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Research Article

Formation of the $[M - 1]^+$ Ions of Methylindoles in APCI Mass Spectrometry

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Indoles are common building blocks of new pharmacologically active chemical entities in drug discovery and development. Due to their poor ionization in electrospray ionization mass spectrometry, atmospheric pressure chemical ionization (APCI) is the method of choice for LC-MS analysis of simple indoles. Three types of ions, including $[M - 1]^+$, $M^{+\bullet}$, and $[M + H]^+$, can be observed in APCI but the relative intensities of these ions may vary depending upon the structural properties of the indoles and the mass spectral source conditions. We report in this paper the observation of $[M - 1]^+$ ions for methylindoles in an Agilent multimode ion source and the investigation into their formation. By means of tandem mass spectrometric experiments performed on a Thermo Fisher Scientific LTQ ion trap mass spectrometer equipped with an APCI source, it was found that $[M - 1]^+$ ions can be generated from $M^{+\bullet}$ ions upon-collision induced dissociation. This suggests that the $[M - 1]^+$ ions might be the in-source fragmentation product of $M^{+\bullet}$ ions. It was proposed that both $[M - 1]^+$ and $M^{+\bullet}$ ions are probably generated through a charge transfer mechanism while $[M + H]^+$ ions are the product of proton transfer. The basicity of the analytes might play an important role in dictating which ionization mechanism is operative. For 3-methylindole, the charge transfer process appears to be more dominant than for 2-methylindole since the former is less basic. As expected, substituting electron withdrawing groups on 3-methylindole, such as fluorine, promotes charge transfer and vice versa. Therefore, it is expected that formation of the $[M - 1]^+$ ions is more pronounced for less basic methylindoles.

1. Introduction

Liquid chromatography-mass spectrometry (LC-MS) has been an increasingly popular tool for both qualitative and quantitative analysis of drugs and their related substances in all stages of drug development owing to its high sensitivity and specificity [1]. This includes, but is not limited to, the analysis of drug metabolites, degradation products, process impurities, extractables/leachables from packaging materials. Several atmospheric pressure ionization (API) techniques, including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), have been successfully interfaced with various mass spectrometric detectors and are widely used in pharmaceutical analysis. However, no single technique ionizes all types of pharmaceutical compounds. Generally, ESI ionizes polar compounds most efficiently and APPI is more suitable for nonpolar compounds, while APCI performs best with moderately polar compounds although significant overlap exists among the three [2]. To improve the throughput while analyzing large numbers of compounds with diverse chemical structures, combined ESI/APCI and APCI/APPI sources have also been developed [3–6]. The advantage of the combined sources in terms of the breadth of ionizable compounds has been investigated recently [4, 6].

Indoles are frequently building blocks for pharmacologically-active drug candidates [7–11], and they have been analyzed by a variety of mass spectrometry techniques including both GC-MS and LC-MS. However, simple indoles are not ionizable by ESI. Therefore APCI and combined ESI/APCI sources are the methods of choice in addition to GC-MS [4]. During the study of some 3-methylindoles and 2-methylindole using an Agilent LC-MSD equipped with a multimode ion source, an intense peak corresponding to

the $[M-1]^+$ ion was observed in the mass spectrum of 3-methylindole and its analogues but not for 2-methylindole. Normally, $[M-1]^+$ ions are not prominent ions in the API mass spectra of polar compounds in protic mobile phase. This observation prompted us to study the mechanism of formation of the $[M-1]^+$ ions in API mass spectrometry. In APCI several reaction mechanisms, such as hydride abstraction and charge transfer, have been proposed in the formation of the $[M-1]^+$ ions of other classes of compounds such as hexane [12, 13]. In the present study, we discovered that $[M-1]^+$ ions of indoles could be generated from $M^{+\bullet}$ ions via collision induced dissociation (CID), suggesting that $[M-1]^+$ ions may be the in-source CID product of metastable $M^{+\bullet}$ ions. The parameters that influence the formation of $[M-1]^+$ ions are also discussed.

