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Liquid Chromatography/Mass Spectrometry Methods for Distinguishing *N*-Oxides from Hydroxylated Compounds

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This study describes the application of liquid chromatography/mass spectrometry (LC/MS) methods for distinguishing between aliphatic and aromatic hydroxylations and between hydroxylations and N-oxidations. Hydroxylations and N-oxidations are common biotransformation reactions of drugs. Electrospray (ESI) and atmospheric pressure chemical ionization (APCI) were used to generate ions from liquid chromatographic effluents. ESI-MS, ESI-MS/MS, APCI-MS, and APCI-MS/MS experiments were performed on several metabolites and derivatives of loratadine (a long-acting and nonsedating tricyclic antihistamine) using an ion trap mass spectrometer (LCQ) and a triple-quadrupole mass spectrometer (TSQ). The observations are as follows: (1) LC/ESI-MS produced predominantly $[M + H]^+$ ions with minor fragmentation. (2) LC/ESI-MS/MS data, however, showed a predominant loss of water from metabolites with aliphatic hydroxylation while the loss of water was not favored when hydroxylation was phenolic. *N*-Oxides (aromatic and aliphatic) showed only a small amount of water loss in the MS/MS spectra. (3) Under LC/APCI-MS conditions, aliphatic hydroxylation could be readily distinguished from aromatic hydroxylation based on the extent of water loss. In addition, *N*-oxides produced distinct $[M + H - O]^+$ ions. These $[M + H - O]^+$ ions were not produced in the APCI-MS spectra of hydroxylated metabolites. (4) Similar to the ESI-MS/MS spectra, the APCI-MS/MS spectra from the $(M + H)^+$ ions of *N*-oxides yielded a small amount of water loss but no $[M + H - O]^+$ ions. These results indicate that LC/APCI-MS can be used to distinguish between hydroxylated metabolites and *N*-oxides.

Background. Drugs and xenobiotics, when exposed to living systems, undergo biotransformation to form polar metabolites that can be readily eliminated from the body. The two principal pathways involved in biotransformation, also known as metabolism, consist of two stages of reaction, namely, phase I and phase II.[1] The phase I reaction consists of oxidation, reduction, and hydrolysis, while phase II processes consist of congugate formations, e.g., glucuronidation, sulfation, amino acid conjugation, etc. Identification of these metabolites is an important part of drug development and registration.

A common phase I oxidative reaction involves hydroxylation or oxidation of heterocyclic atoms, such as nitrogen. The metabolites resulting from the oxidation of nitrogen are known as *N*-oxides. *N*-Oxide metabolites have generated some interest because several *N*-oxides have been shown to be carcinogenic[2,3] and/or to exhibit toxicological effects.[4] The identification of *N*-oxides represents a challenge because both hydroxylation and N-oxidation result in an increase in molecular weight by 16. The molecular ions of these metabolites are indistinguishable by mass spectrometry.

Current Methods: Advantages and Disadvantages. Under electron impact (EI) and chemical ionization (CI) conditions, mass spectra of *N*-oxides exhibit fragment ions corresponding to loss of an oxygen atom from the molecular ion and hydroxylated metabolites exhibit fragment ions corresponding to loss of an OH or H_2O.[5] Thus, distinguishing *N*-oxides from hydroxylated metabolites is possible using EI and CI. Unfortunately, these techniques suffer from lack of sensitivity and inability to couple to a reversed-phase liquid chromatography (LC) system. Therefore, direct analysis of metabolites in biological fluids and extracts is not possible. Further, in the EI spectra of *N*-oxides, which are often thermally labile, molecular ions are absent and $[M - O]^+$ ion is detected as the base peak.[6] Lay et al. examined *N*-oxides of six antihistamines using fast atom bombardment (FAB) mass spectrometry.[7] A characteristic fragment ion corresponding to loss of elemental oxygen was observed only when a nonreductive FAB matrix was used. In the presence of a reducing FAB matrix, such as thioglycerol, the molecular ion is absent, thus providing no information about the molecular weight of the metabolite. Similar to EI and CI methods, FAB is also relatively insensitive, difficult to interface with LC systems, and generates many fragment ions. The presence of fragment ions together with ions of reduced compounds can make the spectra difficult to interpret, and information about minor metabolites can be easily lost when

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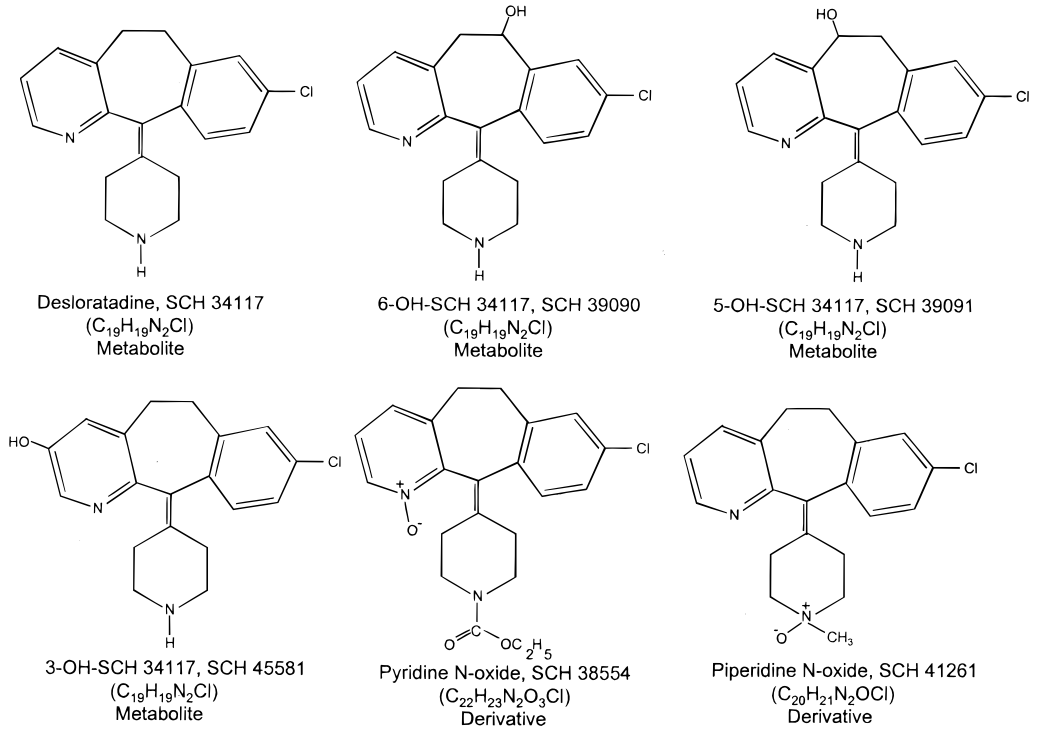


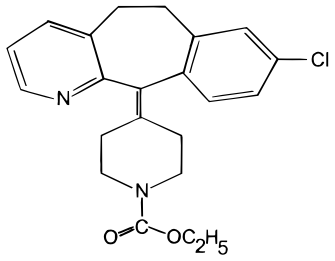
Figure 1. Chemical structures of loratadine, its metabolites and derivatives.

several metabolites are present in the sample. Again, direct analysis of biological fluids or extracts is not possible.

