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IDENTIFICATION OF DESVENLAFAXINE, THE MAJOR ACTIVE METABOLITE OF VENLAFAXINE, IN EXTENDED-RELEASE CAPSULES

Identificação da Desvenlafaxina, o Principal Metabólito da Venlafaxina, em Cápsulas de Liberação Prolongada

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Abstract: In this work, we describe the identification of desvenlafaxine in extended release capsules of venlafaxine (VEN) in acid degradation studies. We developed a stability indicating reverse-phase HPLC method and validated for the analysis of VEN in pharmaceutical formulation. The HPLC method was linear over the range of 0.45-1.05 mg/ml (r^2=0.999). The RSD values for intra- and inter-day precision studies showed good results (RSD < 2%) and accuracy was greater than 99%. The degradation studies in acidic media for 24 h showed two additional peaks, which were further identified by ESI-MS/MS as the desvenlafaxine and the dehydration product of venlafaxine. Furthermore, desvenlafaxine is the major active metabolite of venlafaxine and has recently been approved for treatment of major depressive disorder.

Keywords: Degradation studies; extended-release capsules; desvenlafaxine; ESI-MS/MS.

Resumo: Neste trabalho, descrevemos a identificação da desvenlafaxina em cápsulas de liberação prolongada de venlafaxina (VEN) em estudos de degradação forçada. Um método de CLAE de fase reversa foi desenvolvido e validado para a análise da VEN em formulações farmacêuticas. O método de CLAE desenvolvido mostrou-se linear na faixa de 0,45-1,05 mg/mL (r^2=0.999). Os valores de RSD para os estudos de precisão intra- e inter-dias mostraram bons resultados (RSD < 2%) e a

exatidão foi maior que 99%. Os estudos de degradação em meio ácido por 24h apresentaram dois picos adicionais, que foram identificados por ESI-MS/MS como desvenlafaxina e o produto da desidratação da venlafaxina. Ademais, a desvenlafaxina é o principal metabólito ativo da VEN e foi recentemente aprovado para o tratamento da depressão maior.

Descritores: Estudos de degradação; cápsulas de liberação prolongada, desvenlafaxina, ESI-MS/MS.

Introduction

Venlafaxine (VEN), chemically known as 1-[2-(dimethylamino)-1-(4-methoxy-phenyl)ethyl] cyclohexanol hydrochloride (Fig. 1), a third generation antidepressant (MUTH *et al*., 1986; TZANAKAKI *et al*., 2000), inhibits the reuptake of serotonin, norepinephrine and to a lesser extent dopamine, is a serotonin noradrenalin reuptake inhibitor (MUTH *et al*., 1985; SCHWEITZER *et al*., 1991; ANDREWS *et al*., 1996). In human, VEN is well absorbed and extensively metabolized to desvenlafaxine (*O*-desmethylvenlafaxine), the major active metabolite of venlafaxine, and two less active metabolites *N*-desmethyl and *N,O*-didesmethyl (HICKS *et al*., 1994) (Fig. 1).

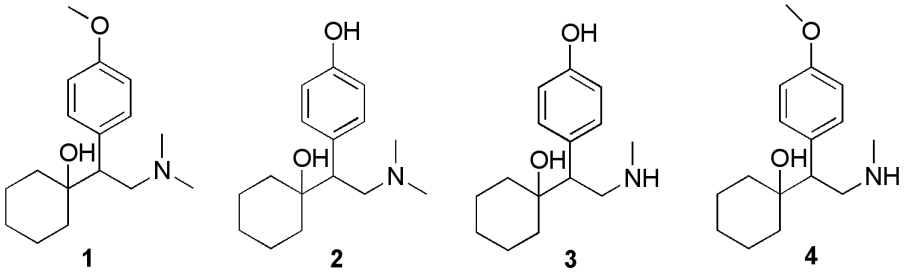

Figure 1. Structures of venlafaxine (1), its active metabolite desvenlafaxine (2), and its other metabolites *N,O*-didesmethyl (3) and *N*-desmethyl (4).

