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Liquid chromatography/tandem mass spectrometry utilizing ion-molecule reactions and collision-activated dissociation for the identification of N-oxide drug metabolites

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ABSTRACT

A liquid chromatography/tandem mass spectrometry (LC/MS^3) method based on ion-molecule reactions and collision-activated dissociation (CAD) is presented for the identification of analytes with the N-oxide functional group directly in mixtures. Tri(dimethylamino)borane (TDMAB) rapidly and selectively derivatizes protonated N-oxides in a modified commercial linear quadrupole ion trap (LQIT) mass spectrometer to yield a distinct product ion (adduct—$(CH_3)_2NH$). The LQIT was outfitted with an external reagent-mixing manifold that allows TDMAB to be mixed with the helium buffer gas used in the trap. The derivatized analytes are readily identified on the basis of a shift of 98 Th (Thomson) relative to the *m/z* value of the protonated analyte. Further probing of the derivatized analytes via isolation followed by CAD can be used to confirm the presence of an N-oxide, and distinguish between aliphatic and aromatic tertiary N-oxides. Since the ion-molecule reaction is fast, these experiments can be accomplished on the same time scale as typical CAD-based MS^n experiments, thus maintaining the duty cycle of the instrument for this type of experiment. To demonstrate real world applicability, the method was tested on real active pharmaceutical ingredients and their derivatives.

1. Introduction

Drug metabolism studies are being performed at early stages in drug discovery to shorten and streamline the drug development process [1]. Rapid identification of drug metabolites can aid medicinal chemists in the development of safe and effective drugs. Mass spectrometry (MS), especially when coupled with high-performance liquid chromatography (HPLC), has proven invaluable in the field of drug metabolism and pharmacokinetic screening, due to its high sensitivity, selectivity and speed [2,3]. Tandem mass spectrometry (MS/MS) utilizing collision-activated dissociation (CAD) has become the technique of choice for structural elucidation of drug metabolites [4–6]. However, CAD alone does not provide enough information to definitively identify a metabolite in many cases. One such example is the oxidation of a tertiary amino group to an N-oxide.

N-oxidation is a common phase I biotransformation for drugs containing a tertiary amino group [7]. N-oxide metabolites are notoriously difficult to identify via standard MS and MS/MS techniques. CAD of these metabolites often does not produce any significant N-oxide functional group specific fragmentation [8–10], and thus only inferences can be drawn as to the structure of the analyte. Further, CAD spectra of isomeric metabolites, such as those resulting from C-hydroxylation in the same molecule, may be indistinguishable from those of the N-oxides [9,11]. Thus, isolation and purification techniques must be employed to obtain a sufficient amount of the pure metabolite to allow structural characterization by NMR, or authentic reference compounds must be synthesized for comparison, both of which detract from the speed of the metabolite profiling process.

Several approaches have been developed to tackle the above analytical challenge. Chemical modification of the sample, such as selective reduction [12], has been used for identification of N-oxides; however, this approach can be time-consuming and hindered by the presence of sample matrix. LC/MS utilizing on-line H/D exchange with a deuterated mobile phase has also been reported [13,14], but this requires replacing all sample solutions and the aqueous mobile phase with D_2O, which can become expensive and may not always be practical. Furthermore, this approach does not allow for the differentiation of N-oxides from tertiary amines. MS methods based on thermal decomposition have also been developed to distinguish N-oxide metabolites from their isomers [8–11,15]. In some cases, however, the thermal conversion of the N-oxide to the parent compound is so extensive that the metabolite itself is not detected [10,15]. Further,

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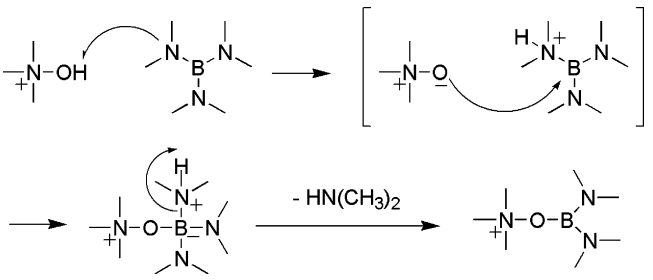


Scheme 1. Mechanism proposed for the reaction between protonated N-oxide containing analyte and neutral TDMAB.

this method does not capitalize on the inherent sensitivity and specificity of MS/MS, and it may be difficult to identify low-level metabolites.

