Short communication

Gas-phase derivatization via the Meerwein reaction for selective and sensitive LC–MS analysis of epoxides in active pharmaceutical ingredients

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ABSTRACT

A gas-phase derivatization strategy is reported by using the gas-phase Meerwein reaction for rapid and direct LC–MS analysis of epoxides, which are potential genotoxic impurities (GTIs) in active pharmaceutical ingredients (APIs). This class-selective ion/molecule reaction occurs between epoxides and the ethylnitrilium ion ($CH_3-C\equiv\overset{+}{N}H \leftrightarrow CH_3-\overset{+}{C}=NH$) that is generated by atmospheric pressure ionizations (when acetonitrile is used as the mobile phase). Density functional theory (DFT) calculations at the B3LYP/6-311+G(d,p) level show that the gas-phase Meerwein reaction is thermodynamically favorable. Commonly used atmospheric pressure ionization techniques including ESI, APCI and APPI were evaluated for optimal formation of the Meerwein reaction products. APCI appears to be the method of choice since it offers better sensitivity and more robust detection under typical LC–MS instrumentation conditions. Quantitative analysis of epoxides can be achieved by either single ion monitoring (SIM) or multiple reaction monitoring (MRM) of the Meerwein reaction products. We demonstrate herein quantitative analysis of two potential GTIs of SB797313 and SB719133 in APIs. The validated methods afford excellent linearity ($r^2 \geq 0.999$), sensitivity (LOD $\leq$ 1 ppm by w/w in 10 mg/mL APIs) and recovery (ranging from 92% to 102%), as well as accuracy ($\leq$2.8% difference) and precision ($\leq$2.2% RSD) based on injections of six prepared standards. This novel strategy is particularly useful when a target analyte is difficult to be directly analyzed by LC–MS (e.g. due to poor ionization) or unstable in the course of solution-phase derivatization.

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1. Introduction

The structurally selective gas-phase ion/molecule reactions have been shown to be a useful tool in biological and pharmaceutical analysis due to their structural specificity, high sensitivity, and unparalleled speed [1–4]. In our previous report, several epoxides were shown to efficiently undergo the gas-phase Meerwein reaction with the ethylnitrilium ion ($CH_3-C\equiv\overset{+}{N}H \leftrightarrow CH_3-\overset{+}{C}=NH$) in the atmospheric pressure ionization sources (Scheme 1) [5]. This structure-selective ion/molecule reaction was demonstrated to be applicable for direct analysis of epoxides using liquid chromatography–mass spectrometry (LC–MS) by single ion monitoring (SIM) or LC–MS/MS via multiple reaction monitoring (MRM). Since the ethylnitrilium ion is generated by protonation of acetonitrile (when it is used as the LC–MS mobile phase) under the typical conditions of atmospheric pressure ionizations, the gas-phase derivatization of epoxides via the Meerwein reaction can be achieved via direct LC–MS analysis.

The occurrence of such an atmospheric pressure ion/molecule reaction, however, is expected to be dependent upon the conditions of atmospheric pressure ionizations [6] such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). This is in analogy to a solution-phase chemical reaction where experimental conditions (such as temperature and reaction time) play important roles. Therefore, in this report we attempt to explore the optimal conditions of atmospheric pressure ionization sources to improve detection of epoxides using the gas-phase Meerwein reaction. This includes the mobile phase flow rate, source temperature, fraction of CH_3CN in the mobile phase, and MS source voltage (the ESI spray voltage, APCI corona discharge needle voltage and APPI repeller voltage).

