# Data-Dependent Neutral Gain $MS^3$: Toward Automated Identification of the N-Oxide Functional Group in Drug Metabolites

Steven C. Habicht, Nelson R. Vinueza, Penggao Duan, Mingkun Fu, and Hilkka I. Kenttämaa

Department of Chemistry, Purdue University, West Lafayette, Indiana, USA

We report here an automated method for the identification of N-oxide functional groups in drug metabolites by using the combination of liquid chromatography/tandem mass spectrometry ($LC/MS^n$) based on ion-molecule reactions and collision-activated dissociation (CAD). Data-dependent acquisition, which has been readily utilized for metabolite characterization using CAD-based methods, is adapted for use with ion-molecule reaction-based tandem mass spectrometry by careful choice of select experimental parameters. Two different experiments utilizing ion-molecule reactions are demonstrated, data-dependent neutral gain $MS^3$ and data-dependent neutral gain pseudo-$MS^3$, both of which generate functional group selective mass spectral data in a single experiment and facilitate increased throughput in structural elucidation of unknown mixture components. Initial results have been generated by using an $LC/MS^n$ method based on ion-molecule reactions developed earlier for the identification of the N-oxide functional group in pharmaceutical samples, a notoriously difficult functional group to identify via CAD alone. Since commercial software and straightforward, external instrument modification are used, these experiments are readily adaptable to the industrial pharmaceutical laboratory. (J Am Soc Mass Spectrom 2010, 21, 559–563) © 2010 American Society for Mass Spectrometry

The identification of metabolites is a critical and challenging task in the drug discovery and development process [1]. Liquid chromatography/tandem mass spectrometry ($LC/MS^n$) based on collision-activated dissociation (CAD) has become the technique of choice for structural elucidation of metabolites [2, 3]. Generally, the $LC/MS^n$ run is preceded by an initial LC/MS experiment to identify the metabolites of interest. These analytes are then targeted for subsequent $MS^n$ by the user. However, in some cases, even after several chromatographic runs, the structures of the metabolites of interest may only be narrowed down to a list of several possibilities. Thus, especially with the need to unambiguously identify metabolites at early stages of drug discovery, new, efficient, and more structurally informative $MS^n$ methods are desired.

The development of data-dependent analysis methods has greatly expedited the process of structural elucidation by increasing the amount of mass spectral data acquired per chromatographic run. In these experiments, software-based decision-making algorithms signal the mass spectrometer to perform $MS^2$ and subsequent $MS^n$ experiments for ions detected in the initial full MS scans (dubbed "survey" scans) based on a specific set of user-defined criteria [4, 5]. In contrast to analyte-targeted $MS^n$ scans, data-dependent scans allow all components of a complex mixture to be interrogated "on-the-fly," requiring little to no prior knowledge of the components present. Many examples can be found in the literature that demonstrate the effectiveness of combining $LC/MS^n$ based on CAD with data-dependent acquisition for rapid drug metabolite characterization [6–11].

We recently reported an $LC/MS^n$ method based on ion-molecule reactions followed by CAD for the identification of the N-oxide functional group in drug metabolites [12]. This experiment used the neutral reagent tri(dimethylamino)borane (TDMAB) to rapidly derivatize N-oxide containing protonated analytes that were then further characterized by their unique fragmentation pattern upon collisional activation. This paper demonstrated the utility of combining ion-molecule reactions and CAD for identifying N-oxide drug metabolites, which are difficult to identify by CAD alone. Here, we present a specific application of this methodology, which takes advantage of the data-dependent scanning features available on many commercial mass spectrometers to automate functional group identification and increase throughput in structural elucidation studies. We believe that this work represents the first

Address reprint requests to Professor H. I. Kenttämaa, Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN, 47907, USA. E-mail: hilkka@purdue.edu



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example of implementing ion-molecule reactions in a data-dependent manner and, hence, emphasizes the value of ion-molecule reactions as a complement to CAD for the characterization of certain metabolites.