Our group has demonstrated the usefulness of gas-phase ion-molecule reactions of tri(dimethylamino)borane (TDMAB) with protonated analytes in the identification the N-oxide functional group in a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer [16]. The general mechanism proposed for the ion-molecule reaction of TDMAB with a protonated N-oxide is shown in Scheme 1. The derivatization product formed in this reaction is easy to identify based on a mass shift of 98 Da and due to the unique isotope ratio of boron (25% ^{10}B relative to ^{11}B). However, the chemical ionization methods used to generate the protonated analytes in these experiments are not amenable to LC/MS, which is often required for trace level analysis of pharmaceuticals in complex sample matrices.

Recently, a few reports have appeared on LC/MS^2 methods based on ion-molecule reactions rather than CAD. Watkins et al. [17] as well as Pyatkivskyy and Ryzhov [18] used pulsed valve assemblies in FT-ICR and quadrupole ion trap instruments, respectively, to introduce neutral reagents into the mass analyzer for ion-molecule reactions. As the analytes of interest eluted from the HPLC column, they were ionized via ESI, isolated in the ion trap and then derivatized by a neutral reagent. While both methods were effective, the specialized instrument configurations and/or modifications required may not be feasible to implement in a pharmaceutical laboratory.

To improve the utility of the above methodology for structural elucidation of unknown compounds within the pharmaceutical setting, we report here an LC/MS^3 method based on ion-molecule reactions combined with CAD for the identification and characterization of the N-oxide functional group in drug metabolites. The apparatus used to introduce TDMAB into the mass analyzer for these experiments is completely external to the instrument and requires no modification of the instrument hardware itself. Hence, this experiment is straightforward to set up and accessible to any laboratory. Further, we believe this to be the first demonstration of using consecutive ion-molecule reactions and CAD to explore the structure of an analyte after it has eluted from an HPLC into a commercial linear quadrupole ion trap (LQIT) mass spectrometer.

2. Experimental

2.1. Materials

Olanzapine and olanzapine N-oxide were provided by Eli Lilly and Company. Loratadine N-oxide and loratadine epoxide were synthesized from loratadine based on a known procedure [19]. 3-Hydroxyloratadine was purchased from Toronto Research Chemicals, Inc. HPLC grade water was purchased from Burdick & Jackson and HPLC grade acetonitrile and methanol were purchased from Mallinckrodt. All other chemicals used were purchased from Sigma–Aldrich and used without further purification.

2.2. Liquid chromatography

A Surveyor HPLC system (Thermo Fisher, San Jose, CA), consisting of an autosampler, thermostatted column compartment, quaternary pump and photodiode array (PDA) detector was used for chromatographic separation. The analytes were separated on a Waters Xbridge C_{18} (150 mm × 2.1 mm i.d., 3.5 μm) column at a column temperature of 35 °C. Mobile phase A consisted of 0.1% (v/v) formic acid in H_2O, and mobile phase B consisted of 0.1% (v/v) formic acid in CH_3CN. 5 μl of each sample solution was loaded onto the column. Separation was accomplished by using a flow rate of 300 μl/min and the following mobile phase gradient (minutes, A:B, v/v): 0, 95:5; 20, 5:95; 25, 5:95; 25.1, 95:5; 30, 95:5. The PDA detector was set to acquire a single channel at a wavelength of 254 nm.

2.3. Mass spectrometry

A modified LTQ linear quadrupole ion trap (LQIT) mass spectrometer (Thermo Fisher, San Jose, CA) equipped with an electrospray ionization (ESI) source and coupled to the HPLC system described in Section 2.2 was used for the LC/MS^n experiments. Sample solutions were prepared at various concentrations (10^{-9} to 10^{-4} M) in a mixture of H_2O and CH_3OH (50:50, v/v). For direct infusion experiments, the solutions were infused at a flow rate of 5/min using the integrated syringe drive of the LQIT. For LC/MS experiments, the HPLC eluent (300 μl/min) was directly introduced into the ESI source. Typical ESI conditions were: needle voltage, 4.0–4.5 kV, sheath gas (N_2) flow, 10–30 (arbitrary units), auxiliary gas (N_2) flow, 0–10 (arbitrary units) and capillary temperature, 275 °C. Voltages for the ion optics were optimized using the tune feature of the LTQ Tune Plus interface.

2.4. Ion-molecule reactions

Neutral TDMAB was introduced into the helium buffer gas line by using an external reagent-mixing manifold as described previously [20]. Briefly, the TDMAB was introduced into the manifold via a syringe drive at 10–20 μl/h where it was diluted into a measured flow of helium (100–500 ml/min) to give mixing ratios of ~10^4–10^5 (He/reagent). The syringe port and surrounding area of the manifold was heated to ~70 °C to ensure rapid evaporation of TDMAB into the flow of helium. Based on our previous results, the neutral reagent is present at less than 0.1% of the background helium and does not interfere with the performance of the LQIT [20,21].