Epoxides are electrophiles that can react with deoxyribonucleic acids (DNAs) to induce genetic mutations [7], thus constituting a structural alert for genotoxicity. Quantitation of such potential genotoxic impurities (GTIs) is challenging and it has attracted increasing attention during pharmaceutical development [8]. Both the European Medicines Agency (EMEA) and the U.S. Food and Drug Administration (FDA) set the 'threshold of toxicological concern' (TTC) to less than 1.5 μg/day in any marketing applications [9,10]. As such quantitative analysis of GTIs at low parts per million (ppm)
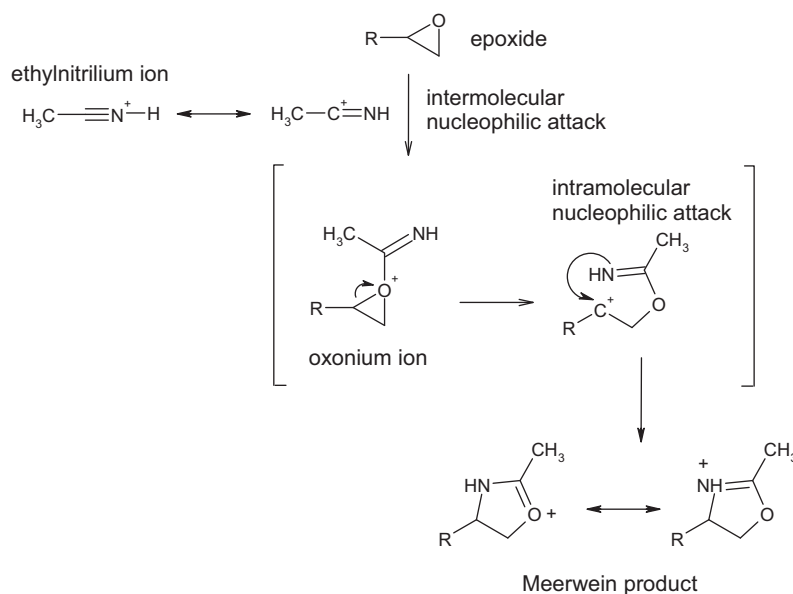
Scheme 1. The gas-phase Meerwein reaction of epoxides with the ethylnitrilium ion.

levels in drug substances and drug products is required. Despite recent advances in developing sensitive methods for individual or selective classes of GTIs, development of a direct and selective but also simple and robust method still remains an ongoing challenge [11–16]. Here we report the use of the gas-phase Meerwein reaction for rapid and direct LC–MS analysis of epoxides, where quantitation of these two potential GTIs at a low ppm level in the active pharmaceutical ingredients (APIs) is discussed.

2. Experimental

2.1. Chemicals

The structures of two epoxides and their corresponding Meerwein reaction products in this study are listed in Fig. 1. SB797313

((R)-2-((5-bromo-2,3-difluorophenoxy)methyl)oxirane) and SB719133 (2-((S)-1-((R)-oxiran-2-yl)allyl)isoindoline-1,3-dione) were synthesized at GlaxoSmithKline (King of Prussia, PA, USA). Trifluoroacetic acid (TFA, 99.0% purity) was purchased from Sigma–Aldrich (Milwaukee, WI, USA) and used without further purification. The HPLC-grade solvents including water (H_2O) and acetonitrile (CH_3CN) were purchased from Honeywell Burdick & Jackson (Morristown, NJ, USA).