2. Experimental

2.1. Reagents. 4-Fluoro-3-methylindole was prepared at GlaxoSmithKline (King of Prussia, PA, USA). 3-Methylindole, 2,3-dimethylindole, 3-indolyacetonitrile, 2-methylindole, and D-tryptophane were obtained from Sigma-Aldrich (Milwaukee, WI, USA). All HPLC grade solvents were purchased from Burdick and Jackson (Muskegon, MI, USA).

2.2. HPLC Analysis. Samples with a concentration of approximately 0.1 mg/mL were injected onto an Agilent 1100 HPLC system directly (Agilent, Palo Alto, CA, USA). Chromatographic separation was achieved on a Luna C18 (2) column, 50 × 2 mm, 3 μ (Phenomenex, Torrance, CA, USA). Mobile phases A and B were water and acetonitrile, respectively, both of which were modified with 0.05% TFA. A linear gradient ramping from 0% B to 95% B over 8 min with a flow rate of 1 mL/min was used for elution.

2.3. Mass Spectrometry. Mass spectral data were acquired on an LC-MSD (model #G1946D, Agilent, Palo Alto, CA, USA) equipped with a multimode ion source in the positive ion mode. Full scan mass spectra were collected from *m/z* 120 to 1000. The capillary voltage was maintained at 2 kV, and the charging voltage was set to 2000 V. The drying gas flow was set between 4 to 13 L/min with a temperature of 350°C. The vaporizer temperature was set to 250°C. The nebulizer pressure was set between 20 to 60 psi. The corona current varied from 1.5 to 4 μA. For comparison, mass spectra were also acquired on an LTQ ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Ion Max APCI source. The LC effluent (1/5 of the 1 mL/min flowed into MS) was nebulized using nitrogen as the sheath gas, auxiliary gas, and sweeping gas with flow rates of 30, 5, and 5 au (arbitrary unit), respectively. The vaporizer temperature was set at 400°C, and the corona discharge current was set at 4 μA. The capillary voltage was set to 20 V with the temperature at 200°C. The tube lens voltage was set at 75 V. CID experiments were performed in the collision cell using helium as the collision gas with an isolation width of 2. All data were analyzed using Xcalibur 1.4.

3. Results and Discussion

3.1. Behaviours of Methylindoles in the Agilent Multimode Ion Source (Essentially APCI Mode). Simple indoles such as 4-fluoro-3-methylindole do not ionize in positive ion ESI mode; therefore, 4-fluoro-3-methylindole was tested on an Agilent LC-MSD using a multimode ion source. It afforded a base peak at *m/z* 148 (Figure 1(a)) corresponding to an $[M-1]^+$ ion while the protonated molecule peak of *m/z* 150 $[M+H]^+$ was of only half as intense. The peaks at higher masses, at *m/z* 164 and *m/z* 189, were tentatively assigned as the oxidation product and the CH_3CN adduct of *m/z* 148, respectively. In order to explore the generality of the formation of the $[M-1]^+$ ions and its underlying mechanisms, both 3-methylindole and 2-methylindole were analyzed. Interestingly, for 3-methylindole, a strong peak at *m/z* 130 corresponding to the $[M-1]^+$ ions was observed in addition to the protonated molecule at *m/z* 132 $[M+H]^+$ of almost equal intensity (Figure 1(b)). In the mass spectrum of 2-methylindole, however, the protonated molecule $[M+H]^+$ at *m/z* 132 was the base peak while the $[M-1]^+$ ion (*m/z* 130) was not observable (Figure 1(c)). This suggests that 3-methylindole prefers to form the $[M-1]^+$ ions.

We then examined ionization behaviour of other indole derivatives in the Agilent multimode ion source. As expected, both *m/z* 144 ($[M-1]^+$) and *m/z* 146 ($[M+H]^+$) were observed for 2,3-dimethylindole and the intensity of the $[M-1]^+$ ions was approximately half of the $[M+H]^+$ ions (Figure 1(d)). In the mass spectrum of 3-indolyacetonitrile (Figure 1(e)), on the other hand, extensive in-source fragmentation and gas-phase ion-molecule reactions were observed. The peak at *m/z* 146 which could be the monooxygenation adduct of the ions with *m/z* 130 was observed as the base peak. The second most intense peak at *m/z* 155 is presumably the $[M-1]^+$ ions, while the protonated molecule peak at *m/z* 156 $[M+H]^+$ was absent. Another indole examined was D-tryptophane which contains an amino functional group on the alkyl side chain offering a primary protonation site. In the mass spectrum of D-tryptophane (Figure 1(f)), the protonated molecule with *m/z* 205 $[M+H]^+$ was observed as the base peak, and the $[M-1]^+$ ions (*m/z* 203) are not detectable. It was of interest to observe a common ion of *m/z* 130 for all 3-methylindole (Figure 1(b)), 3-indolyacetonitrile (Figure 1(e)), and D-tryptophane (Figure 1(f)).