Ion-molecule reactions were performed using the advanced scan features of the LTQ Tune Plus interface. The analyte ion was isolated using a 2–3 Th (Thomson) window and a q value of 0.25, and then allowed to react with TDMAB for varying periods of time (by adjusting the activation time in the Tune Plus interface) before being ejected from the trap and detected. CAD experiments were performed by isolating the reaction product using a 3–5 Th window and a q value of 0.15–0.25, then applying an appropriate activation voltage (generally 10–20% of the normalized collision energy (NCE) [22] for the analyte, as defined by the Tune Plus interface) for 30 ms in the presence of helium. Pulsed q collision-activated dissociation (PqCAD) [23] experiments were performed by isolating the reaction product using a 3–5 Th window and a q value of 0.7, and then applying an appropriate activation voltage (generally 20–30% of the NCE for the analyte) for 100 μs in the presence of helium. Xcalibur 2.0 software was used for both data acquisition and processing.

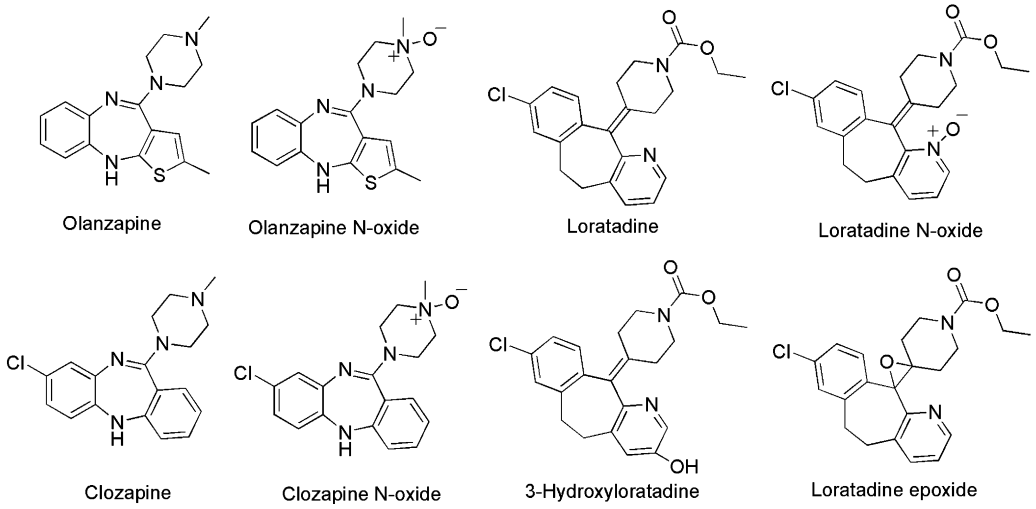


Fig. 1. Drugs and derivatives used to demonstrate the feasibility of the method.

3. Results and discussion

3.1. Method development

As mentioned in Section 1, TDMAB has been shown to be an effective reagent for the identification of the N-oxide functionality in protonated analytes by using an FT-ICR mass spectrometer [16]. The diagnostic reaction involves deprotonation of the protonated analyte by TDMAB, followed by addition of the neutral analyte to protonated TDMAB and elimination of dimethylamine (Scheme 1). Initial experiments were performed to demonstrate that this methodology can be successfully adapted to a LQIT mass spectrometer. Fig. 1 shows several drugs (olanzapine (Zyprexa®), clozapine (Clozaril®) and loratadine (Claritin®)) and their derivatives used to test the method. For example, a 10 μg/ml solution of each olanzapine and its derivative, olanzapine N-oxide, were directly infused into the ESI source of the LQIT. Both compounds formed abundant protonated molecules upon ESI, as expected. For each compound, the protonated molecule was isolated and allowed to react with TDMAB for 30 ms–10 s (the maximum reaction time allowed by the instrument software). The diagnostic product ion was only observed for protonated olanzapine N-oxide in as little as 30 ms. This was the only product ion observed in the mass spectrum (Fig. 2b), in agreement with the results obtained using the FT-ICR instrument [16]. Since the reaction was successfully observed after 30 ms, this reaction time was used in the LC/MS^n experiments described in the following sections (since this is also the typical activation time used in CAD experiments, and thus the duty cycle is preserved). It should be noted here that even after reaction times much longer than this, the diagnostic product ion was not observed for protonated olanzapine (Fig. 2a).