2.2. Sample preparation

The API sample solutions were prepared at 10 mg/mL in water/acetonitrile (1:1 by v/v) solution. Typical standard solutions at 10 ng/mL of SB797313 and SB719133 were prepared by dissolving the neat solid materials and then diluted several times in the
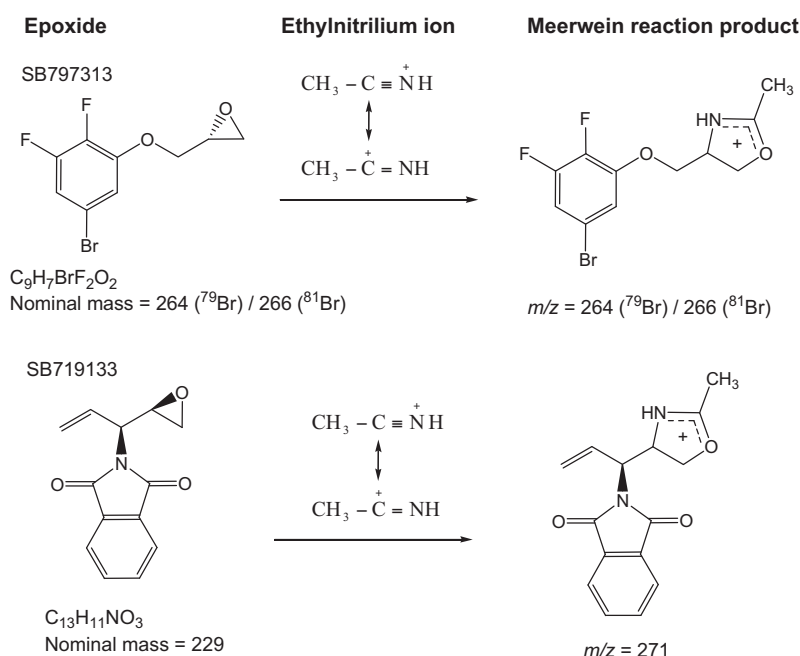

Fig. 1. Chemical structures of two epoxides of SB797313 (top) and SB719133 (bottom) and their corresponding Meerwein reaction products in the study.

water/acetonitrile (1:1) to the desired concentrations. The spiked solutions were prepared by dissolving 10 mg of the individual solid API samples in 1 mL of the corresponding 10 ng/mL standard solutions to give a spiking level of 1 ppm (by w/w).

2.3. LC–MS optimization

For investigating the optimal conditions of the gas-phase Meerwein reaction, the LC–MS experiments were performed on an Agilent 1100 LC system (Agilent Technologies, Wilmington, DE, USA) coupled to a Waters Q-TOF Premier quadrupole orthogonal acceleration time-of-flight mass spectrometer controlled by Mass-Lynx 4.1 (Waters Corporation, Manchester, UK). Using a Q-TOF rather than a quadrupole instrument was primarily due to the availability of all three ionization sources (ESI, APCI and APPI) in a single instrument in the authors' lab for direct comparison. The temperature of ESI desolvation gas and APCI/APPI probe was set at 500 °C to maximize desolvation of the LC eluent. The desolvation gas flow rate and sample cone voltage are 600 L/h and 30 V, respectively. For the APPI source, the Krypton (Kr) lamp was used to generate 10.6 eV photons. If not specified, the source temperature as well as the ESI spray, APCI corona discharge, and APPI repeller voltages were 120 °C, 4.0 kV, 1.0 kV and 1.0 kV, respectively (although some parameters might not represent the optimal values for the Meerwein reaction). The chromatographic separation was achieved using a Luna C18 column (50 mm × 2.0 mm, 3 μm; Phenomenex, Torrance, CA, USA) with a column temperature kept at 40 °C. Mobile phases A and B are 0.01% TFA in water and 0.01% TFA in acetonitrile, respectively. The HPLC gradient was ramped from 25%B to 75%B linearly in 4 min. If not specified, the mobile phase flow rate was set to 1 mL/min of which 200 μL/min was splitted into the atmospheric pressure ionization source of a mass spectrometer by an Acurate splitter (LC Packings, Sunnyvale, CA, USA).