3.2. Mechanistic Study of $[M-1]^+$ Ion Formation of Methylindoles by Ion Trap MS. In an APCI source mobile-phase solvents are removed by vaporization and vacuum while the analytes are ionized in the gas phase by a plasma generated from the discharge of a corona needle [14]. The ionization process consists of a series of complicated gas-phase chemical reactions. In the positive ion mode, the $[M+H]^+$ and $M^{+\bullet}$ ions are produced through "proton transfer" (1) and "charge transfer" (2), respectively [15]. Interestingly, prominent $[M-1]^+$ ions have been observed in APCI analysis of certain non-polar compounds, such as chloroethanes [16], alkanes [12, 13] using non-polar solvents, and hydroxyl polycyclic aromatic hydrocarbons (PAH) with SFC solvents

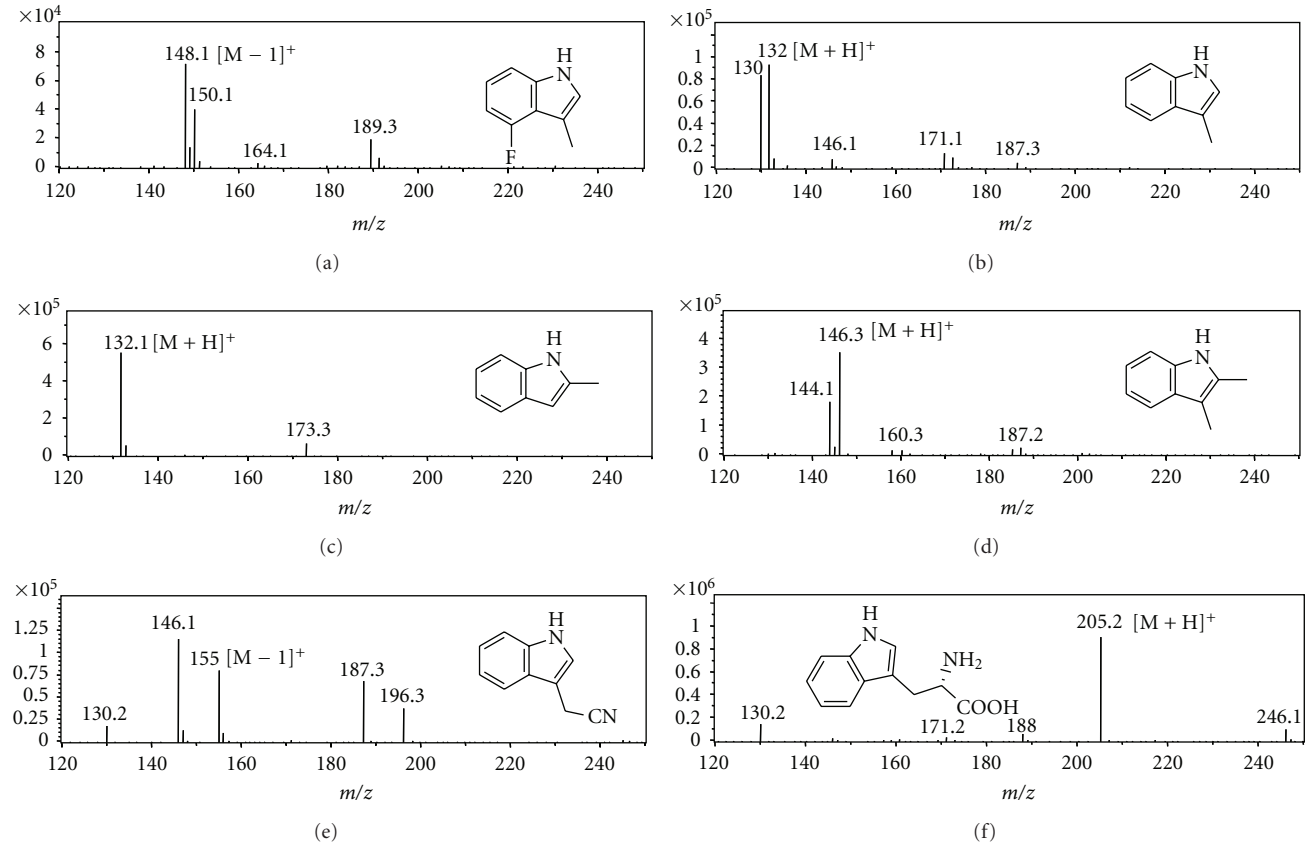


Figure 1: Full scan mass spectra obtained on an Agilent LC-MSD equipped with a multimode ion source in the positive ion mode (corona current, 2.5 μA; drying gas flow, 5 L/min; nebulizer pressure, 40 psi). (a) 4-Fluoro-3-methylindole; (b) 3-methylindole; (c) 2-methylindole; (d) 2,3-dimethylindole; (e) 3-indolyacetonitrile; (f) D-tryptophane.

