Research Article

Pseudo-MS³ Approach Using Electrospray Mass Spectrometry (ESI-MS/MS) to Characterize Certain (2E)-2-[3-(1H-Imidazol-1-yl)-1-phenylpropylidene]hydrazinecarboxamide Derivatives

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Received 23 October 2013; Accepted 2 December 2013; Published 5 January 2014

Academic Editor: Irene Pandori

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An approach for the use of in-source fragmentation with electrospray ionization followed by product ion scan in a triple quadrupole mass spectrometer system is described. This approach is based on the elucidation of the various fragmentation pathways by further dissociation of each fragment ion in the ion spectrum. This can be achieved predominately, by combining fragmentor voltage induced dissociation (in-source fragmentation) with subsequent collision-induced dissociation; this process can be referred to as pseudo-MS³ scan mode. This technique permitted unambiguous assignment and provided sufficient sensitivity and specificity. It is advantageous for structure elucidation of unknown compounds. We investigate the possibility of using in-source fragmentation with the diverse novel chemical entities encompassing different substituents. This process was intended to improve the qualitative capability of tandem mass spectrometry simulating the MS³ of ion trap for studying fragmentation mechanisms. The approach is to implement the investigated technique as a well established tool for the characterization of new pharmacologically important chemical entities. The data presented in this paper provided useful information on the effect of different substituents on the ionization/fragmentation processes and can be used in the characterization of (2E)-2-[3-(1H-imidazol-1-yl)-1-phenylpropylidene]-hydrazinecarboxamide derivatives **3a-h**.

1. Introduction

It has always been known that the use of a triple quadrupole mass spectrometer system (QqQ) for quantitative analysis is superior to that of any ion trap mass spectrometry systems. This is due to the fact that one can always utilize the collision-induced dissociation (CID) in the QqQ which usually produces more abundant fragment ions than those shown in case

of resonance excitation in the ion trap (IT) [1]. However, the opposite has always remained correct with regard to qualitative analysis. On the other hand, electrospray ionization mass spectrometry (ESI-MS)—as a robust technique for quantification studies—has been excessively applied for the analysis of different types of compounds such as oligosaccharides, glycoproteins, oligonucleotides, and drug metabolites [2–7]. In electrospray ionization (ESI), ions fragmentation may take

FIGURE 1: Chemical structures of nafimidon (A), denzimol (B), arylsemicarbazones C, and the test compounds 3a-h.

place inside the ion source (in-source fragmentation) prior to reaching the mass analyzer. Although this method of in-source fragmentation has low specificity, as most of the ions in the ion source are fragmented simultaneously, it has been reported by different scientists [8–17]. Elaborate ion spectra can be acquired from a single stage MS² scan using QqQ. By contrast, generating a similar number of fragment ions in IT demands various fragmentation stages (MSⁿ) utilizing CID through the in-trap resonance excitation, leaving it to be time consuming and less sensitive. Additionally, the low mass cutoff in product ion scans represents another drawback of IT. This is due to the fact that both precursor ion and the lowest *m/z* fragment ion have to be stable simultaneously inside the IT [1, 18]. In this study, we use in-source fragmentation (ISF) prior to product ion (MS²) scan to elucidate the structure of certain (2*E*)-2-[3-(1*H*-imidazol-1-yl)-1-phenylpropylidene]-hydrazinecarboxamide derivatives 3a-h using electrospray ionization tandem mass spectrometry (ESI-MS/MS). The use of this approach was designed to boost the structure elucidation power of QqQ to mimic the MS³ of ion trap avoiding the IT drawbacks mentioned above.

Compounds 3a-h are considered as hybrid structures incorporating both imidazole moiety, a pharmacophore of alkylimidazole anticonvulsants (nafimidon (A) and denzimol (B), Figure 1) and arylsemicarbazone moiety, a pharmacophore of arylsemicarbazone anticonvulsants with the general structural formula C (Figure 1) [19–22]. The test compounds 3a-h displayed anticonvulsant activity in the preliminary anticonvulsant screening assays [23]. Accordingly, in the light of interesting structural and biological results the situation definitely urges some additional research in their analyses.

2. Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

2.1. Chemistry

2.1.1. General Procedure for Preparation of the Ketones 1a-c. The appropriate acetophenone (200 mmol), dimethylamine hydrochloride (270 mmol), and paraformaldehyde (90 mmol) were heated to reflux in absolute ethanol (35 mL) in the presence of catalytical amount of concentrated hydrochloric acid (0.5 mL). Reflux of the reaction mixture was continued under stirring for two hours, the mixture was cooled, and acetone (200 mL) was added. The formed Mannich base hydrochlorides were precipitated, filtered off, and dried. Subsequently, Mannich base hydrochlorides (100 mmol) were dissolved in water (100 mL) and imidazole (200 mmol) was added. The reaction mixture was heated to reflux for five hours, cooled and the precipitated solids were collected by filtration to give ketones 1a-c [24–26] which were pure enough to be used in the next step.

2.1.2. General Procedure for the Synthesis of Arylsemicarbazides 2a-e. A solution of the appropriate aniline derivative 1a-e (20 mmol) in CH₂Cl₂ (20 mL) was added dropwise to a stirred solution of ethyl chloroformate (10 mmol) in CH₂Cl₂ (5 mL). The reaction mixture was stirred at room temperature for 0.5 hr (2a and 2c-e) and 24 hrs (2b). After completion of the reaction, the reaction mixture was filtered and the filtrate was washed with 1 N HCl, dried (Na₂SO₄), and evaporated under reduced pressure to give the respective crude carbamates. A mixture containing the formed carbamates (10 mmol) and hydrazine hydrate (10 mL) was heated to reflux for 1 hr (2e), 2 hrs (2d), and 24 hrs (2a-c). The reaction

SCHEME 1: Synthetic protocol to achieve test compounds **3a–h**. Reagents and conditions: (i) ethanol, acetic acid, RT, 18 hrs or semicarbazide hydrochloride, anhydrous sodium acetate, ethanol, RT, 18 hrs for **3h**.

mixture was cooled and filtered to yield the corresponding crude arylsemicarbazides **2a–e** [27]. The crude products were used as such for the next reactions.

2.1.3. General Procedure for the Synthesis of the Test Compounds 3a–g. A solution containing the appropriate arylsemicarbazide **2a–e** (10 mmol), appropriate ketone **1a–c** (10 mmol), and few drops of glacial acetic acid in ethanol (15 mL) was stirred at room temperature for 18 hrs. The reaction mixture was rotovapped and the residue was crystallized from ethanol to give the title compounds **3a–g** [23].

2.1.4. Synthesis of (2E)-2-[1-(4-Bromophenyl)-3-(1H-imidazol-1-yl)propylidene]hydrazinecarboxamide (3h). A solution containing **1a** (4.3 mmol), semicarbazide hydrochloride (4.3 mmol) and anhydrous sodium acetate (4.3 mmol), in absolute ethanol (40 mL) was stirred at room temperature for 18 hrs. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was crystallized from ethanol to give the title compound **3h** [23].

2.2. Mass Spectrometry

2.2.1. Reagents and Solvents. HPLC water was purified using cartridge system (Milford, Bedford, USA). Ultrapure water of 18 $\mu\Omega$ was obtained from Milli-Q Plus Purification System (Millipore, Bedford, MA, USA). Acetonitrile (ACN) HPLC grade was purchased from BDH Laboratory Supplies (Poole, UK).

2.2.2. LC-MS/MS. An Agilent 6410 triple quadrupole mass spectrometer (Agilent technologies, USA) equipped with an electrospray ionization interface (ESI) coupled to an Agilent 1200 HPLC (Agilent Technologies, USA) was used.

Agilent 1200 series system consists of G1311A binary pump, G1322A degasser, G1367B HIP-ALS autosampler, and G1316A thermostated column compartment. A connector is used instead of the column to allow direct injection of samples. Mobile phase consists of water and acetonitrile (ACN) (1:1) running for 3 minutes with a flow rate of 0.4 mL/min. Compounds **3a–h** were prepared by weighing the solid substances to 1 mg·mL⁻¹ in ACN. Test solutions for MS were prepared by diluting the stock solutions with ACN/H₂O mixture (1:1). 10 μ L of each sample was injected into the LC-MS/MS. MS parameters were optimized for each compound by varying fragmentor voltage of the ion source for scan mode and collision energy for product ion mode. Analytes concentrations of 20 to 50 μ g·mL⁻¹—depending on the ions intensities—were used for optimization of the ionization conditions and fragment ion spectra. For screening of mass signals of the different compounds and identifying of the parent ions for MS/MS experiments, MS/MS scans were performed in the mass range of m/z 100–600. Because of the flow rate dependency of the ESI process, ion source specific parameters were readjusted. The ESI was operated in positive mode. The source temperature was set to 350°C and ion spray voltage was 4.5 kV.