Parker et al.[8] evaluated the applicability of LC/thermospray ionization mass spectrometry for alkaloid *N*-oxides. Under thermospray conditions, *N*-oxides spectra exhibited fewer protonated molecular ions and more abundant fragment ions corresponding to loss of an oxygen atom and water from the protonated molecular ion. Unfortunately, thermospray also suffers from many of the disadvantages of EI, CI, and FAB. Additionally, each of the above techniques requires 1−2 μg of isolated compound to obtain an interpretable mass spectrum. In most cases, obtaining pure 1−2 μg of a minor metabolite is at best difficult.

Present Investigation. The recent development of electrospray ionization (ESI)[9] and atmospheric pressure chemical ionization (APCI)[10] techniques has dramatically increased the application of mass spectrometry in the pharmaceutical industry, more importantly for the identification and quantitation of drugs and metabolites in biological fluids and extracts.[11] The factors that fueled this increase include the ability to (1) couple easily to HPLC systems with a wide range of flow rates and solvent systems, (2) detect low nanogram- or picogram-level materials, and (3) generate molecular ions of polar and nonpolar compounds. ESI-LC/MS/MS and APCI-LC/MS/MS are now routinely used for the identification of metabolites directly from biological fluids and extracts.

We evaluated atmospheric pressure ionization and collision-induced dissociation (MS/MS) for distinguishing between hydroxylated metabolites and *N*-oxide derivatives of loratadine. Loratadine (SCH 29851), a long-acting and nonsedating tricyclic



Loratadine, SCH 29851

antihistamine,[12] is rapidly absorbed, extensively metabolized, and rapidly excreted in urine and feces. The biotransformation of loratadine involves decarboethoxylation to desloratadine (SCH 34117), hydroxylation in the 3 (SCH 45581), 5 (SCH 39091), and 6 (SCH 39090) positions, and glucuronide conjugation (Figure 1).

LC/ESI-MS, LC/APCI-MS, LC/ESI-MS/MS, and LC/APCI-MS/MS spectra obtained on the ion trap mass spectrometer were compared to aid in identification of these metabolites and several *N*-oxide derivatives. For further comparison, LC/ESI-MS/MS spectra were obtained using a triple-quadrupole mass spectrometer.

EXPERIMENTAL SECTION

All compounds were obtained from the Department of Chemical Research at Schering-Plough Research Institute. A 40 ng/μL solution was prepared in methanol, and 200 ng of each analyte

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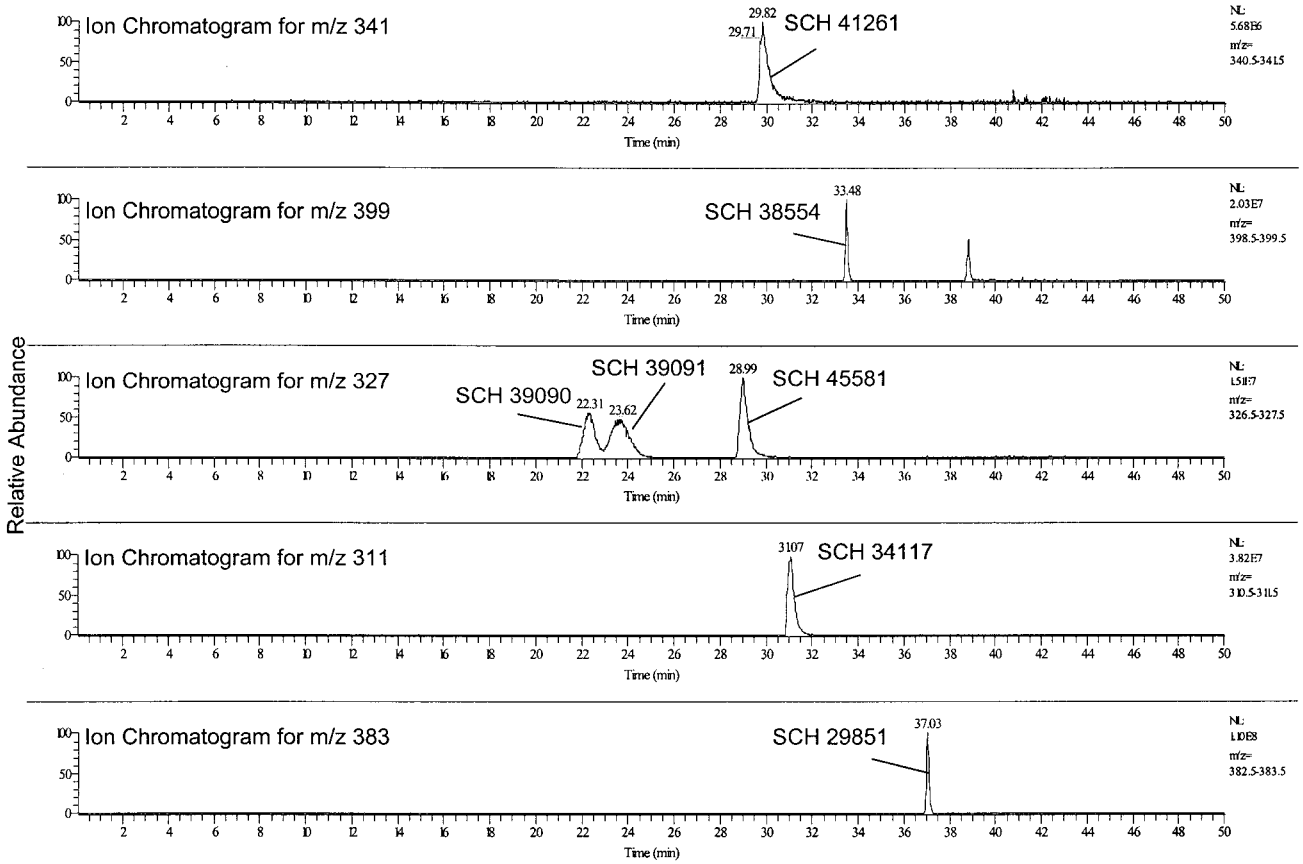


Figure 2. Representative LC/ESI-MS ion chromatograms of loratadine and metabolite and derivative standards (200 ng each on column) obtained using a LCQ ion trap mass spectrometer.

was injected for analysis. Ammonium acetate (ACS grade) was purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile and methanol were from Burdick and Jackson (Muskegon, MI). Water was purified using the Millipore Milli-Q_{plus} water purification system (Bedford, MA).