CAD has been previously shown to generally allow the distinction of aliphatic and aromatic tertiary N-oxides that have been derivatized by TDMAB in a FT-ICR mass spectrometer [16]. Several of the model compounds studied in this previous work were also examined in the LQIT during this study, and shown to behave similarly (data not shown). Derivatized aliphatic tertiary N-oxides fragment by the characteristic loss of a neutral molecule of 116 Da, corresponding to $HOB(N(CH_3)_2)_2$ (possibly formed via the mechanism shown in Scheme 2a). This result was confirmed for a real drug compound by isolating the ion-molecule reaction product of protonated olanzapine N-oxide (*m*/*z* 427) and subjecting it to CAD. The resulting mass spectrum is shown in Fig. 2c. The only fragment ion observed has a *m*/*z* value of 311, which corresponds to the diagnos-

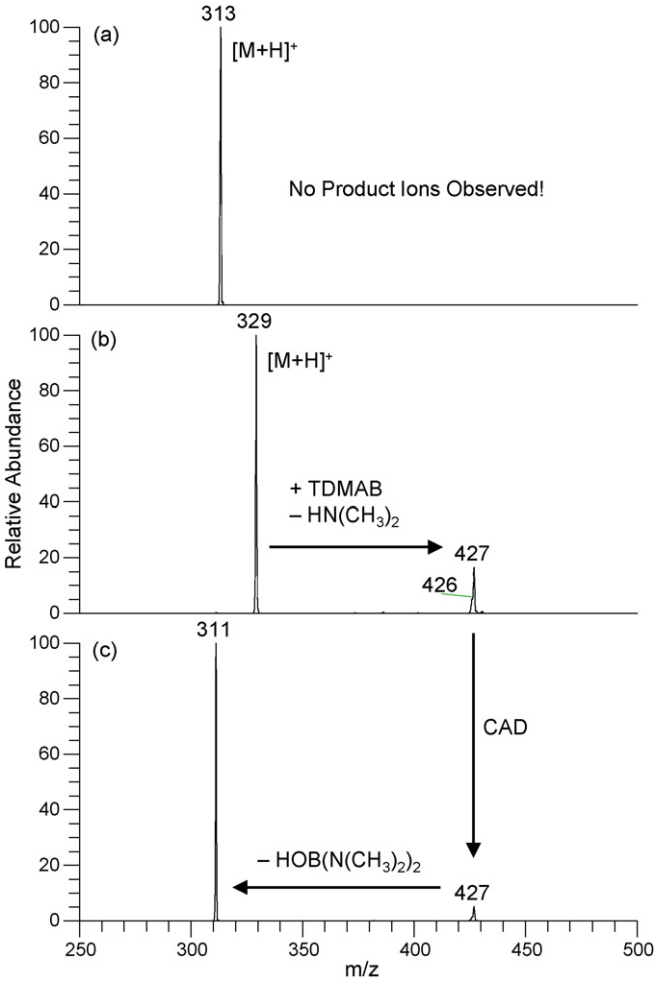
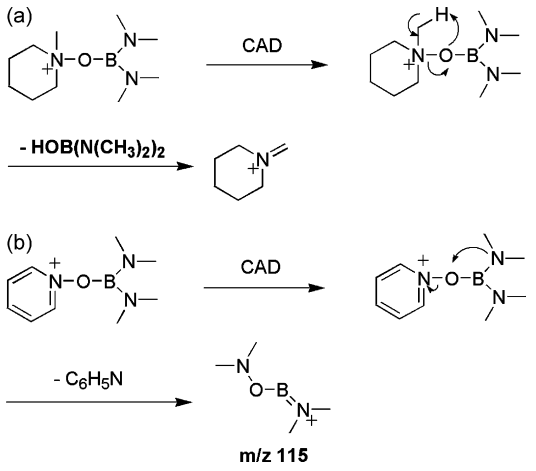


Fig. 2. (a) A mass spectrum obtained for olanzapine after positive mode ESI, isolation of the protonated molecule and exposure to TDMAB for 5 s. No derivatization product is observed even at this long reaction time. (b) A mass spectrum obtained for olanzapine N-oxide after positive mode ESI, isolation of the protonated molecule and exposure to TDMAB for 50 ms. (c) A mass spectrum obtained after isolation of the TDMAB derivatization product of olanzapine N-oxide followed by CAD.



Scheme 2. Fragmentation pathways proposed upon CAD of TDMAB-derivatized (a) aliphatic N-oxide and (b) aromatic N-oxide.

tic loss of $HOB(N(CH_3)_2)_2$ from the TDMAB-derivatized olanzapine N-oxide.