To overcome the reduced specificity issue of ISF, an MS² scan took place prior to ISF to determine each compound's related fragment ions. Fragmentor voltage was optimized to produce adequate in-source fragmentation; values of 100, 120, 140, 160, 180, and 200 V were tested to obtain the fragments of each compound in the scan spectra. The optimum fragmentor voltage to generate in-source fragments was 200 V. Furthermore, the collision energy used for product ion (MS²) analysis was also optimized by varying collision energy values (4, 6, 8, 10, 12, 14, 16, 18, and 20 eV) and was set to 20 eV to attain the fragment ions.

(a)

(b)

(c)

(d)

FIGURE 2: Continued.

FIGURE 2: (a) ESI mass spectrum of compound **3d** $[M + H]^+$ ion (m/z 442.10). (b) MS^2 spectrum of m/z 442.10. (c) In-source fragmentation of compound **3d**. (d) MS^2 spectrum of m/z 374.10. (e) MS^2 spectrum of m/z 271.10. (f) MS^2 spectrum of m/z 214.20.

3. Results and Discussion

3.1. Chemistry. The test compounds **3a–h** have been successfully achieved as illustrated in Scheme 1. Thus, the appropriate ketone **1a–c** [24–26] was allowed to react with the appropriate semicarbazide **2a–e** [27] and/or semicarbazide hydrochloride to produce the corresponding semicarbazones **3a–h** [23].

3.2. Mass Spectrometry. An initial MS/MS scan followed by a product ion scan of each compound was performed to distinguish the parent ion peaks and the fragment ions of compounds **3a–h**. The data obtained played a guidance role prior to the pseudo- MS^3 process for the same compounds. The highly sensitive product ion spectra of compounds **3a–h** acquired from a one stage product ion scan with abundant ions and no low mass cutoff are represented in Figures 2 and 3. The ISF step showed numerous fragments including the daughter ion peaks produced by MS/MS scans, which in turn were used as precursor ions for the pseudo- MS^3 step.

A pattern of seven major fragments (**I–VII**) was observed following in-source fragmentation (ISF) for all compounds **3a–h** regardless of the different substituents either in “X” or “R” position of the main nucleus. A common fragment (**I**) was observed for the substituted compounds **3a–h** with

$[M + H]^+$ at m/z (266.21, 300.30, 280.22, 374.10, 364.10, 358.10 and 268.12, resp.) as shown in Scheme 2. These $[M + H]^+$ values suggested that this fragment is characterized by the removal of imidazole ring. Upon exposure to further MS/MS fragmentation, fragment (**I**) showed defined pattern of $[M + H]^+$ at m/z (250.83, 203.21, and 173.10 for X = Br, OCH_3 , and H, resp.) suggesting the breakage of the amide linkage. The latter (**II**) was also a common fragment of in-source fragmentation that finally produced a fragment of $[M + H]^+$ at m/z 77.10 for a benzene ring. Two other common fragments (**III** and **IV**) were seen after ISF and a result of MS/MS of fragment (**I**). One fragment (**III**) with $[M + H]^+$ at m/z (207.98, 160.20, and 130.12 for X = Br, OCH_3 , and H, resp.) was suggested to have lost an imidazole ring and an $HN-CO-NH-R$. While the other fragment (**IV**) with $[M + H]^+$ at m/z (225.10, 177.21, and 147.12 for X = Br, OCH_3 , and H, resp.) was perhaps characterized by the removal of the imidazole ring in addition to an $HCO-NH-R$ moiety. Additionally, fragment (**III**) was also seen in MS/MS spectrum of fragment (**IV**). Fragment (**V**) commonly appeared by ISF of the main nucleus with $[M + H]^+$ at m/z (319.10, 271.10, and 241.10 for X = Br, OCH_3 , and H, resp.) which assumed a loss of an $R-NH$ moiety followed by removal of imidazole ring and $HN-NH-CHO$ after product ion scan. Moreover, fragments (**VI** and

(a)

(b)

(c)

(d)

FIGURE 3: Continued.