The HPLC system consisted of an Alliance model 2690 pump (Waters Corp., Milford, CA) with an autoinjector, model 2487 UV detector (Waters Corp.), an Inertsil C8 guard column (Metachem Technologies, Torrance, CA), and a 150 × 4.6 mm Inertsil C8 analytical column (Metachem Technologies) maintained at 40 °C. The mobile phase consisted of 1 mM ammonium acetate solution adjusted to pH 6 with acetic acid (A) and acetonitrile (B). A stepwise gradient elution was run with A/B ratios of 90/10, 72/28, 10/90, 10/90, 90/10, and 90/10 at 0, 25, 40, 50, 51, and 65 min, respectively, at a constant flow rate of 1.0 mL/min. For all LC/ESI-MS experiments, the column effluent was split to divert 15−25% into the mass spectrometer and the balance was analyzed by a radiometric flow detector or sent to waste. For all APCI experiments, the total column effluent (1 mL/min) was sent to the mass spectrometer.

LC/MS (scan range 100−800 Da) and LC/MS/MS experiments were performed using either an ion trap (LCQ, Finnigan MAT, San Jose, CA) mass spectrometer equipped with either an ESI or APCI ion source or a triple-quadrupole mass spectrometer (TSQ, Finnigan MAT) equipped with an ESI source. Operating conditions for ESI in electrospray ionization mode were as follows: spray voltage, 4500 V; capillary temperature, 350 °C; sheath gas (N_2), 80 PSI; auxiliary gas (N_2), 40 mL/min; collision gas (Ar), 1.5−3 mTorr. The collision energy used for LC/MS/MS experiments on the triple-quadrupole mass spectrometer was 25 or 35 eV. ESI conditions on the ion trap mass spectrometer were as follows: spray voltage, 5.0−6.6 kV; capillary temperature, 250−270 °C; capillary voltage, 5−6 V; tube lens offset, 35−45 V; sheath gas (N_2), 70 PSI; auxiliary gas (N_2) 3 mL/min. APCI conditions on the ion trap mass spectrometer were as follows: discharge current, 6 μA; vaporizer, 450 °C; capillary temperature, 150−175 °C; capillary voltage, 5−6 V; tube lens offset, 50−60 V; sheath gas (N_2), 80 PSI; auxiliary gas (N_2), 10 mL/min; sample flow rate, 1 mL/min. For all MS/MS experiments, the precursor isolation window was set at 1 amu and the collision energy was set at 15−20%.

RESULTS AND DISCUSSION

LC/ESI-MS Experiments. All of the standards (Figure 1) generated protonated molecular ions under LC/ESI-MS conditions. The retention times of SCH 39090 (6-OH desloratadine), SCH 39091 (5-OH desloratadine), SCH 45581 (3-OH desloratadine), SCH 41261 (piperidine-*N*-oxide), desloratadine, SCH 38554 (pyridine-*N*-oxide) and loratadine were 22.3, 23.6, 29.0, 29.8, 31.1, 33.5 and 37.0 min, respectively (Figure 2). In addition to predominantly protonated precursor ions, characteristic fragment ions were observed at relatively low abundance. For the parent drug, loratadine, ions corresponding to $[M + H]^+$ and $[M + H - C_2H_5OH]^+$ were detected at m/z 383 and 337, respectively (Figure 3). For the metabolite formed through decarboethoxylation (desloratadine), ions corresponding to $[M + H]^+$, $[M + H -$

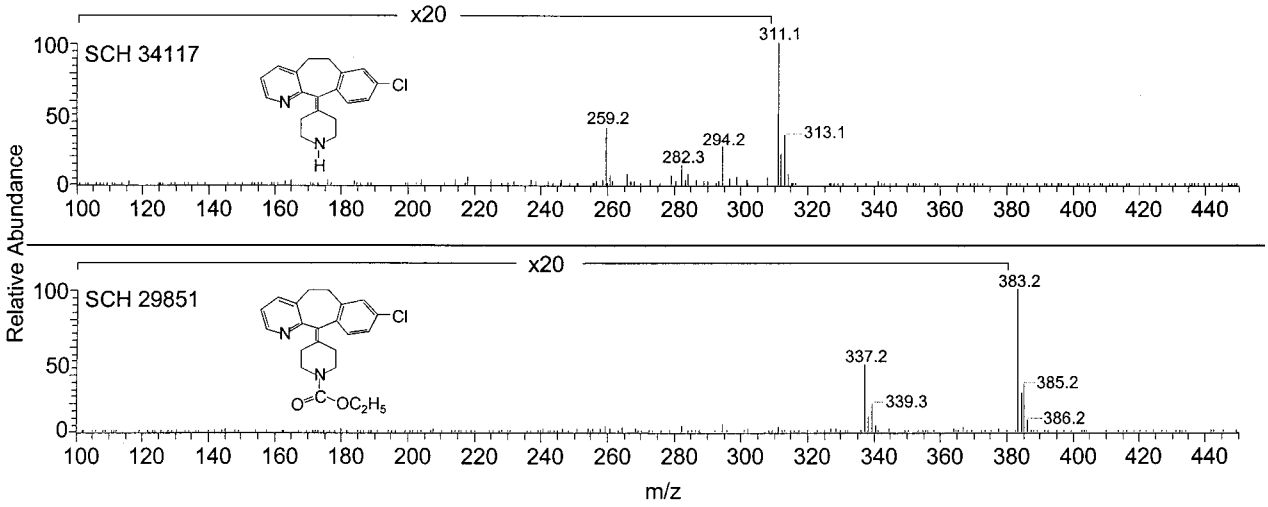


Figure 3. LC/ESI mass spectra of loratadine (bottom panel) and desloratadine (top panel) standards obtained using the ion trap mass spectrometer.