2.4. Quantitative analysis of epoxides

Quantitation methods for two epoxides by SIM and MRM were developed on an Agilent 6140 single quadrupole and Agilent 6410 triple quadrupole mass spectrometer, respectively. The former one was controlled by ChemStation while the later one was controlled

by MassHunter Workstation (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was achieved using a Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters Corporation, Milford, MA, USA) with temperature kept at 40 °C. Mobile phases A and B are 0.01% TFA in water and 0.01% TFA in acetonitrile, respectively. The HPLC gradient was ramped from 0%B to 60%B linearly in 10 min to achieve a complete separation of SB797313 and SB719133 from their corresponding APIs. The flow rate was set as 0.5 mL/min, of which 100 μL/min was splitted into the APCI source by an Acurate splitter. For the spiked samples of epoxides with APIs, the HPLC eluent was diverted to the waste from 0 to 7.6 min to prevent the main peak from entering the ion source. The APCI-MS conditions are: APCI corona discharge voltage, 2 kV; nitrogen drying gas flow rate and temperature, 12 L/min and 400 °C, respectively; dwell time, 880 ms. In addition, the APCI-MS/MS conditions are: CID collision gas, nitrogen; collision energy for MRM, 18 eV.

2.5. Theoretical calculations

Theoretical calculations were performed based on density functional theory (DFT) using Becke's three-parameter hybrid functional with the Lee, Yang and Parr correlation (B3LYP) and the 6-311+G(d,p) basis set [17]. The relative potential energies were obtained based on structural optimization and frequency analysis followed by single-point calculations. The Gaussian 2003 program (revision D.02) was used for all calculations [18].

3. Results and discussion

3.1. The effect of the mobile phase flow rates

The effect of the HPLC flow rates on the gas-phase Meerwein reaction of SB797313 in the ESI, APCI and APPI sources was investigated and the results are illustrated in Fig. 2(a). It appears that the formation of the gas-phase Meerwein reaction product under the ESI condition is dramatically enhanced when the HPLC flow rate is at low μL/min. This suggests that desolvation is key to the gas-phase Meerwein reaction occurring in the ESI source that generates ions from evaporation of an aerosol of charged liquid droplets
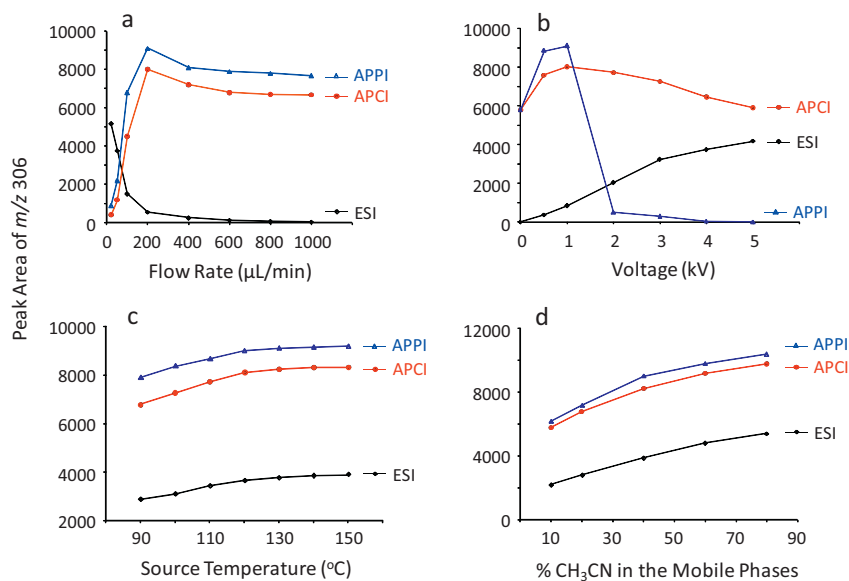

Fig. 2. Effects of (a) the HPLC mobile phase flow rates; (b) the voltages applied to the ESI spray needle, APCI corona discharge needle and APPI repeller; (c) the ESI, APCI, and APPI source temperatures; and (d) the percentages of CH$_3$CN in the mobile phase on the gas-phase Meerwein reaction of SB797313 with the ethylnitrilium ion. Refer to Section 2.3 for other experimental conditions.

[19–21]. However, decreasing the flow rate by splitting will also decrease the absolute amounts of epoxides entering the ESI source to react with the ethylnitrilium ion.