[17]. Carroll et al. explained the formation of the $[M - 1]^+$ peaks in the APCI spectrum of hexane and proposed that the $[M - 1]^+$ ions are formed through hydride abstraction from molecular hexane by $C_4H_9^+$ ions [13]. Bell et al. proposed that the $[M - 1]^+$ ions of cyclohexane were produced by charge transfer (3) [12]

$$M + n(H_2O)H^+ \longrightarrow [M + H]^+ + nH_2O \quad (1)$$

$$M + {N_2}^{+\bullet} \longrightarrow M^{+\bullet} + N_2 \quad (2)$$

$$N_2^+ + \text{cyclo-}C_6H_{12} \longrightarrow \text{c-}C_6H_{11}^+ + H^+ + N_2 \quad (3)$$

Moyano et al. proposed the structures of $[M - 1]^+$ ions of hydroxyl polycyclic aromatic hydrocarbons as corresponding protonated quinones after loss of H_2 from the corresponding $[M + H]^+$ ions [17].

In order to explore the formation of the $[M - 1]^+$ ions of the methylindoles, 2-methylindole, and 3-methylindole, they were also analyzed by LTQ using an Ion Max APCI source. It was surprising to observe that the expected $[M - 1]^+$ peaks at *m/z* 130 were much smaller than the protonated molecular ions at *m/z* 132 for 3-methylindole in contrast to the results obtained on the Agilent multimode ion source. APCI ionization may proceed through two

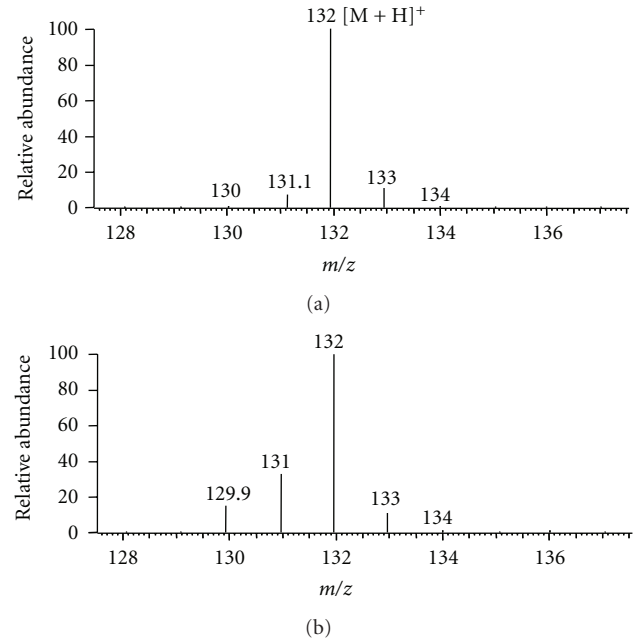


Figure 2: APCI mass spectra of 2-methylindole (a) and 3-methylindole (b) obtained on a LTQ ion trap mass spectrometer using acetonitrile as the mobile phase.