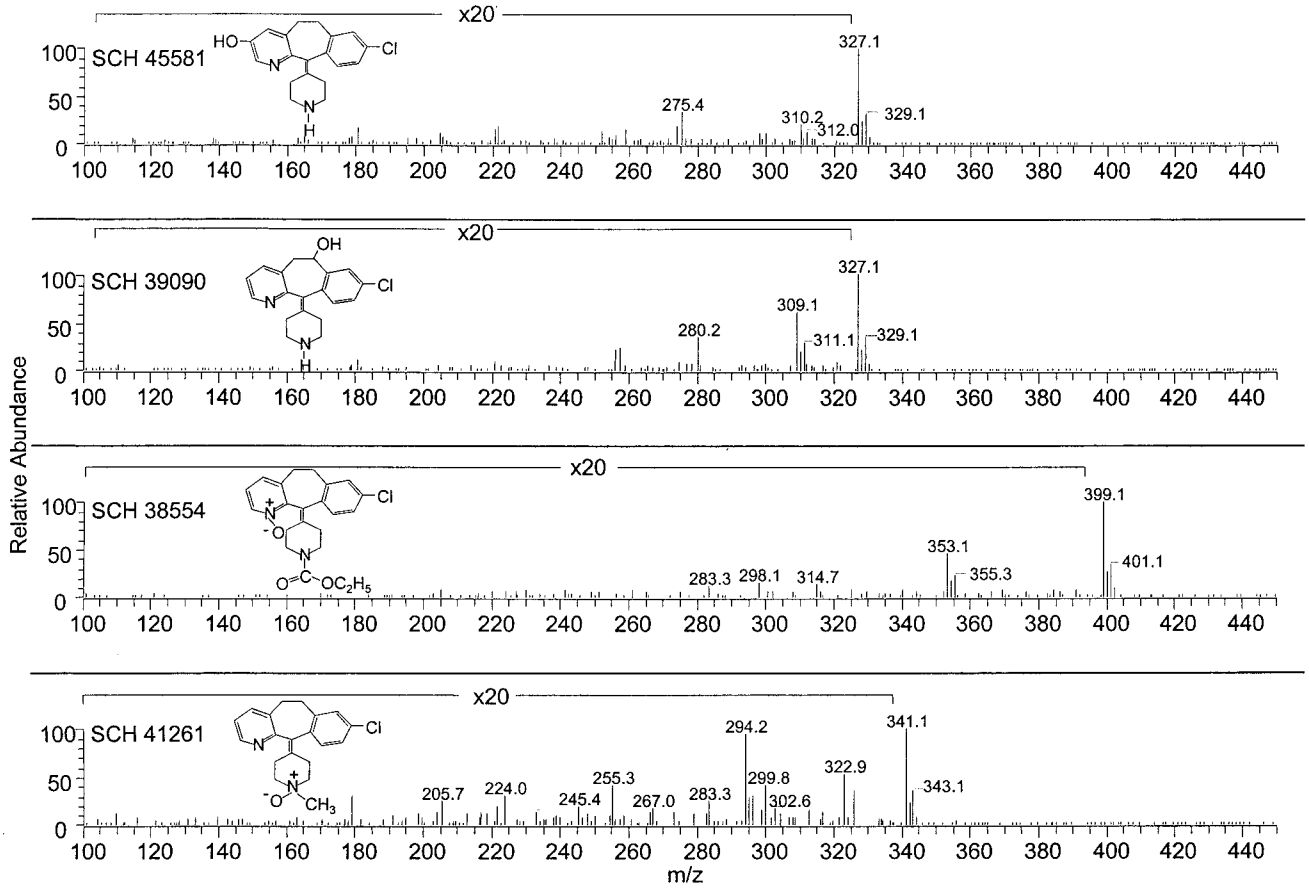


Figure 4. LC/ESI mass spectra of piperidine-*N*-oxide, pyridine-*N*-oxide, 6-OH desloratadine, and 3-OH desloratadine (from bottom to top) standards obtained using the ion trap mass spectrometer.

$NH_3]^+$, $[M + H - NH{=}CH_2]^+$, and $[M + H - NH_3 - Cl]^{\bullet+}$ were detected at m/z 311, 294, 282, and 259, respectively (Figure 3). For the hydroxylated metabolites, 5-OH desloratadine (spectrum is similar to that of 6-OH desloratadine) and 3-OH desloratadine, protonated molecular ions were detected at m/z 327 (Figure 4). Examination of LC/ESI mass spectra indicates that the spectrum of 3-OH desloratadine could be readily distinguished from those of 6-OH desloratadine and 5-OH desloratadine because the formation of m/z 309 (loss of water) was not favored when hydroxylation was on the aromatic ring (3-OH desloratadine). The mass spectrum (Figure 4) of the pyridine *N*-oxide derivative (SCH 38554) contained protonated molecular ion (m/z 399) and a fragment ion corresponding to $[M + H - C_2H_5OH]^+$ at m/z 353 (Figure 4). The mass spectrum (Figure 4) of piperidine *N*-oxide derivative (SCH 41261) contained protonated molecular ion (m/z 341) and fragment ions corresponding to $[M + H - H_2O]^+$ and $[M + H - H_2O - NH{=}CH_2]$ at 323 and 294, respectively. Unlike in EI, CI, and FAB ionization mass spectrometry methods,[5–7]