In contrast, derivatized aromatic N-oxides are characterized by the formation of a fragment ion of *m/z* 115 upon CAD (possibly via the mechanism shown in Scheme 2b) [16]. Indeed, after isolation of the ion-molecule reaction product (*m/z* 497) of protonated loratadine N-oxide and CAD, the fragment ion of *m/z* 115 was formed. However, if the ion of *m/z* 497 is isolated at the standard *q* value of 0.25, the low-mass cut-off for the LQIT is *m/z* 135, and thus the diagnostic fragment ion (*m/z* 115) is not detected. There are two approaches available to solve this problem. First, the *q* value can be lowered to 0.15, which decreases the low-mass cut-off to *m/z* 80 and allows the detection of the fragment ion. This approach requires knowledge of the *m/z* values of the likely ion-molecule reaction products so that optimal *q* and collision energy values can be chosen. Another solution is the use of PqCAD or a similar method, high-amplitude short-time excitation (HASTE) [24] CAD. These methods involve the application of a very short, high-amplitude resonance excitation pulse to excite the ion at a high *q* value, after which the *q* value is quickly dropped to trap the resulting fragment ions. Thus, these methods do not suffer from the limitation of the low-mass cut-off. The mass spectrum measured after isolation of the ion-molecule reaction product of protonated loratadine N-oxide followed by PqCAD is shown in the Supplementary material. The fragment ion characteristic for aromatic N-oxides is clearly observed at *m/z* 115.

3.2. LC/MS^2

Once the feasibility of the LC/MS^2 method on the LQIT was demonstrated, the possibility of performing this analysis on the chromatographic time scale was examined. Several mixtures were prepared, each consisting of a drug and its derivative(s), and introduced into the mass spectrometer after HPLC separation. The LQIT was set to isolate the protonated molecule of each analyte and allow it to react with the neutral reagent. For each analysis, a selected ion chromatogram (SIC) refers to data collected in real time. An extracted ion chromatogram (XIC) refers to a chromatogram that was created during data analysis by searching a SIC for the presence of a particular ion via the software.

Consider, for example, a mixture of clozapine (0.125 mg/ml) and its N-oxide (0.100 μg/ml). The concentration of clozapine was chosen based on the assay procedure listed in the USP monograph for clozapine tablets [25]. The concentration of clozapine N-oxide was chosen to provide an approximately 0.1–1% area response relative to clozapine in the UV chromatogram, to properly evaluate the ability of the method to identify low-level impurities. The chromatograms obtained from LC/MS^2 analysis of the Clozapine mixture are shown in Fig. 3. The MS^2 SICs of the protonated molecule for each compound (obtained after isolation of the protonated molecule and exposure to TDMAB) clearly shows a peak corresponding to the protonated molecule. However, when examining the XICs corresponding to the ion-molecule reaction product of interest (i.e., $MH^+ + TDMAB - HN(CH_3)_2$), only the N-oxide containing analyte displays the expected peak. These results were confirmed by examining the full mass spectra associated with each peak in the MS^2 chromatograms. The mass spectrum corresponding to the peak of protonated clozapine in the MS^2 SIC shows the protonated molecule of *m/z* 327, but no ion-molecule reaction products. However, the mass spectrum corresponding to the peak of protonated clozapine N-oxide in the MS^2 SIC shows not only the protonated molecule of *m/z* 343, but also the ion-molecule reaction product of *m/z* 441. Similar results were observed for a mixture of olanzapine and olanzapine N-oxide prepared at the same concentrations.

Data obtained for another example, a mixture of loratadine (0.4 mg/ml [26]) and three isomeric impurities (each present at 0.4 μg/ml), are shown in Fig. 4. The N-oxide, epoxide and hydroxyl impurities were chosen to demonstrate the selectivity of the MS^2 experiment. The MS^2 SIC of protonated loratadine (obtained after