## Experimental

The experiments were performed in a modified linear quadrupole ion trap (LQIT) mass spectrometer (LTQ, Thermo Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source and coupled to a HPLC system (Surveyor, Thermo Scientific). Clozapine, clozapine N-oxide, and TDMAB were acquired from Sigma-Aldrich (St. Louis, MO, USA), hydroxyloratadine was acquired from Toronto Research Chemicals (Toronto, ON, Canada), loratadine N-oxide was synthesized [12], and olanzapine and olanzapine N-oxide were provided by Eli Lilly and Co. (Indianapolis, IN, USA). Sample solutions were prepared at various concentrations ($10^{-9}$ to $10^{-6}$ M) in a 50/50 (vol/vol) mixture of $H_2O$ and $CH_3OH$. The analytes were separated by reversed-phase HPLC and ionized via ESI by using conditions described elsewhere [12]. Xcalibur 2.0 software was used for both data acquisition and processing.

LC/$MS^n$ experiments were carried out by using the data-dependent feature of Xcalibur 2.0, via the regime depicted in Figure 1. The most abundant ion in the full MS scan was isolated automatically (isolation width 2 Th; q value 0.25) and allowed to react (30 ms) with TDMAB (introduced into the helium bath gas via an external manifold, as described previously [13]). If the desired reaction product (neutral gain of 98 Da) was observed among the three most abundant peaks in the $MS^2$ scan, it was automatically isolated (isolation width 5 Th; q value 0.15–0.25) and subjected to CAD. CAD involved application of an appropriate activation voltage (generally 10%–20% of the normalized collision energy [14] for the analyte) for 30 ms in the presence of helium. Following the acquisition of a CAD ($MS^3$) spectrum, the instrument automatically advanced to the next full scan. If the desired ion-molecule reaction product was not detected in the above experiment ($MS^2$ scan), the instrument also advanced to the next full MS scan. This cycle was repeated until completion of the experiment. At the conclusion of the experiment, the $MS^3$ spectra were inspected by the user for the presence of diagnostic fragment ions [12], which further confirm the presence of the N-oxide functional group and provide additional structural information.

Although most of the instrument settings for the above data-dependent experiment are similar to those typically employed, it is necessary to note a few important exceptions. The first (and perhaps obvious) exception is that a negative number is entered in the neutral loss trigger box, thus signaling a neutral gain experiment. Second, the charge state of ions for the activation event must be set to +2, even though most small molecule analyses, such as this, focus on singly-charged

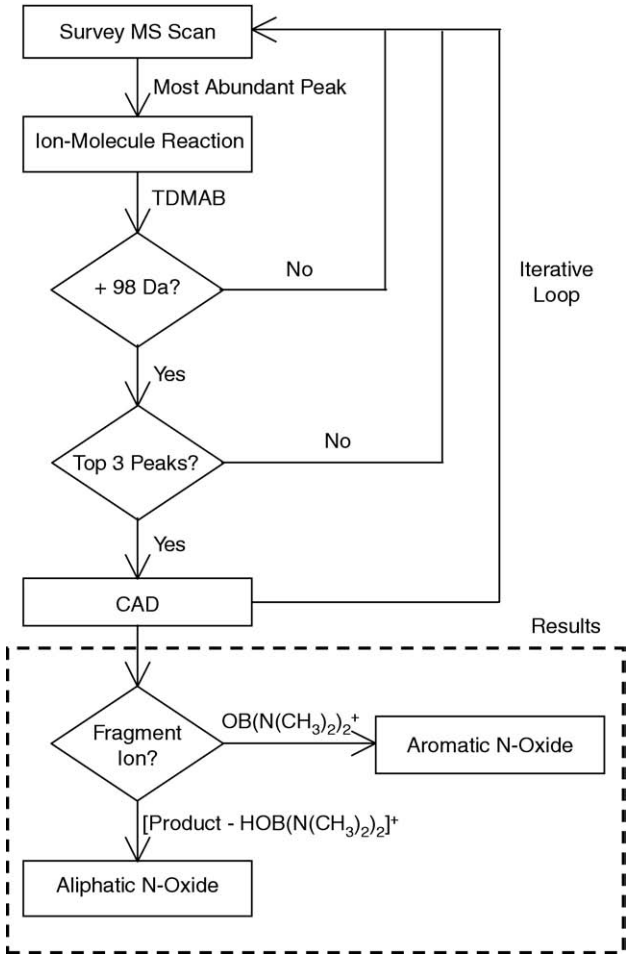


**Figure 1.** Flow chart depicting the data-dependent strategy for identifying N-oxide containing drug metabolites. This process can be adapted for a variety of functional group selective ion-molecule reactions by identification of the relevant neutral gain and determination of the diagnostic fragmentation pattern(s).