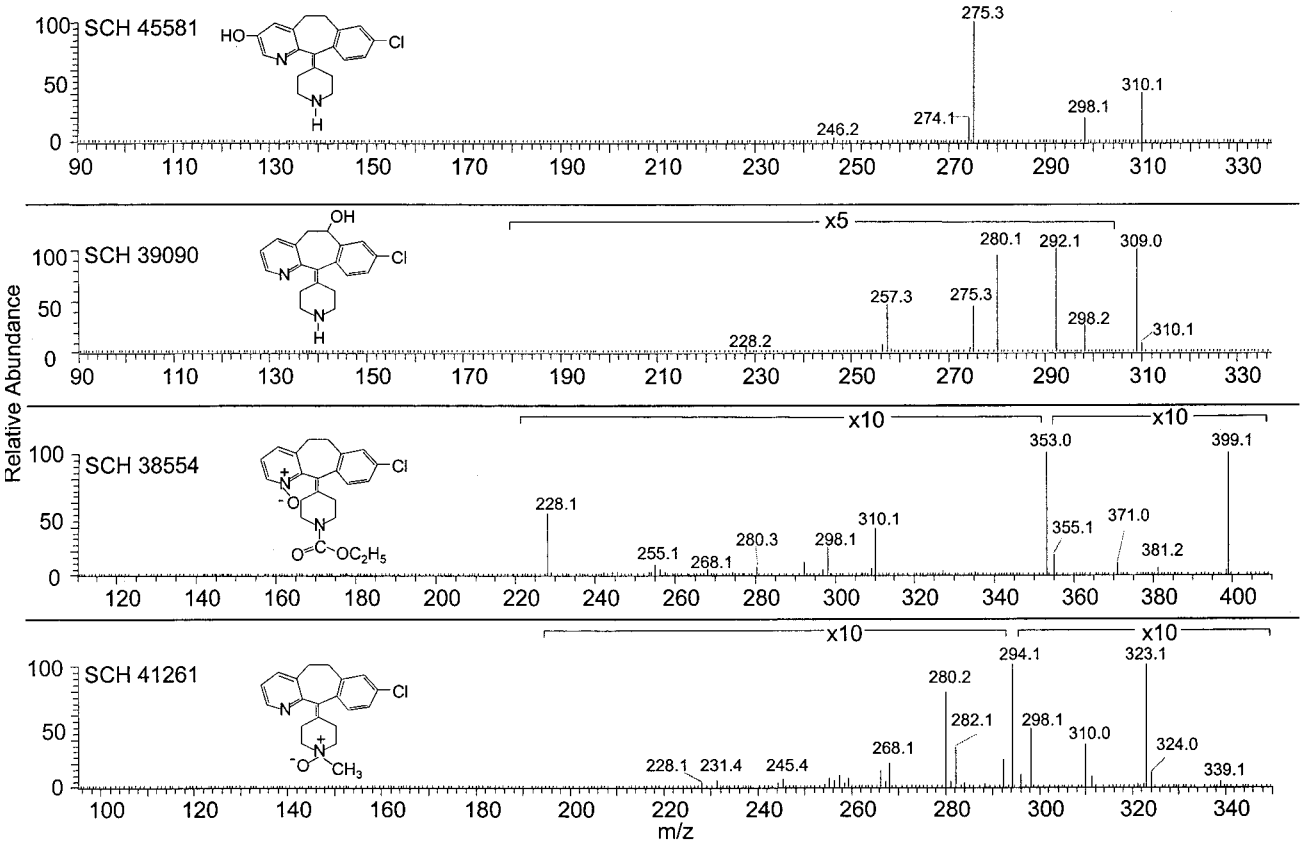
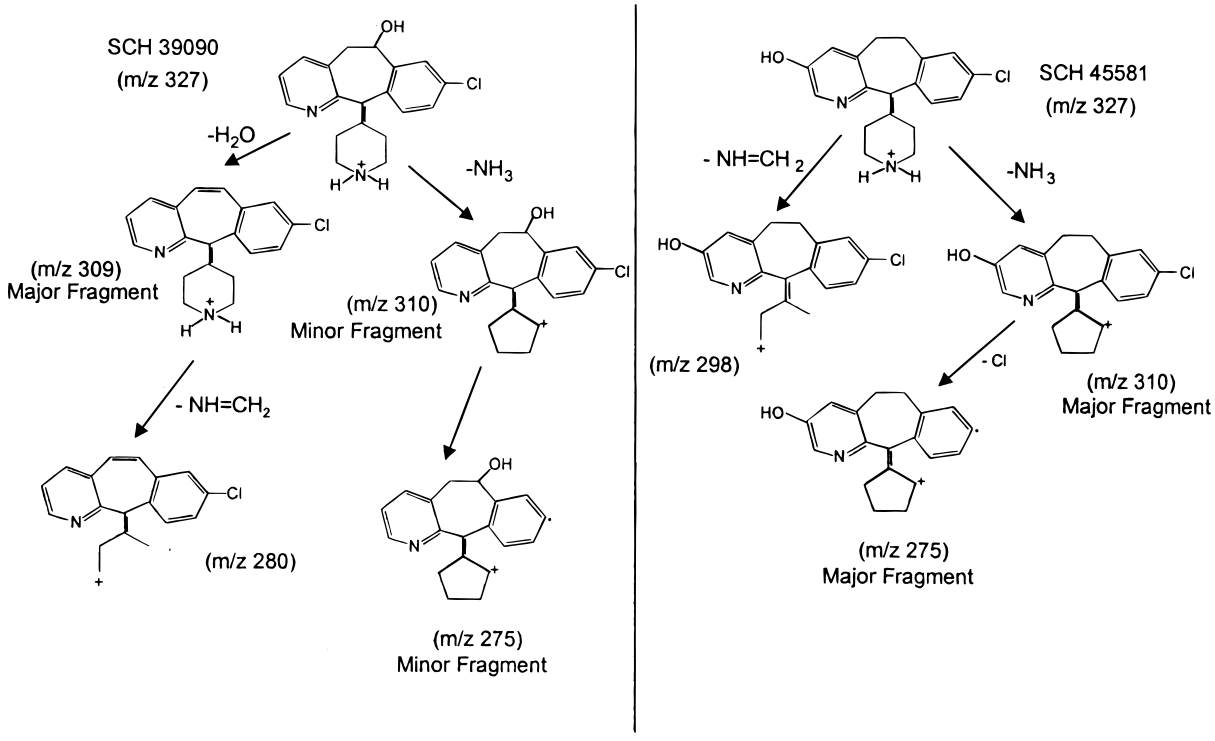


Figure 5. LC/ESI-MS/MS spectra of piperidine-*N*-oxide, pyridine-*N*-oxide, 6-OH desloratadine, and 3-OH desloratadine (from bottom to top) standards obtained using the ion trap mass spectrometer.

Scheme 1. Fragmentation Pathway of SCH 6-OH Desloratadine and 3-OH Desloratadine[a]



[a] These fragment ions were observed in both LC/ESI-MS and LC/ESI-MS/MS experiments

where the presence *N*-oxide metabolites are often missed due to thermal instability of the *N*-oxide metabolites, under ESI-MS conditions abundant protonated molecular ions were observed for *N*-oxides. However, LC/ESI mass spectra of the *N*-oxides and hydroxylated metabolites (Figure 4) did not provide any evidence to distinguish *N*-oxides from hydroxylated metabolites.