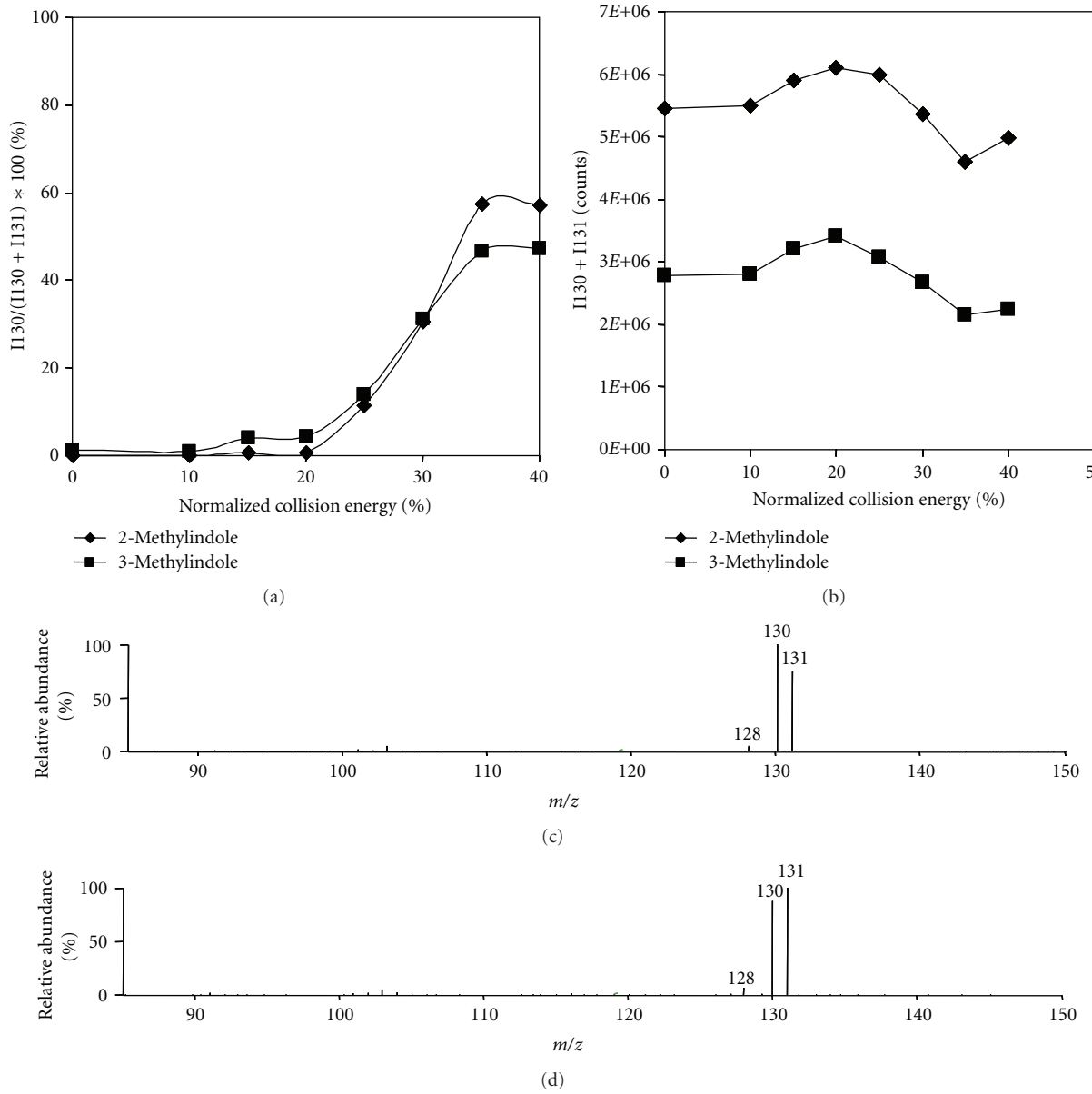


Figure 3: Relationship between the *m/z* 130 $[M-1]^+$ ions and *m/z* 131 $M^{+\bullet}$ ions of 2-methylindole and 3-methylindole studied by collision induced dissociation. (a) Collision energy resolved production curves of *m/z* 130 during CID of *m/z* 131. I130 and I131 are the absolute intensity of *m/z* 130 and *m/z* 131 ions, respectively. (b) Collision energy resolved total intensity (sum) of the *m/z* 130 and *m/z* 131 ions of 2-methylindole and 3-methylindole. (c) and (d) CID spectra of the *m/z* 131 ions of 2-methylindole and 3-methylindole, respectively, at 35% normalized collision energy.