APCI and APPI, on the other hand, differ from ESI in the way that ionization occurs. Typically, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated tube in the APCI/APPI probe before interacting with the creating ions by the APCI corona-discharged electrons or the APPI photons [22]. Efficient delivery of thermal energy to achieve maximum evaporation of the liquid and analyte droplets in a short time frame (less than 1 ms) is crucial to the APCI and APPI sources where the spray is emitting from a near-sonic nozzle and traversing only a few centimeters. Higher gas flow produces a more uniform distribution of temperature across the vaporizing region in the APCI and APPI sources. As such, it reduces incomplete evaporation of solvents and solute(s) even when a relatively high flow rate is used. Higher flow rate of the eluent appears to favor the formation of the Meerwein reaction product. However, further increasing the HPLC flow rate (beyond $200\,\mu L/mL$) leads to incomplete desolvation and hence compromises the gas-phase Meerwein reaction.

3.2. The effect of the ESI spray, APCI corona discharge and APPI repeller voltages

The effect of the ESI spray, APCI corona discharge needle and APPI repeller voltages on the formation of the gas-phase Meerwein reaction was evaluated. The results are summarized in Fig. 2(b). A higher ESI spray voltage would result in smaller size of droplets and higher charge density on the surface of droplets, thereby facilitating desolvation process. This is consistent with the experimental results. As for the APCI source, the onset applied APCI corona discharge voltage is essential in terms of generating the ethylnitrilium ion. As displayed in Fig. 2(b), the Meerwein reaction product reaches maximum at about 1 kV. However, further increasing the APCI corona discharge voltage (beyond 1 kV) results in a slight decrease of the Meerwein reaction product. This is presumably due to the higher energy deposition that results in more fragmentation of the Meerwein reaction product. Different from APCI and ESI, the APPI repeller voltage is not directly involved in the ionization process. Instead, it helps push the ions generated at atmospheric pressure to the cone at the region of low vacuum. Since a higher APPI repeller voltage can disturb the equilibrium state of the ethylnitrilium ion, the Meerwein reaction is significantly reduced when the APPI repeller voltage is increased beyond 2 kV.

3.3. The effect of the ion source temperatures

The effect of the ion source temperatures (ranging from 80 to 150 °C) was investigated and the results are displayed in Fig. 2(c). Higher temperature implies higher average kinetic energy of molecules and hence more ion/molecule collisions per unit time. Therefore, it is expected that higher temperature in the ion source would lead to higher yield of the Meerwein reaction product. This trend was observed in all three ionization sources, ESI, APCI and APPI. Although the ethylnitrilium ion and the epoxide molecules are pre-heated at 500 °C by the desolvation gas (the ESI source) or the APCI/APPI probe (the APCI/APPI source), an increase in the source temperature still plays an important role in improving the yield of the Meerwein reaction product.

3.4. The effect of the percentages of CH₃CN in the mobile phase

The effect of the percentages of CH_3CN in the mobile phase on the formation of the Meerwein reaction product in the MS source was examined. At atmospheric pressure the mean free path between collisions for an ion/molecule reaction is short [23,24].
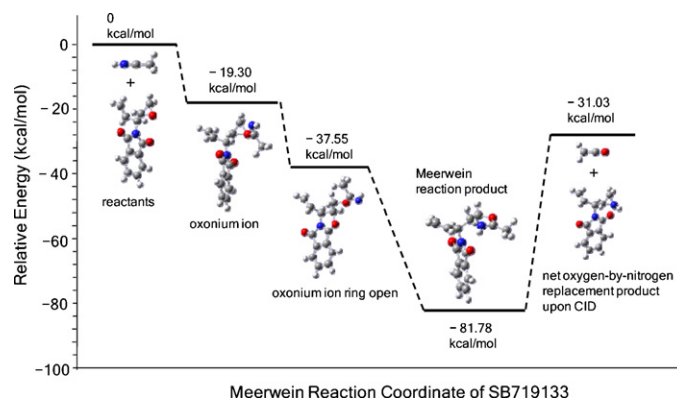

Fig. 3. The DFT calculated potential energy surface diagram for the gas-phase Meerwein reaction of SB719133 with the ethylnitrilium ion followed by the elimination of ethenone upon CID.