ions. This is because the upper limit of the mass range (actually the highest *m/z* value detected) for singly-charged ions in data-dependent experiments is automatically set to only ~10% higher than the *m/z* value of isolated ion, in the interest of maintaining a fast duty cycle. Under these conditions, the reaction product ions of most ion-molecule reactions (e.g., the product ions formed in the reactions of interest here) are not detected. However, when the ion charge state is set to +2, the instrument will set the high mass limit to approximately twice that of the *m/z* value of isolated ion, in anticipation of the formation of singly-charged fragment ions (some of which may have an *m/z* value up to twice that of the isolated ion) upon CAD. Finally, although not the case for this experiment, ion-molecule reactions that require reaction times longer than the typical 30 ms allotted for $MS^2$ experiments (under "activation time") necessitate a shorter ion injection time or less signal averaging per data-dependent experiment [5].

To examine whether the time of the duty cycle of the above data-dependent experiments can be decreased,

the $MS^3$ experiments were converted into $MS^2$ experiments by subjecting the ion-molecule reaction product ions formed in the trap to CAD without isolation (pseudo-$MS^3$) [15]. In a pseudo-$MS^3$ experiment, a precursor ion (the protonated analyte) is isolated (isolation width 2 Th; q value 0.15) and subjected to CAD (typical normalized collision energy of 10%–15%) in the presence of TDMAB ($MS^2$), which will produce both ion-molecule reaction products and fragment ions. Rather than isolating any ion-molecule reaction product ions for CAD (conventional $MS^3$), pseudo-$MS^3$ was performed by setting the instrument to activate ion-molecule reaction product ions corresponding to a neutral gain of 98 Da (at the same normalized collision energy as $MS^2$) in the presence of all ions in the trap. Removal of the isolation step allows the time of the duty cycle of the $MS^3$ experiment to be decreased; however, the trade-off is that activation must be applied to the precursor ion during the ion-molecule reactions due to software limitations. Thus, the spectra obtained from this experiment represent a composite of three separate MS experiments: CAD of the protonated analyte ($MS^2$), ion-molecule reaction of the protonated analyte ($MS^2$), and CAD of the desired ion-molecule reaction product of the protonated analyte ($MS^3$). It should be noted that this same approach would most likely be used to enact this $MS^3$ experiment on a triple quadrupole instrument.

## Results and Discussion

### *Data-Dependent Neutral Gain $MS^3$*

A mixture of the antipsychotic drug clozapine and its N-oxide metabolite was initially examined to demonstrate the feasibility of combining data-dependent acquisition with $MS^3$ using functional group-selective ion-molecule reactions followed by CAD in the LQIT on the chromatographic time scale. The concentration of clozapine N-oxide (~1 μg/mL) in the mixture was ~1% (wt/wt) that of the endogenous drug clozapine, to mimic situations that may be encountered in the pharmaceutical setting. As the drug eluted from the column, it was ionized by ESI and mass analyzed (MS survey scan), after which the protonated molecule (the most abundant ion) was automatically isolated and exposed to TDMAB for 30 ms ($MS^2$). Since the targeted neutral gain (98 Da) was not observed, the instrument automatically skipped the $MS^3$ scan and instead progressed to the next survey MS scan. Similarly, as the N-oxide eluted from the column, it was ionized by ESI and mass analyzed, and then the protonated molecule (*m/z* 343) was isolated and exposed to TDMAB for 30 ms ($MS^2$; Figure 2). However, in this case, the diagnostic ion-molecule reaction product (i.e., $MH^+$ + TDMAB − $HN(CH_3)_2$) was detected at *m/z* 441. Thus, this ion was isolated and subjected to CAD ($MS^3$). Manual examination of the $MS^3$ spectrum (Figure 2c) showed a dominant peak at *m/z* 325, corresponding to a neutral loss of 116 Da from the ion-molecule reaction product. This loss