competing mechanisms, proton transfer and charge transfer. Limiting the availability of proton in the ion source could promote the charge transfer mechanism and suppress the proton transfer mechanism. Thus, if the $[M-1]^+$ ions were generated by the charge transfer mechanism as suggested by other studies, reducing the water content (source of protons) would increase the intensity of the $[M-1]^+$ ion peak. To test this hypothesis, both indoles were dissolved in acetonitrile and infused into the LTQ directly. To our surprise, the relative intensity of $[M-1]^+$ peak increased only slightly. However, this slight increase was accompanied by the obvious increase in relative intensity of the $M^{+\bullet}$ peak. As expected, the $[M-1]^+$ peaks were observed for both compounds, but the $[M+H]^+$ peaks at *m/z* 132 are the base peaks (Figure 2). Different from those spectra obtained on LC-MSD (Figures 1(b) and 1(c)), it was of great interest to observe significant *m/z* 131 peak corresponding to the $M^{+\bullet}$ ions (Figure 2), which is more intense than $[M-1]^+$ peaks. A tempting question to ask is whether or not the $[M-1]^+$ ions are secondary (or product) ions of $M^{+\bullet}$ ions.

The above hypothesis was investigated by taking advantage of the tandem mass capability of the Thermo Fisher

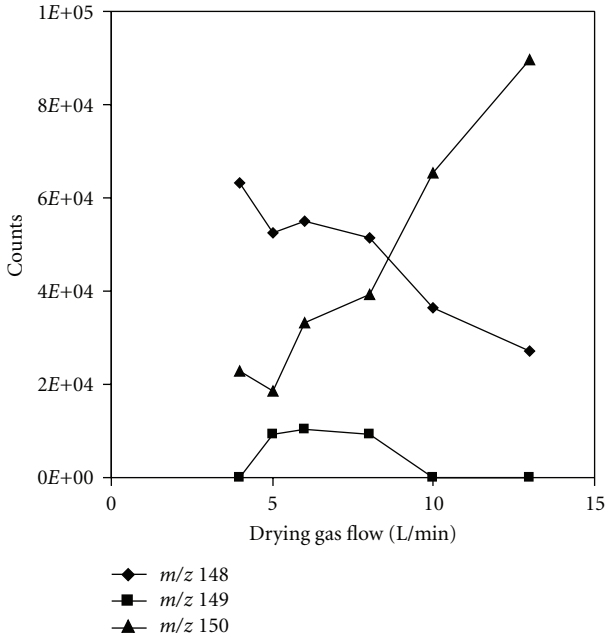


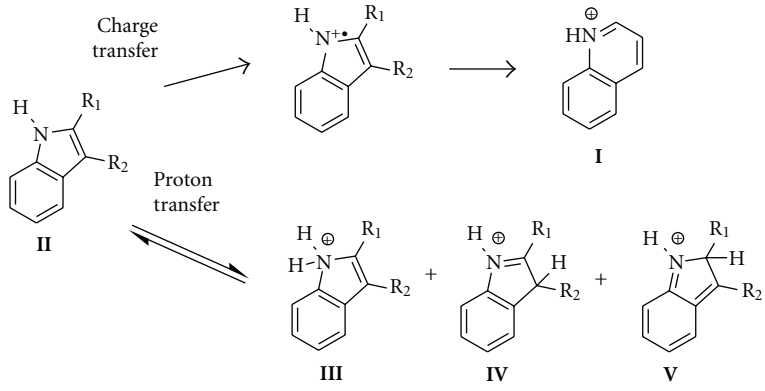
Figure 4: Effects of drying gas flow on the intensity of *m/z* 148 $[M-1]^+$, *m/z* 149 $M^{+\bullet}$, and *m/z* 150 $[M+H]^+$ ions of 4-fluoro-3-methylindole studied on an Agilent LC-MSD equipped with the multimode ion source (nebulizer pressure, 40 psi; corona current, 2.5 μA).