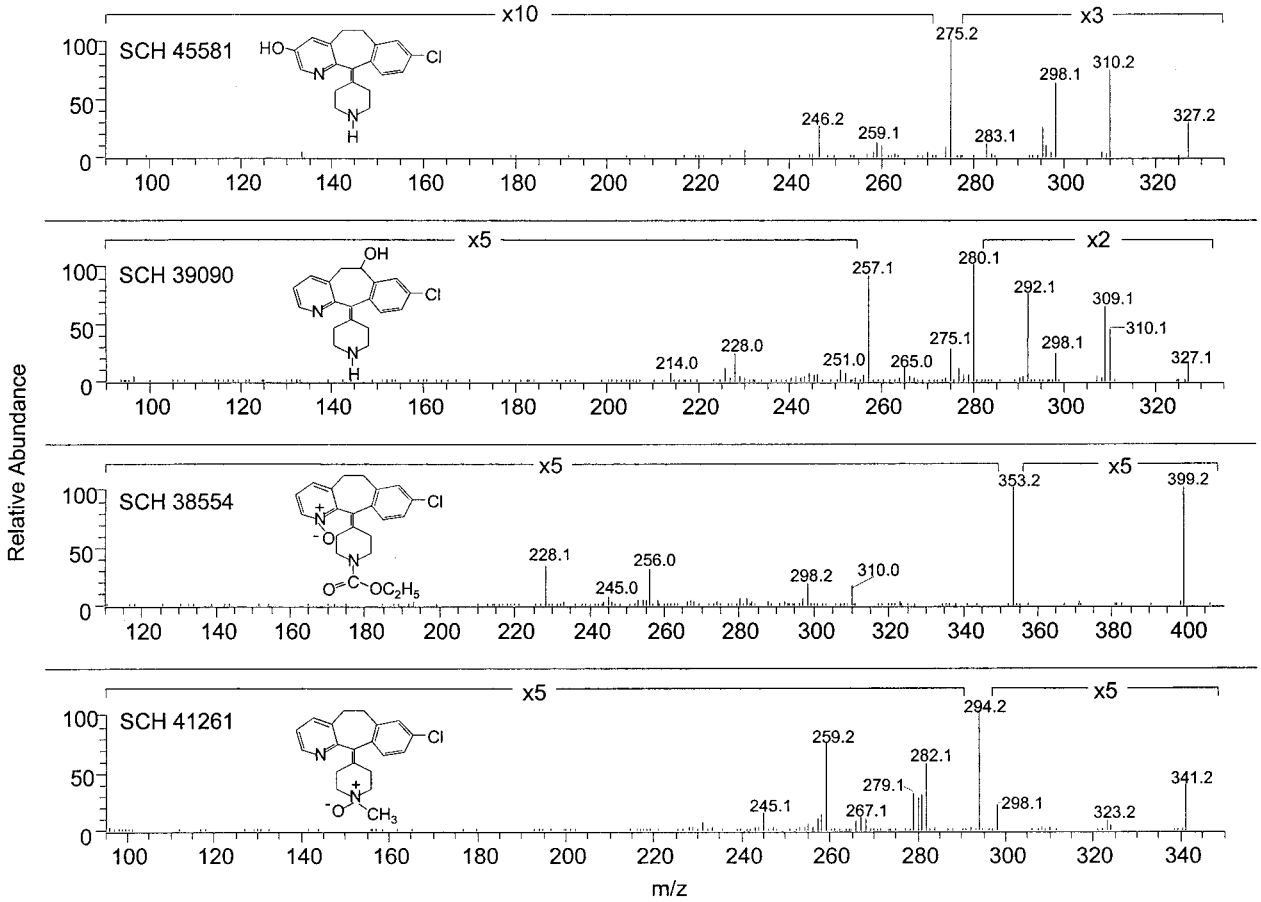


Figure 6. LC/ESI-MS/MS spectra of piperidine-*N*-oxide, pyridine-*N*-oxide, 6-OH desloratadine, and 3-OH desloratadine (from bottom to top) standards obtained using the triple-quadrupole mass spectrometer.

LC/ESI-MS/MS Experiments. Because the minor fragment ions in the ESI-MS spectra did not yield any information to aid in the distinction between the hydroxylated and *N*-oxide metabolites, we evaluated the LC/ESI-MS/MS spectra of the six standards (Figure 1) using the LCQ mass spectrometer. Under the MS/MS conditions, abundant fragment ions were obtained from the protonated molecular ions of loratadine. The major fragment ion detected corresponded to $[M + H - C_2H_5OH]^+$ at *m*/*z* 337 (MS/MS spectrum not shown). Similarly, protonated molecular ions of desloratadine also fragmented to give MS/MS spectrum similar to that of LC/MS spectrum, *albeit* in higher abundance. Prominent fragment ions included those corresponding to $[M + H - NH_3]^+$, $[M + H - NH{=}CH_2]^+$, and $[M + H - NH_3 - Cl]^{\bullet+}$ at *m*/*z* 294, 282, and 259, respectively. The LC/MS/MS spectrum of 3-OH desloratadine could be readily distinguished from that of 6-OH desloratadine (or 5-OH desloratadine) because the formation of *m*/*z* 309 (loss of water) was not as highly favored when hydroxylation was on the aromatic ring (3-OH desloratadine) (Figure 5 and Scheme 1).

The LC/ESI-MS/MS spectra of $[M + H]^+$ ions of the two *N*-oxides obtained on the LCQ mass spectrometer are provided in Figure 5. Piperidine-*N*-oxide fragmented to form ions of *m*/*z* 323, 310, 294, 282, and 280 corresponding to $[M + H - H_2O]^+$, $[M + H - OCH_3]^{\bullet+}$, $[M + H - CH_3NH_2(O)]^+$, $[M + H - C_2H_5NO]^+$, and $[M + H - H_2O - CH_3 - N{=}CH_2]^+$, respectively. Protonated molecular ions of pyridine-*N*-oxide fragmented to form *m*/*z* 353, 310, 298, and 228 corresponding to $[M + H - C_2H_5OH]^+$, $[M + H - C_2H_5CO_2NH_2]^+$, $[M + H - C_2H_5CO_2N{=}CH_2]^+$, and $[C_{14}H_{11}NCl]^+$, respectively. Similar to LC/ESI-MS spectra, the LC/ESI-MS/MS spectra of the $[M + H]^+$ ions of the two *N*-oxides did not provide any direct evidence for distinguishing *N*-oxides from hydroxylated metabolites.

Next, we evaluated the use of a triple-quadrupole mass spectrometer with these compounds. The LC/MS spectra of the seven standards obtained using the ESI technique were similar to those obtained using the LCQ mass spectrometer. Fragment ions observed in the TSQ-LC/MS/MS spectra at 25 eV collision energy (Figure 6) were also similar to those obtained under LCQ-LC/MS/MS conditions, although the relative intensities of the fragment ions were different. Similar to LCQ-LC/MS/MS spectra, the aromatic hydroxylation could be easily distinguished from the aliphatic hydroxylation but *N*-oxides could not be distinguished from the hydroxylated compounds. The MS/MS spectra obtained on the TSQ at 35 eV collision energy also did not provide any unique fragmentation pattern for distinguishing *N*-oxides from hydroxylated metabolites.