So a large number of gas-phase reactions occur quickly to reach an equilibrium state where the most stable species predominate. The high density of reagent ions results in high ion/molecule reaction efficiencies, allowing epoxides to react with the ethylnitrilium ion rapidly given that the Meerwein reaction is thermodynamically favorable [5]. Therefore, it is anticipated that higher percentage of CH_3CN in the mobile phase would result in higher density of the ethylnitrilium ion to give rise to higher yield of the Meerwein reaction product. This relation was observed in all three ionization sources, ESI, APCI and APPI as shown in Fig. 2(d). It is worth noting that most epoxides elute at HPLC gradient ranging from 20% to 80% CH_3CN, thereby providing an adequate source of the ethylnitrilium ion for the Meerwein reaction.

3.5. Energetics of the gas-phase Meerwein reaction of epoxides

The energetics of the gas-phase Meerwein reaction of SB719133 with the ethylnitrilium ion were calculated using DFT and the potential energy surface diagram is depicted in Fig. 3. Similar to SB797313 [5], the Meerwein reaction of SB719133 with the ethylnitrilium ion is highly exothermic (by −81.78 kcal/mol) and hence the reaction is thermodynamically favorable. This is possibly owing to the more stable five-membered ring of the Meerwein reaction products than the three-membered ring of epoxides (reactants) [25,26]. In contrast to SB797313, however, the Meerwein reaction of SB719133 shows that the intermediate via ring opening of the oxonium ion has lower potential energy than the oxonium ion by −18.25 kcal/mol (Fig. 3). This is probably due to the formation of an additional five-membered ring. The DFT calculation also reveals that the dissociation of the Meerwein reaction product of
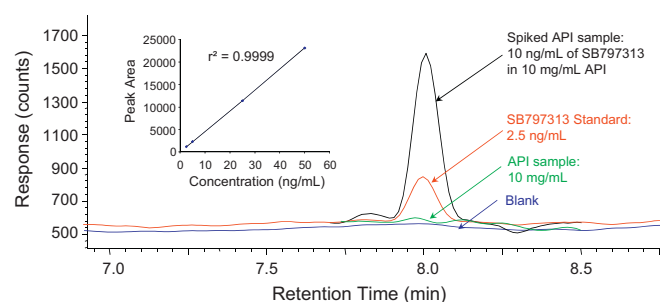

Fig. 4. Using the LC–APCI-MS method, overlay SIM chromatograms of m/z 306 were obtained corresponding to a blank, an API sample at 10 mg/mL, a standard solution of SB797313 at 2.5 ng/mL, and a spiked sample of SB797313 at 10 ng/mL in its corresponding API at 10 mg/mL. The insert is a calibration curve of the standard solutions of SB797313 at concentrations ranging from 2.5 to 50 ng/mL.

Table 1
Summary of validation results using SIM and MRM.

Epoxide	Sensitivity (S/N)[a]		Linearity (r^2)[b]		Accuracy (% difference)[c]		Precision (%RSD)[c]		Recovery (%)[a]	
	SIM	MRM	SIM	MRM	SIM	MRM	SIM	MRM	SIM	MRM
SB797313 [d]	20	21	0.9999	0.9990	1.2	−2.2	1.6	1.8	102	96
SB719133 [e]	10	12	0.9990	0.9991	−1.8	−2.8	2.2	2.0	96	92

[a] At 1 ppm of the specific epoxide relative to 10 mg/mL of its corresponding API.

[b] Concentration ranging from 2.5 to 50 ng/mL.

[c] Based on injections of six prepared standard samples at 10 ng/mL.