(e)

(f)

(g)

FIGURE 3: (a) ESI mass spectrum of compound **3c** $[M + H]^+$ ion (m/z 348.20). (b) MS^2 spectrum of m/z 348.20. (c) In-source fragmentation of compound **3c**. (d) MS^2 spectrum of m/z 280.22. (e) MS^2 spectrum of m/z 241.10. (f) MS^2 spectrum of m/z 183.10. (g) MS^2 spectrum of m/z 173.10.

VII) with $[M + H]^+$ at m/z (262.10 and 214.20 for $X = Br$ and OCH_3 , resp. for fragment **VI**) and $[M + H]^+$ at m/z 183.10 for fragment **VII**. Both fragments were identified after ISF and presumed to involve the loss of an $HN-NH-CO-NH-R$ moiety. Alternatively, a fragment appeared only as a result of MS/MS of fragment (**VI**) in case of $X = Br$ with $[M + H]^+$ at m/z 121.20 assuming the loss of the imidazole ring and

releasing a CH_2-CH_2 moiety. However, in case of fragment **VII** where $X = H$, a typical rearrangement to form a tropylium ion was suggested, producing a fragment after MS/MS with $[M + H]^+$ at m/z 118.14. All ion peaks together with their corresponding proposed structures obtained from ISF and MS^2 scans for compounds **3a-h** are shown in Scheme 2 and are also summarized in Table 1.

TABLE 1: Multistage MS data of compounds **3a–h** by ESI-MS/MS.

Product ions of protonated compounds 3a–h $[M + H]^+$ (m/z)								
	3a (334.10)	3b (368.30)	3c (348.20)	3d (442.10)	3e (432.10)	3f (448.10)	3g (426.2)	3h (336.10)
Major fragments of in-source fragmentation	173.10	173.10	173.10	—	—	—	—	—
	183.10	183.10	183.10	—	—	—	—	—
	—	—	—	214.20	214.20	—	—	—
	—	—	—	—	—	225.10	225.10	225.10
	241.10	241.10	241.10	—	—	—	—	—
	—	—	—	—	—	250.83	250.83	250.83
	—	—	—	—	—	262.10	262.10	262.10
	266.21	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	268.12
	—	—	—	271.10	271.10	—	—	—
	—	—	280.22	—	—	—	—	—
	—	300.30	—	—	—	—	—	—
	—	—	—	—	—	319.10	319.10	319.10
	—	—	—	—	364.10	—	358.10	—
	—	—	—	374.10	—	—	—	—
	—	—	—	—	—	379.92	—	—
Product ion	77.10	77.10	77.10	—	—	—	—	—
	103.10	103.10	103.10	—	—	—	—	—
	118.14	118.14	118.14	—	—	—	—	—
	—	—	—	121.20	121.20	—	—	—
	130.12	130.12	130.12	—	—	—	—	—
	—	—	—	132.97	132.97	—	—	—
	147.12	147.12	147.12	—	—	—	—	—
	—	—	—	160.20	160.20	—	—	—
	173.10	173.10	173.10	—	—	—	—	—
	—	—	—	177.21	177.21	—	—	—
	—	—	—	—	—	181.20	181.20	181.20
	—	—	—	203.21	203.21	—	—	—
	—	—	—	214.11	214.11	—	—	—
	—	—	—	—	—	207.98	207.98	207.98
	—	—	—	—	—	225.10	225.10	225.10
	—	—	—	—	—	—	—	239.90
	—	—	—	—	—	250.83	250.83	250.83
	—	—	—	—	—	262.10	262.10	262.10
	—	—	—	—	—	—	329.80	—
	—	—	—	—	—	351.21	—	—

SCHEME 2: Proposed fragmentation pattern of compounds **3a-h** conducted with pseudo-MS³ scan mode in ESI-MS/MS.

4. Conclusions

ESI-QqQ has proven to be an efficient technique for the structural elucidation of compounds **3a-h**. The approach described in this study comprehensively studied the fragmentation pathways of compounds **3a-h** utilizing the integration of ISF with the collision-induced dissociation. Those fragmentation pathways of compounds **3a-h** were elaborated by further dissociating individual fragment ions in the MS² scan mode. Substructures of all fragment ions were unambiguously identified. Sensitive ion spectra of compounds **3a-h** were acquired from one stage product ion scan with abundant ions and absence of the low mass cutoff. The described technique is characterized by its simplicity, sensitivity, and time saving and can be used to identify unknown compounds, metabolites, impurities, and drug degradates.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University

for funding this work through the Research Group Project no. RGP-VPP-322.

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