LC/APCI-MS Experiments. To assess the stability of the *N*-oxide derivatives and the hydroxylated metabolites under APCI conditions, the set of seven standards was investigated using LC/APCI-MS on the LCQ mass spectrometer. Surprisingly, the simple LC/MS spectra of these standards in APCI revealed distinct differences between the mass spectra of hydroxylated compounds and those of *N*-oxide derivatives. Shown in Figure 7 are LC/APCI-MS spectra of 3-OH desloratadine, 6-OH desloratadine, pyridine-

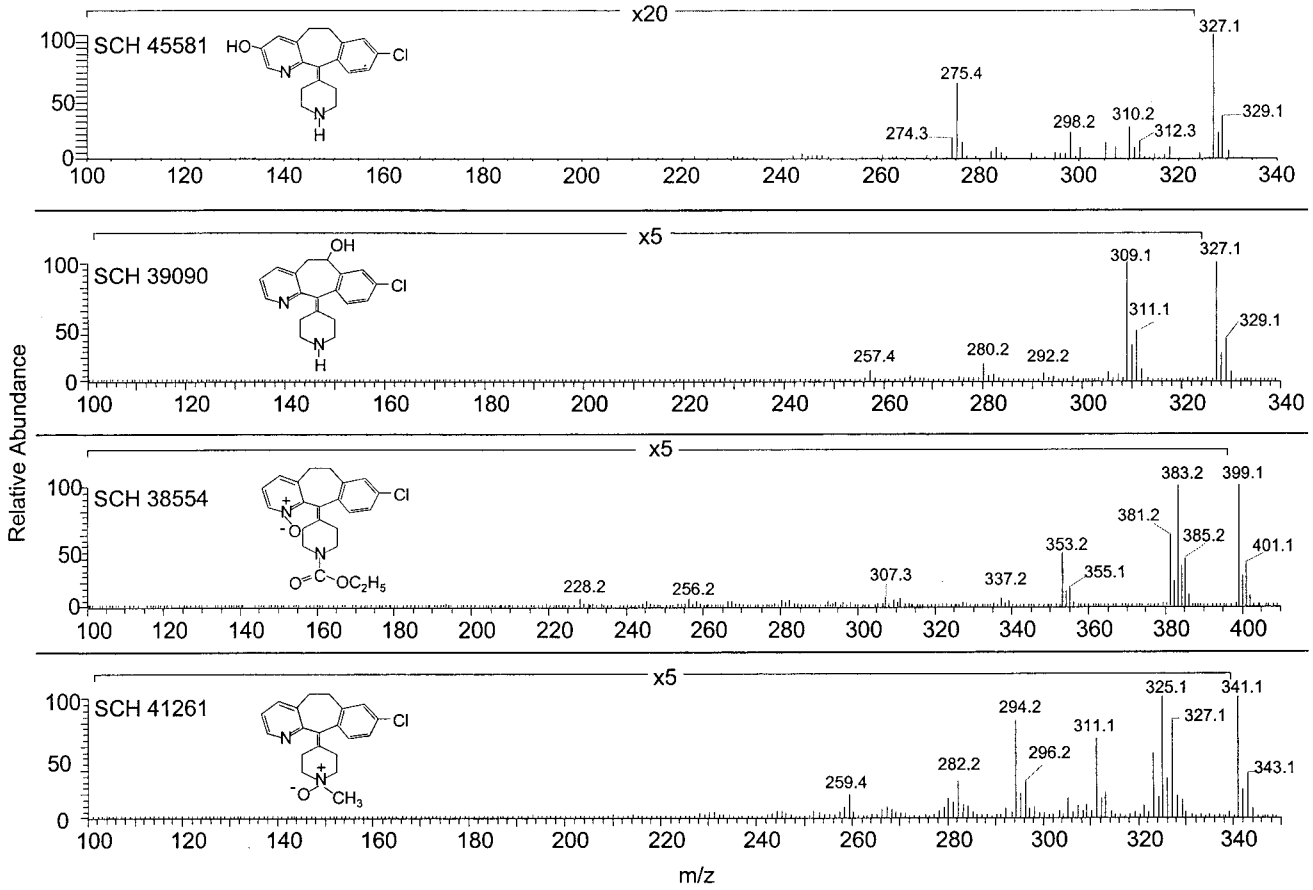
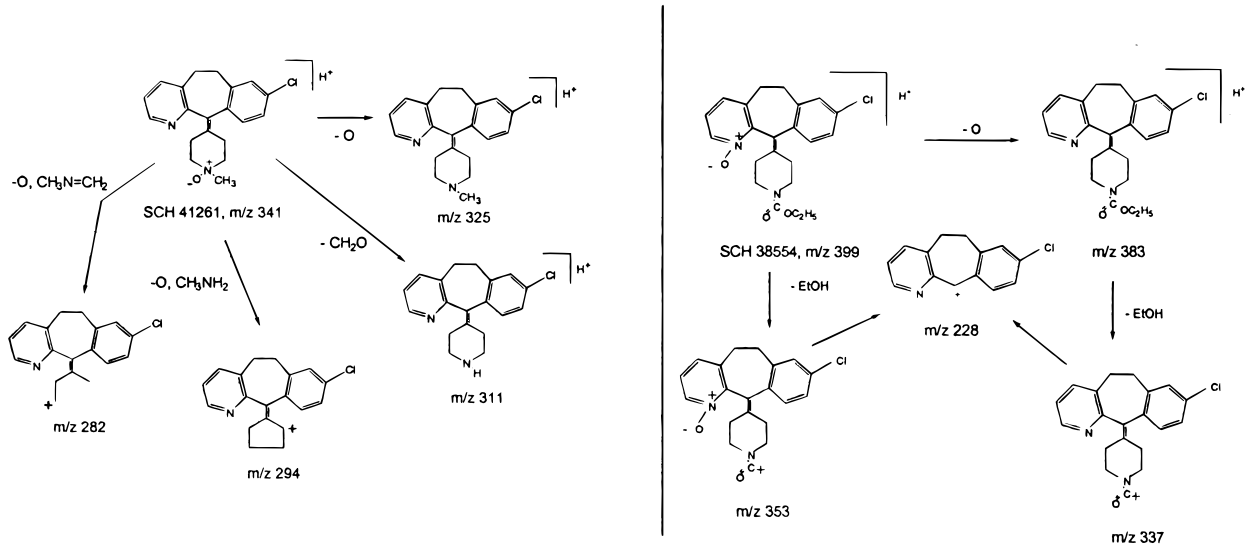


Figure 7. LC/APCI mass spectra of piperidine-*N*-oxide, pyridine-*N*-oxide, 6-OH desloratadine, and 3-OH desloratadine (from bottom to top) standards obtained using the ion trap mass spectrometer.

