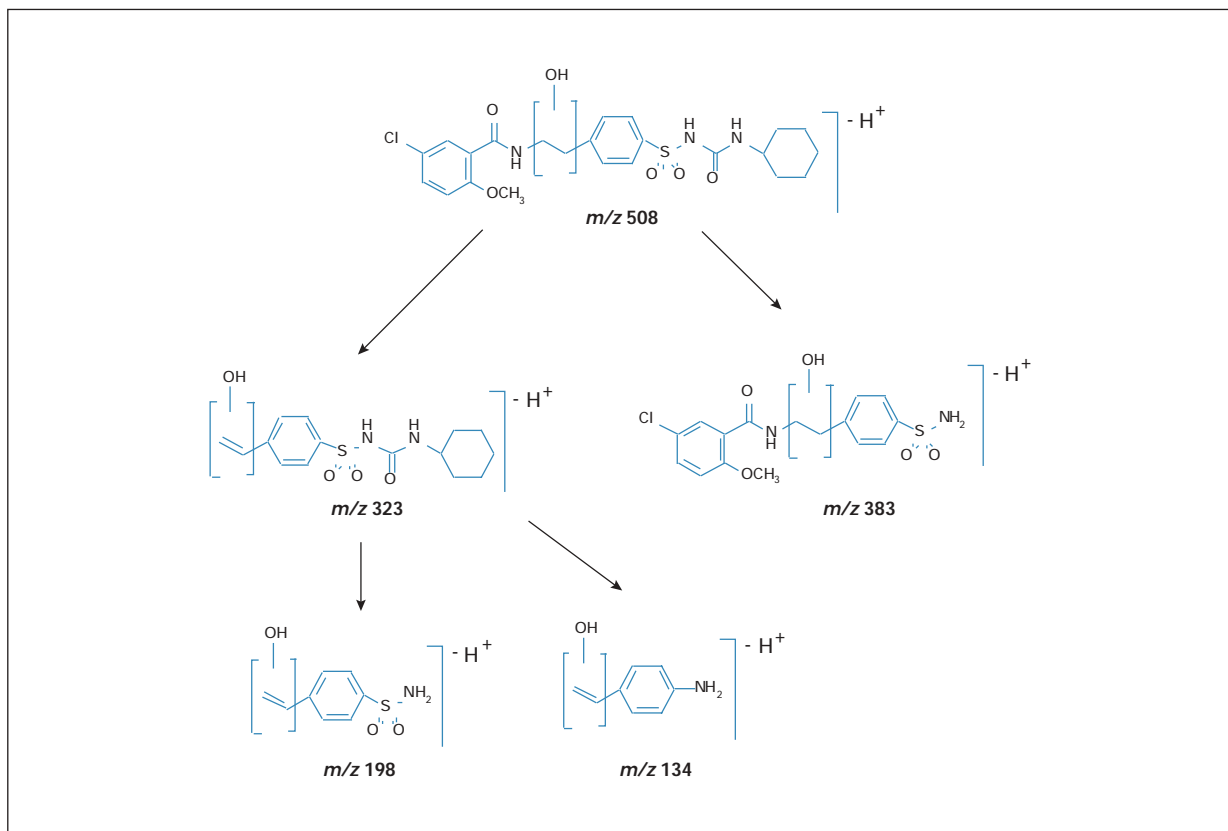


Figure 6. Possible structures of metabolite at 23.87 min



Scheme 2



Scheme 3

Conclusions

With the use of Data-Dependent MS/MS²/MS³ analyses seven metabolites of glyburide were structurally characterized within two LC/MS analyses. This approach not only afforded molecular weight, retention time, and structural information with greater specificity than LC/MS and LC/MS/MS using a triple quadrupole, but reduced the length of analysis time.

References

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