[d] For quantitation of SB797313, the ion of m/z 306 (^{79}Br) and the MS/MS transition of m/z 306 → 168 were selected for the SIM and MRM experiments, respectively.

[e] For quantitation of SB719133, the ion of m/z 271 and the MS/MS transition of m/z 271 → 212 were selected for the SIM and MRM experiments, respectively.

SB719133 needs to overcome a energy barrier of 50.75 kcal/mol to give rise to the net oxygen-by-nitrogen replacement product along with ethenone. The calculated energetics for the net oxygen-by-nitrogen replacement is in agreement with the reported value for the net oxygen-by-sulfur replacement [25].

3.6. Quantitative analysis of epoxides SB797313 and SB719133 in APIs

Derived from the above results, the APCI source was selected for developing a quantitative method for analysis of SB797313 and SB719133 in APIs. This is because the APCI source is readily available in most analytical laboratories equipped with LC–MS instrumentation (in comparison to the less popular APPI source). In addition, the occurrence of the gas-phase Meerwein reaction in the APCI source appears to be less prone to the corona discharge voltage (more robust signal), in contrast to the APPI source where the reaction is susceptible to the APPI repeller voltage. Although the APPI source provides slightly better sensitivity than the APCI source, APCI was chosen in this case.

Since SB797313 and SB719133 elute after their corresponding APIs, the matrix effect (e.g. from APIs) was assessed prior to the validation of the LC–APCI-MS method. It was found that lower recoveries (<85%) were consistently obtained. This is probably due to the existence of the residual API molecules in the APCI source can scavenge protons from the ethylnitrilium ion and hence avert the Meerwein reaction product of the epoxides eluting after APIs.

To avoid the occurrence of this undesired proton transfer reaction, the HPLC eluent was diverted to the waste from 0 to 7.6 min to prevent the API molecules from entering into the APCI source.

On the basis of our current practice by using a fresh calibration curve for analysis of GTIs [9–16], the LC–APCI-MS method was validated by assessing the intra-day linearity, accuracy, precision, and recovery. Representative SIM chromatograms of the Meerwein reaction product of a blank, an API sample at concentration of 10 mg/mL, a standard solution of SB797313 at concentration of 2.5 ng/mL, and a spiked sample are shown in Fig. 4. The spiked sample corresponds to 10 ng of SB797313 in 10 mg/mL API that is 1 ppm by w/w. The insert in Fig. 4 displays a linear curve at concentrations of SB797313 ranging from 2.5 to 50 ng/mL with $r^2 > 0.999$. The method was validated and the validation results are summarized in Table 1. The method affords excellent sensitivity, accuracy, precision and recovery. SB719133 was also tested and similar results were obtained (Table 1). The LC–APCI-MS method was further applied to actual sample analysis where trace amounts of the epoxides were detected at a level of below 'threshold of toxicological concern' (TTC) (data not shown). Therefore this method was able to determine unknown amounts of trace epoxides when the application to analysis of real samples is a necessity.