Scheme 2. Fragment Ions Observed in the APCI Mass Spectra of Piperidine-*N*-oxide and Pyridine-*N*-oxide (Figure 7)



N-oxide, and piperidine-*N*-oxide. Clearly, the major fragment ion from *N*-oxides (piperidine-*N*-oxide and pyridine-*N*-oxide) was due to the loss of an oxygen atom (Scheme 2) while the prominent fragment ion from the aliphatic hydroxylated compound (6-OH desloratadine) was due to loss of H_2O. The aromatic hydroxylated compound (3-OH desloratadine) did not lose water, but indicated a loss of NH_3 (m/z 310), typical of this series of compounds. The distinct fragmentation of *N*-oxides was also observed in the LC/APCI-MS spectra of several other *N*-oxide metabolites from biological extracts (data not reported here).

LC/APCI-MS/MS Experiments. We also investigated these standards under LC/APCI-MS/MS conditions on the LCQ mass spectrometer. Surprisingly, the distinct differences observed under LC/MS conditions between the mass spectra of hydroxylated

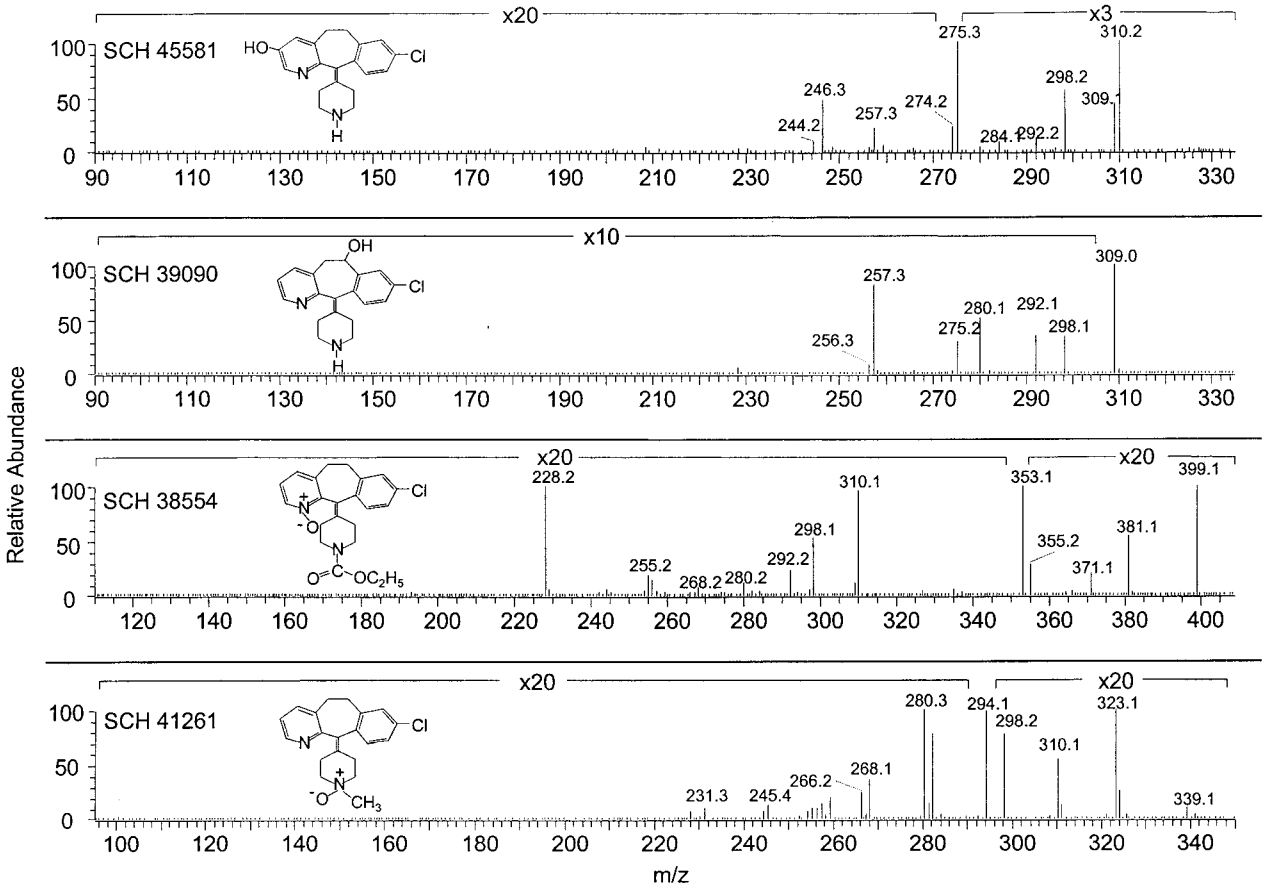


Figure 8. LC/APCI-MS/MS spectra of piperidine-*N*-oxide, pyridine-*N*-oxide, 6-OH desloratadine, and 3-OH desloratadine (from bottom to top) standards obtained using the ion trap mass spectrometer.

compounds and those of *N*-oxide derivatives were absent in the MS/MS spectra (Figure 8). The APCI-MS/MS spectra of the molecular ions of these compounds were similar to that of ESI-MS/MS spectra described above.

CONCLUSION

The present investigation demonstrates that LC/APCI-MS is most useful for distinguishing *N*-oxides and hydroxylated metabolites. ESI-MS may be used as a primary tool for the determination of molecular weights of unknown metabolites. Because of limited fragmentation, the $(M + H)^+$ ions stand out. LC/MS/MS, on the other hand, provides abundant structure-specific fragment ions. The results from the three experiments, when combined, provide the most useful information for structural determination of unknown metabolites.

The formation of $[M + H - O]^+$ ions from *N*-oxides in APCI mass spectra is most likely due to the thermal degradation of the neutral *N*-oxide molecules prior to APCI and/or thermally assisted fragmentation of $[M + H]^+$ ions following ionization. This conclusion is supported by the fact that, in APCI-MS/MS, $[M + H]^+$ ions did not fragment to form $[M + H - O]^+$ ions. This finding is in agreement with that recently reported by Slegel and Karancsi.[13] These authors also noted the loss of an oxygen atom from *N*-oxide metabolites in APCI and attributed this loss to thermal decomposition. Further, the thermal degradation of *N*-oxides in GC/MS is well documented.[5] The detailed experimental results that include comparisons of data from APCI-MS, ESI-MS, and MS/MS presented here clearly establishes the advantage of using APCI-MS for the detection of *N*-oxide metabolites.

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