Scientific LTQ ion trap mass spectrometer. The $M^{+\bullet}$ ions (*m/z* 131) of both indoles were isolated and then fragmented by CID experiments to examine the formation of the *m/z* 130 $[M-1]^+$ ions. From the spectra obtained at various collision energy, the total intensity of the $[M-1]^+$ ions at *m/z* 130 and the $M^{+\bullet}$ ions at *m/z* 131 can be summed and then plotted against the collision energy (Figure 3(b)). Figure 3(b) shows that the total ion intensity remains constant (almost a flat line), which provided a valid basis for the calculation of the percentage of *m/z* 130 peaks over the total intensity. The percent *m/z* 130 ions was then plotted against the collision energy (Figure 3(a)). For 2-methylindole, the *m/z* 130 $[M-1]^+$ peak is essentially absent when the normalized collision energy is at 0%, but it starts to increase significantly at 20% normalized collision energy. For 3-methylindole, on the other hand, the *m/z* 130 $[M-1]^+$ peak starts to increase significantly at the 10–15% normalized collision energy. When 35% normalized collision energy was applied, the *m/z* 130 $[M-1]^+$ and 131 $M^{+\bullet}$ ions have almost the equal intensity, which is the case for both 2-methylindole (Figure 3(c)) and 3-methylindole (Figure 3(d)). Generation of the $[M-1]^+$ ion appears to correlate with ramping of the collision energy which supports the hypothesis that $[M-1]^+$ peaks might be generated through fragmentation of the $M^{+\bullet}$ ions. During ionization similar gas-phase reactions, namely in-source CID, could cause the formation of the $[M-1]^+$ ion of methylindoles.

In EI-MS, the $[M-1]^+$ ions at *m/z* 130 were base peak for both 2-methylindole and 3-methylindole, where the $[M-1]^+$ ions of both compounds were proposed to have the same structure, a quinolinium ion **I** (Scheme 1), which was established by both stable isotope labelling and ion kinetic energy studies [18–20]. The CID results obtained on the LTQ seem to suggest that the transition from *m/z* 131 to *m/z* 130 of 2-methylindole may require higher activation energy than that of 3-methylindole. Similarly, the $M^{+\bullet}$ ions could be converted to $[M-1]^+$ ions by in-source CID, and the extent of this conversion would depend on a combination of the internal energy of the ions, the activation energy of the fragmentation reaction, and the voltage settings of skimmers [21, 22].

3.3. Competitive Formation of the $[M-1]^+$ and $[M+H]^+$ Ions of Substituted Indoles in an Agilent Multimode Ion Source. The above data suggest that the $[M-1]^+$ ions of the methylindoles could be generated by charge transfer which is in competition with a second mechanism, proton transfer. It is reasonable to hypothesize that its formation would be affected by the structures of the methylindoles in terms of ionization potential and proton affinity in addition to the source conditions (which will be discussed in the next section) [23]. It would be of interest to understand why charge transfer reaction was more pronounced for 3-methylindole than that of 2-methylindole in the Agilent multimode ion source. The ionization energy (IE) of 3-methylindole has been previously determined as 7.5243 ± 0.0001 eV, while the IE of 2-methylindole is 7.6708 ± 0.0001 eV [24]. Although the IE of 3-methylindole is ∼0.15 eV lower than that of 2-methylindole, this subtle difference is not likely to be the underlying reason for promoting 3-methylindole in favour of charge transfer. An alternative possibility is that 2-methylindole has higher proton affinity than 3-methylindole. Andonovski et al. reported that the proton affinity of indoles correlates well with their solution basicity; that is, more basic indoles have better proton affinity [25]. Both 2-methylindole and 3-methylindole are weak bases, and can be protonated only in very strong acids in solution. 2-Methylindole, however, is relatively more basic than 3-methylindole as indicated by their pKa's at −0.28 and −4.55, respectively [26]. Methylindoles could be protonated at three positions (1, 2, or 3) competitively (Scheme 1), and the importance of individual protonated structure (**III**, **IV**, **V**) is influenced by the substitution. It was suggested that the stability of the ions is affected by the substitution positions of the methyl group [27], and the solution basicity of indoles closely relates to the positions of methyl substitution (Scheme 1) [26]. Interestingly, the importance of charge transfer of 2,3-dimethylindole (Figure 1(d)) lies between 2-methylindole and 3-methylindole since the pKa (−1.49) falls between the two, 3-methylindole at −4.55 and 2-methylindole at −0.28. The relative intensity of the $[M-1]^+$ ion (i.e., the importance of charge transfer mechanism) is in decreasing order of 3-methylindole > 2,3-dimethylindole > 2-methylindole, which is in agreement with their solution basicity.