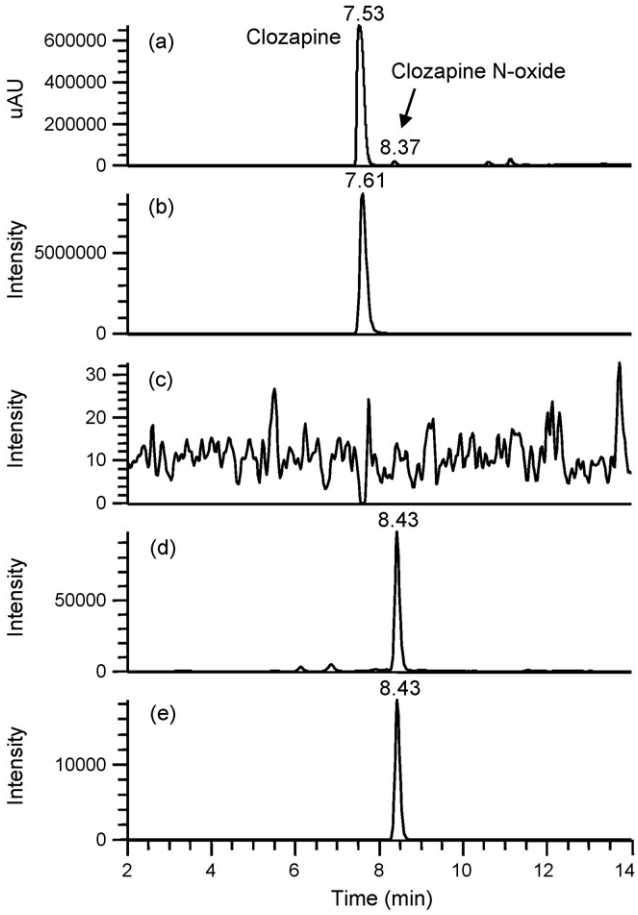


Fig. 3. (a) UV chromatogram of a mixture of clozapine (7.53 min) and clozapine N-oxide (8.37 min). (b) MS^2 selected ion chromatogram (SIC) for protonated clozapine (*m/z* 327). (c) Extracted ion chromatogram (XIC) for the expected ion-molecule reaction product (*m/z* 425) of protonated clozapine. No peaks are observed. (d) MS^2 SIC for protonated clozapine N-oxide (*m/z* 343). (e) XIC for the expected ion-molecule reaction product (*m/z* 441) of protonated clozapine N-oxide. A peak is observed at the same retention time as clozapine N-oxide.

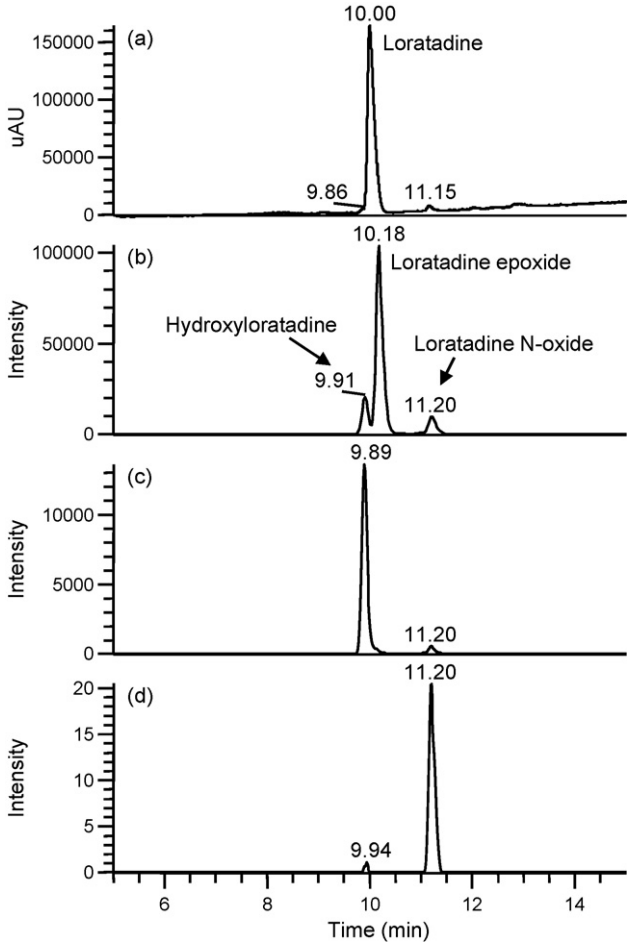


Fig. 4. (a) UV chromatogram of a mixture of 3-hydroxyloratadine (9.91 min), loratadine (10.00 min), loratadine epoxide (10.18 min) and loratadine N-oxide (11.20 min). (b) MS^2 selected ion chromagram (SIC) for protonated isomeric loratadine derivatives (*m*/*z* 399). Three peaks are observed at retention times of 9.91, 10.18 and 11.20 min. (c) MS^3 SIC of the expected ion-molecule reaction product (*m*/*z* 497) of the protonated loratadine derivatives. Two peaks are observed at the same retention times as 3-hydroxyloratadine and loratadine N-oxide, while no peak is observed for loratadine epoxide. (d) Extracted ion chromatgram (XIC) of the fragment ion (*m*/*z* 115) characteristic for aromatic N-oxides. Only one significant peak is observed, corresponding to loratadine N-oxide.