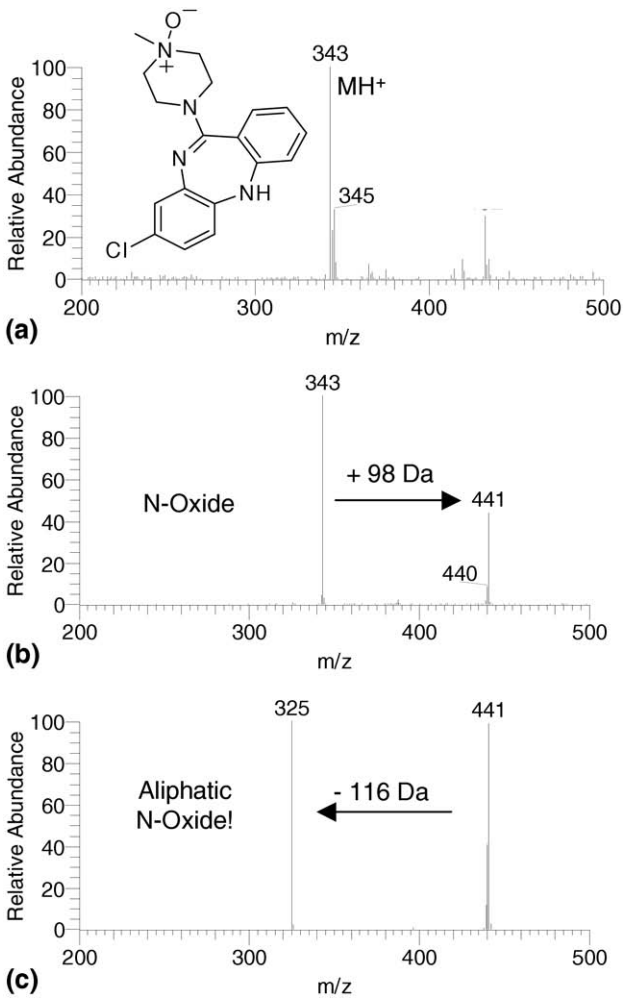


**Figure 2.** (**a**) Mass spectrum obtained for clozapine N-oxide after positive mode ESI. (**b**) $MS^2$ spectrum obtained for clozapine N-oxide after isolation of the protonated molecule (*m/z* 343) and exposure to TDMAB for 30 ms. The observed neutral gain of 98 Da (*m/z* 441) corresponds to formation of the expected ion-molecule reaction product and indicates the presence of an N-oxide. (**c**) $MS^3$ spectrum obtained after CAD of the TDMAB derivatization product of clozapine N-oxide (*m/z* 441). The neutral loss of 116 Da, corresponding to loss of $HOB(N(CH_3)_2)_2$, indicated by the ion of *m/z* 325 is characteristic for tertiary aliphatic N-oxides.

corresponds to the molecule $HOB(N(CH_3)_3)_2$, which is indicative of an aliphatic tertiary N-oxide (Figure 1). Similar results were obtained when this methodology was applied to mixtures of the antipsychotic drug olanzapine and the antihistamine loratadine with their N-oxide derivatives. It should be noted here that these experiments have the same duty cycle as the traditional $MS^3$ experiments based on CAD alone, with about 3–5 $MS^3$ spectra acquired per HPLC peak.