Since the clinical use of VEN has become increasingly common, various methods are reported in literature for its analyses in human plasma or serum including GC (WILLE *et al*., 2007), HPLC (VU *et al*., 1997; TITIER *et al*., 2003; WASCHGLER *et al*., 2004), LC-MS (BHATT *et al*., 2005; LIU, CAI and LI, 2007) and CE (RUDAZ *et al*., 2000). However, all of these methods are applied to biological fluids and utilized for VEN drug monitoring metabolism and pharmacokinetics studies. For determining VEN in pharmaceutical formulations, just three stability-indicating methods using HPLC have been described (MAKHIJA and VAVIA, 2002; RAO and RAJU, 2006; BERNARDI *et al*., 2009). Additionally, the in vitro determination of gastric and intestinal stability of VEN was studied (ASAFU-ADJAYE *et al*., 2007). However, only one of these previous studies elucidated the structure of one degradation product generated in acidic media by applying LC-MS (ASAFU-ADJAYE *et al*., 2007).

The active metabolite, desvenlafaxine, was recently approved by the US Food and Drug Administration (FDA) for the treatment of adult patients with major depressive disorder (DESVENLAFAXINE, 2008). Moreover, desvenlafaxine is also being investigated as the first non-hormonal treatment for vasomotor symptoms attributed to menopause (ARCHER *et al*., 2009).

International Conference on Harmonisation (ICH) guideline Q1A (R2) requires the stress testing to be carried out to elucidate the inherent stability characteristics of active substances (ICH, 2005). Moreover, structural elucidation of degradants is an essential part of drug development for a new

chemical entity. Information on degradant structures can lead to the development of new compounds with improved stability and facilitates the design of more stable formulations (WU, 2000).

LC-UV is still the most commonly used technique for the detection and quantitative determination of degradants. However, when unknown degradants are discovered in stability or during stress studies, LC-UV is of little or no use for the identification step. Therefore, LC-MS is often used to determine the molecular mass and MS-MS is used to provide structural characterization (BERNARDI, 2000).

In this work, we describe the degradation pattern of venlafaxine (VEN) in extended release capsules and the identification of its main degradation products by ESI-MS/MS. We developed a stability indicating reverse-phase HPLC method and validated for the analysis of VEN in pharmaceutical formulation.

Experimental

Materials and Reagents

The capsules containing 75 mg of VEN extended-release pellets were obtained from commercial sources within their shelf life period. VEN reference standard (>99.9%) was purchased from European Pharmacopoeia. The chemicals used were purchased from commercial sources and were as follows: acetonitrile (UV and HPLC grade), triethylamine (AR) and monobasic sodium phosphate (AR) were purchased from J. T. Baker (Phillipsburg, NJ, USA), methanol (HPLC grade) from Tedia (Rio de Janeiro, Brazil) and ultrapure water (0.45 μm) was obtained using a Milli-Q Gradient System (Millipore, Bedford, MA, USA).

Preparation of Standard and Sample Stock Solutions

The reference stock solution of VEN was prepared by accurately weighing (37.5 mg) and dissolving in a 50 ml volumetric flask with methanol to obtain a concentration of 0.75 mg/ml of analyte.

To prepare the sample stock solution, the pellets from the capsules containing 75 mg of VEN were accurately weighed and crushed to fine powder. An appropriate amount was transferred into a 50 ml volumetric flask with 25 ml of methanol. This flask was kept in an ultrasonic bath for 15 min. The volume was completed with the same diluent obtaining a final concentration of 1.5 mg/ml of the active pharmaceutical ingredient. The solutions were filtered through a 0.45 μm membrane filter before injection.

Instrumentation and Chromatographic conditions

The analysis was performed on a Waters LC system (Alliance 2695, USA), which consisted of LC-10AD pump, a photodiode array (PDA) detector (2996 Waters), a SLA-10ADVP system controller, a DGU-14A degasser and a Rheodyne 7725i manual injector with a 10 μl loop. Separations were obtained on a XTerra C8 column (150×4.6 mm i.d., 5 μm) (Waters, USA) kept at 40 °C. The LC system was operated isocratically using a mobile phase disodium hydrogen phosphate buffer 40 mM containing triethylamine (pH 6.8) and acetonitrile (75:25, v/v) at flow rate of 1.0 ml/min (runs of 15 min). The mobile phase was filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, US) and degassed by

an ultrasonic bath. The injections were carried out through a 10 μl loop. The analytes were detected and quantified by UV detection at a wavelength of 225 nm.