Substitution of electron withdrawing groups decreases the proton affinity, thus lowering the importance of the proton transfer mechanism and increasing the importance of charge transfer mechanism. As expected, charge transfer is more important (than proton transfer) for both 4-fluoro-3-methylindole (Figure 1(a)) and 3-indolyacetonitrile



Scheme 1: Ionization pathways of 2-methylindole and 3-methylindole.

(Figure 1(e)) than for 3-methylindole. On the other hand, increasing proton affinity by adding a proton seeking group would inhibit the charge transfer dramatically as demonstrated by D-tryptophane (Figure 1(f)).

3.4. Effects of Source Parameters of the Agilent Multimode Ion Source on Formation of $[M-1]^+$ *Ions.* Using 4-fluoro-3-methylindole (Figure 1(a)) as a model compound, the effects of source parameters of the Agilent multimode ion source on formation of the $[M-1]^+$ ions were investigated by changing various parameters. Individual ramping of the nebulizer pressure, corona current, and temperature parameters did not seem to affect the relative intensity of the $[M-1]^+$ peak. Drying gas flow, however, appeared to impact the intensity of the $[M-1]^+$ peak drastically. The intensities of peaks *m/z* 148 $[M-1]^+$, *m/z* 149 $M^{+\bullet}$, and *m/z* 150 $[M+H]^+$ were obtained from combined mass spectra and were plotted against drying gas flow, respectively (Figure 4). When the drying gas flow was ramped from 4 to 13 L/min, the intensity of the *m/z* 148 ion decreased drastically with concomitant increase of the intensity of the *m/z* 150 $[M+H]^+$ ion. Similar experiments were performed using the Ion Max APCI source, and this drying gas effect was less obvious. This observation appears to be in agreement with the competitive ionization between the two mechanisms in the gas phase, charge transfer, and proton transfer in the specific source. In the Agilent multimode ion source, increasing drying gas flow seems to promote proton transfer while inhibiting charge transfer. It has been demonstrated in other studies that trace amounts of moisture in gas flow have been implicated in enhanced proton transfer for APCI [12]. Nonetheless, the underlying mechanism of the effect of drying gas on the formation of the $[M-1]^+$ ion is not well understood.

4. Conclusion

The ionization of a group of simple indoles was investigated by APCI mass spectrometry. Three types of ions, $[M-1]^+$, $M^{+\bullet}$, and $[M+H]^+$ were observed. For certain methylindoles, the $[M-1]^+$ ions were the based peaks using the Agilent multimode ion source. It has been demonstrated using ion trap mass spectrometry that $[M-1]^+$ ions can be generated from $M^{+\bullet}$ ions upon CID suggesting that $[M-1]^+$ ions are possibly formed through in-source CID of $M^{+\bullet}$ ions. It has been proposed that charge transfer might be responsible for the formation of the $[M-1]^+$ ions. The relative significance of the two competitive process, charge transfer and proton transfer, is affected by the basicity of the methylindoles. In addition, it appears that generation of the $[M-1]^+$ ions from the less basic methylindoles is enhanced in the Agilent multimode ion source compared to Thermo Fisher Scientific Ion Max APCI source. Further characterization of the two ion sources is warranted, especially for the relatively new Agilent multimode ion source.

Acknowledgments

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