### *Data-Dependent Neutral Gain Pseudo-$MS^3$*

Pseudo-$MS^3$ experiments were performed to determine whether the duty cycle of the data-dependent $MS^3$ experiment could be decreased. Conversion of the $MS^3$ experiment into a $MS^2$ experiment (pseudo-$MS^3$) was

found to produce a mass spectrum that is a composite of the $MS^2$ and $MS^3$ spectra. Applying this technique to the analyses described above requires judicious choice of the activation energy and, in some cases, the activation q value. The activation energy must be chosen such that the protonated analyte undergoes minimal fragmentation, but the ion-molecule reaction product still fragments, since the same normalized collision energy is used for both experiments due to software limitations. The activation q value is an important factor when analyzing tertiary aromatic N-oxides, since the characteristic fragment ion of *m/z* 115 may be near or below the low-mass cutoff for the isolated analyte.

The pseudo-$MS^3$ spectra acquired for an artificial mixture of the hydroxyl and N-oxide metabolites of loratadine (~1 μg/mL of each analyte) are shown in Figure 3. The experiment was performed using a normalized collision energy of 10% and a q value of 0.15 for CAD. Both spectra display the ion-molecule reaction product of *m/z* 497 and an abundant fragment ion of *m/z* 353, which is the dominant fragment ion for both protonated molecules upon CAD. However, expansion of the low mass region of these mass spectra allows the differentiation of the two isomers. The TDMAB-derivatized 3-hydroxyloratadine does not display any functional group-specific fragmentation, while TDMAB-derivatized loratadine N-oxide produces the fragment ion of *m/z* 115 that is characteristic of aromatic N-oxides (Figure 1). By using the pseudo-$MS^3$ approach, the number of spectra that could be acquired per HPLC peak increased up to about 8.

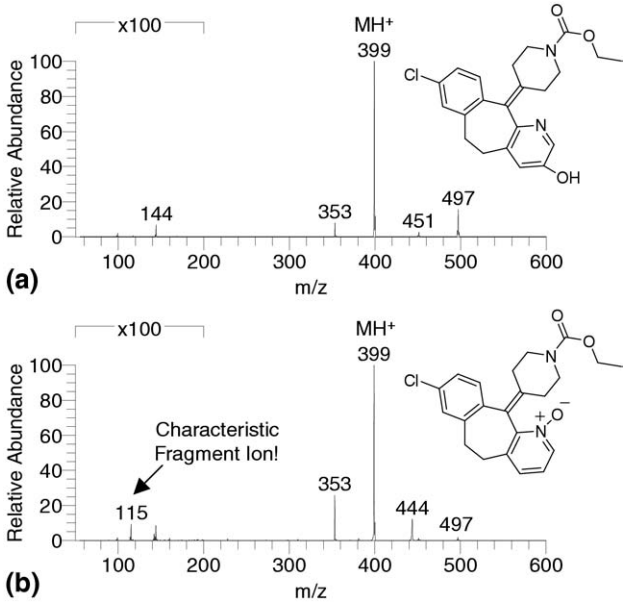


**Figure 3.** Pseudo-$MS^3$ spectra obtained by isolating the protonated analyte (*m/z* 399) of (**a**) 3-hydroxyloratadine and (**b**) loratadine N-oxide, followed by exposure to TDMAB (q value 0.15; normalized collision energy 10%). Following this step, the ion-molecule reaction product (*m/z* 497) was subjected to CAD (normalized collision energy 10%) in the presence of all ions in the trap (i.e., without isolation). Inspection of the mass spectra reveals that only the derivatized aromatic N-oxide displays the characteristic fragment ion of *m/z* 115.

## Conclusions

The combination of data-dependent acquisition with LC/$MS^3$ based on ion-molecule reactions and CAD increases the throughput of N-oxide metabolite characterization by allowing for automated functional group identification. Although this methodology was specifically demonstrated for the N-oxide functional group, it can be applied to other metabolites via judicious choice of the neutral reagent (i.e., by choosing neutral reagents specific to other functional groups). Since these experiments facilitate the identification of specific biotransformations "on-the-fly," they allow for quick and efficient determination of metabolic "hot spots" during drug discovery. This type of an analytical method is readily implemented by using commercial software platforms, though careful attention must be paid to certain experimental parameters. The results obtained using pseudo-$MS^3$ experiments (i.e., combining $MS^2$ and $MS^3$) on the LQIT provides the impetus for adapting these methods to triple quadrupole instruments. These experiments are currently underway in our laboratories.

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