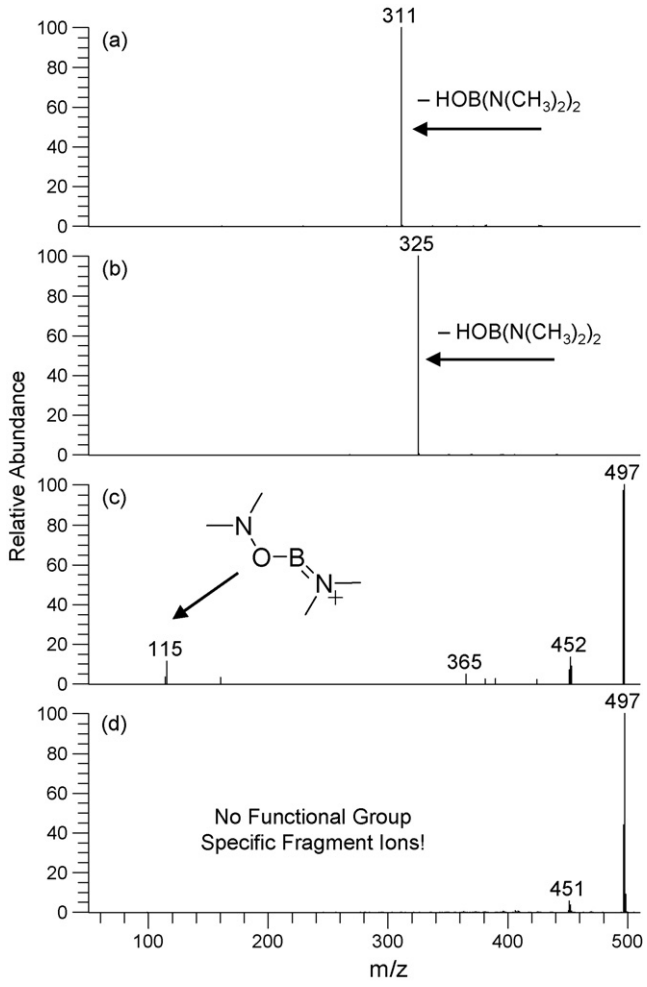


Fig. 5. Mass spectra obtained after isolation and PqCAD of: (a) the product ion (*m*/*z* 427) of protonated olanzapine N-oxide, (b) the product ion (*m*/*z* 441) of protonated clozapine N-oxide, (c) the product ion (*m*/*z* 497) of protonated loratadine N-oxide and (d) the product ion (*m*/*z* 497) of protonated 3-hydroxyloratadine. The neutral loss diagnostic of derivatized aliphatic N-oxides is clearly observed for the two derivatized aliphatic N-oxides (a,b), while the derivatized aromatic N-oxide displays the fragment ion characteristic of derivatized aromatic N-oxides (c). No functional group specific fragmentation is observed for the hydroxylated pyridine; the only fragment ion observed (*m*/*z* 451) corresponds to loss of ethanol, which is the same primary loss observed for underivatized 3-hydroxyloratadine (d).

isolation of the protonated molecule and exposure to TDMAB) shows a peak corresponding to the protonated molecule, but the XIC of the expected ion-molecule reaction product does not display any peaks (not shown). The MS^2 chromatogram of the isomeric loratadine derivatives (obtained after isolation of the protonated molecules and exposure to TDMAB) shows three peaks, as expected. The XIC for the ion of *m*/*z* 497 (the ion-molecule reaction product of interest), however, displays only two peaks, which correspond to the hydroxy- and N-oxide containing isomers. Again, examination of the tandem mass spectra associated with the three peaks in the chromatogram of the protonated analytes confirms the result. Since both the hydroxy- and N-oxide containing analytes form the expected ion-molecule reaction product, an additional stage of MS must be performed to differentiate these isomers.

3.3. LC/MS^3

To further the amount of structural information this method can provide, ion-molecule reactions were combined with CAD in an LC/MS^3 experiment. The same mixtures used to demonstrate the LC/MS^2 methodology were used for these experiments. For example, as one of the N-oxides eluted from the column, it was ionized by ESI (MS), isolated and exposed to TDMAB for 30 ms (MS^2), after which the diagnostic ion-molecule reaction product was isolated and subjected to PqCAD (MS^3). The MS^3 spectra acquired for each of the three N-oxide metabolites and the one hydroxy metabolite used in this study are shown in Fig. 5. The two derivatized aliphatic N-oxides (olanzapine N-oxide and clozapine N-oxide) were clearly identified by a neutral loss of $HOB(N(CH_3)_2)_2$ upon CAD of the diagnostic TDMAB ion-molecule reaction product. The TDMAB-derivatized 3-hydroxyloratadine did not display any functional group specific fragmentation, while TDMAB-derivatized loratadine N-oxide produced the fragment ion at *m*/*z* 115 that is characteristic of aromatic N-oxides.