Quantitation of these two epoxides by MRM was also evaluated where selection of MRM transitions and optimization of collision energies were required. Typical MS/MS spectra of the Meerwein reaction products of SB797313 and SB719133 are displayed in Fig. 5. The MRM transitions of m/z 306 → 168 (Fig. 5(a)) and m/z
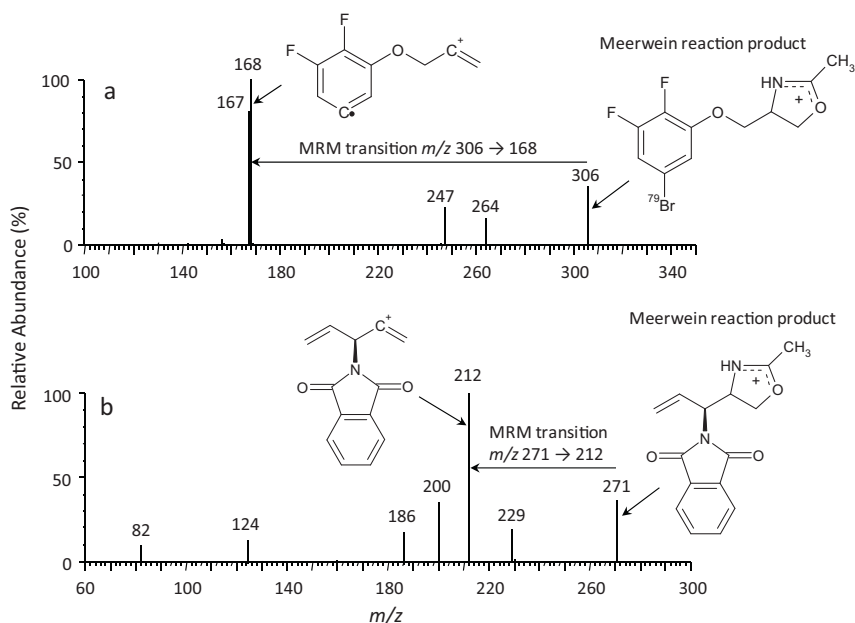

Fig. 5. Typical MS/MS spectra of the gas-phase Meerwein reaction products of (a) SB797313 (^{79}Br) and (b) SB719133 with the ethylnitrilium ion. The CID activation energy to cause MS/MS fragmentation was chosen as 18 eV with nitrogen as the collision gas.

271 → 212 (Fig. 5(b)) were chosen for quantitation of SB797313 and SB719133, respectively. The validation results are tabulated in Table 1. The MRM method gives similar linearity, sensitivity, recovery, accuracy and precision in comparison with the SIM method. However, it is expected that more selective MRM method is advantageous when dealing with more complex sample matrix.

4. Conclusions

A gas-phase derivatization strategy for rapid and direct LC–MS analysis of epoxides has been developed using the gas-phase Meerwein reaction. This structure-specific ion/molecule reaction readily occurs between the epoxides and the ethylnitrilium ion that is generated during ESI, APCI and APPI mass spectrometry (when acetonitrile is used as the mobile phase). Similar to SB797313, the DFT calculation at the B3LYP/6-311+G(d,p) level of theory shows that the gas-phase Meerwein reaction of SB719133 is highly exothermic (by -81.78 kcal/mol). Among three commonly used atmospheric pressure ionization techniques including ESI, APCI and APPI, APCI is the preferred choice due to better sensitivity and more robust detection under routine LC–MS instrumentation settings. However, the Meerwein reaction is very sensitive to the APPI repeller voltage. Elevating ionization source temperature enhances the Meerwein reaction. Although higher content of acetonitrile in the mobile phase leads to higher Meerwein reaction product, the typical HPLC gradient ranging from 20 to 80% CH_3CN provides sufficient detection sensitivity. The validated LC–APCI-MS method was tested for analysis of trace epoxides and produced results that have accuracies and precision comparable to the solution-phase derivatization method [15].

It is worth mentioning that the gas-phase Meerwein reaction is not limited to the ethylnitrilium ion. Tetramethylurea (TMU) and thio-TMU can be used as dopants (e.g. by post-column addition) to generate (thio)acylium ions $(CH_3)_2NC^+=O$ and $(CH_3)_2NC^+=S$ that were shown to undergo the same reaction with (thio)epoxides [26]. Compared to the solution-phase derivatization approach [15], the gas-phase derivatization can occur under the 'interference-free' condition after chromatographic separation (i.e. an impurity of interest is separated from an active pharmaceutical ingredient prior to derivatization reaction). Reactive desorption electrospray ionization (reactive DESI) could be an alternative approach but yet to be explored [27,28]. It is the authors' opinion that gas-phase derivatization via ion/molecule reactions for selective detection of trace impurities is underutilized in pharmaceutical analysis.

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