3.4. Specificity

Various results illustrating the specificity of the ion-molecule reaction used here toward the N-oxide functionality in simple analytes have been reported previously [16]. The experiments described in Sections 3.1–3.3 revealed a fast reaction with TDMAB for protonated N-oxide containing drug derivatives and no reac-

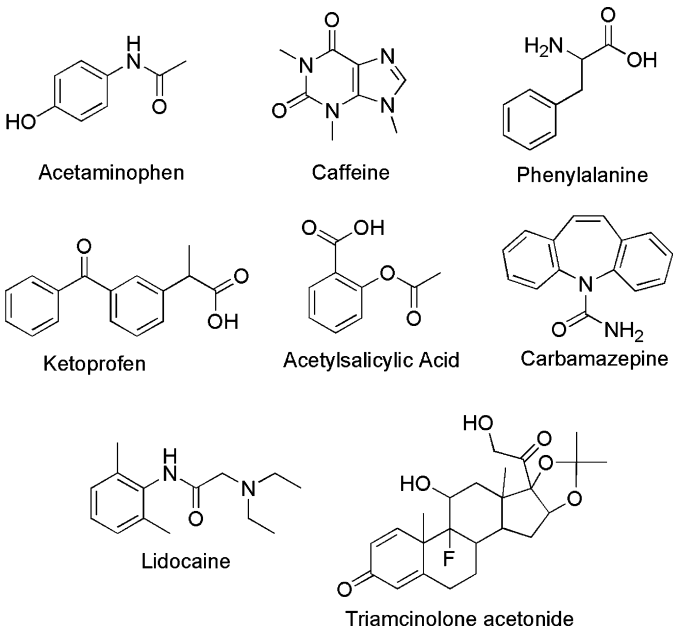


Fig. 6. Small molecules used to demonstrate the specificity of the method.

tion for the three endogenous drug compounds studied, even at substantially higher concentrations. To further probe the selectivity of the reaction, several additional compounds that do not contain the N-oxide functionality were analyzed (Fig. 6). These analytes were chosen as representatives of different types of small molecules that are often encountered in pharmaceutical industry. Upon exposure to TDMAB, none of the protonated analytes displayed the ion-molecule reaction product of interest, except for protonated acetaminophen, carbamazepine, and, to a lesser extent, lidocaine, all of which contain the amido functionality. These analytes display not only the TDMAB derivatization product, but also a dominant product ion of m/z 144, which corresponds to protonated TDMAB. This product ion was not observed for protonated N-oxides and hence may allow the differentiation between amides and N-oxides.

In order to unambiguously demonstrate the ability to differentiate between amides and N-oxides, the amides were examined further by using the full MS^3 experiment proposed here. Isolation of the derivatized amide and subsequent CAD resulted in a loss of $HN(CH_3)_2$. This fragmentation pattern is different than that observed for derivatized N-oxides (Section 3.1); thus, the N-oxide functionality can be identified via this methodology. An example of this behavior is provided in Supplementary material.

4. Conclusions

LC/MS^3 based on consecutive ion-molecule reactions and CAD of protonated analytes provides a powerful method for structural elucidation of pharmaceutical impurities in mixtures. The method demonstrated here was shown to be highly specific for the N-oxide functional group, allow characterization of the type of N-oxide present (aromatic or aliphatic), and was successfully demonstrated at low concentrations. These desired analytical qualities, combined with the straightforward experimental setup, make this experiment readily adaptable within the pharmaceutical laboratory. In addition, since this method is based on fast gas-phase derivatization within the mass spectrometer, it may be readily coupled to any existing LC/MS^n method without the need to develop new chromatographic parameters or sample preparation procedures. By developing a library of functional group specific neutral reagents, this method could allow more definitive structural characterization of metabolites than current MS methods, thus expediting the selection of drugs with favorable pharmacokinetic properties.

These results also suggest additional applications for this and related methodology. For example, LC/MS^2 utilizing ion-molecule reactions may be useful for quantitative "neutral gain" monitoring of metabolites in high-throughput applications. Further, the use of data-dependent experiments could provide for automated functional group identification and increase the speed of impurity identification. These experiments are currently being developed in our laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.09.047.

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