Method Validation

The method was validated following the parameters such as specificity, linearity, precision, accuracy, limits of detection and quantitation and robustness, following the ICH guidelines (ICH, 2005). Furthermore, stability-indicating capability was determined by forced degradation conditions also in accordance with ICH (ICH, 2005).

Specificity

The specificity of the method was evaluated with regards to interference due to the presence of excipients in the pharmaceutical formulation. The placebo samples consisted of all the excipients without the active substance. Then, the specificity of the method was established by determining the peak purity of VEN in samples using a PDA detector, ranging between 190-400 nm.

Linearity and Limits of Detection and Quantitation

The calibration curves were prepared in mobile phase and analyzed at five different VEN reference substance concentrations corresponding to 60%, 80%, 100%, 120% and 140% of the nominal analytical concentration. The procedure was carried out in duplicate for each concentration. The analyte peak area ratios obtained (dimensionless numbers) were plotted against the corresponding concentrations of the analytes (expressed as mg/ml) and the calibration curves were set up by means of the least-square method. In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), mobile phase was injected twenty times. The LOD was calculated to be three times the noise value and ten times the noise gave LOQ, according to USP guidelines (USP, 2007).

Precision

The precision was determined using the parameters of repeatability (intra-day) and intermediate precision (inter-day). In order to evaluate the repeatability of the method six samples were determined during the same day for the concentration of VEN at 0.75 mg/ml. The intermediate precision (inter-day) was studied comparing the assay of VEN from two different days at concentration 0.75 mg/ml. They were expressed as relative standard deviation (RSD) of the measurements.

Accuracy

Accuracy was calculated as the percentage of recovery at three different VEN concentrations of 0.60, 0.75 and 0.90 mg/ml, corresponding to 80, 100 and 120% of the nominal analytical concentration. The procedure was carried out in triplicate for each concentration.

Robustness

In this study, the chromatographic parameters (retention time, theoretical plates, tailing factor, retention factor and repeatability) were evaluated using both sample and reference substance solutions (0.75 mg/ml) changing flow rate (0.9, 1.0 and 1.1 ml/min), buffer concentration (74, 75 and 76%) and pH (6.7, 6.8 and 6.9) of mobile phase, and columns (XTerra C8 column, two different lots).

The stability of reference and sample solutions was also evaluated at the moment they were prepared and after 2, 4, 6, 8, 10, 12 and 15 h. The stability of these solutions was

verified by performing the experiment and observing any change in the chromatographic pattern, comparing with freshly prepared solutions. The RSD of the assay was calculated for the study period during solution stability experiments.

Forced degradation studies

Forced acid, base and oxidative degradation studies on 5.0 ml aliquots of 1.5 mg/ml of venlafaxine hydrochloride standard solution in methanol were performed in triplicate by adding 2M hydrochloric acid, 2M sodium hydroxide or 3% hydrogen peroxide to the drug solutions to achieve a concentration of 0.75 mg/ml. The studies in alkaline and acid condition were incubated at 60 ± 5 ºC for 24 h. The samples were cooled to room temperature and neutralized. Oxidative condition was obtained by treating the drug with 3% hydrogen peroxide at room temperature for 3 h, protected from light.

Following the degradation period, all samples were prepared for analysis as previously described. For peak purity test, a PDA detector was used in scan mode (190 - 400 nm) and the purity factor was observed.

MS Analysis

The degradation products were evaluated by HPLC, samples peaks were collected from a semi-preparative HPLC using the mobile phase described before.

The samples of forced degradation studies were introduced into the electrospray ion source by direct infusion (ACN:H_2O (1:1, v/v) and 0.1% of formic acid) using the integrated syringe pump at flow rates ranging from 2 to 10 μl/min identified. The mass spectra were acquired in the positive ion mode with the source and analyzer parameters optimized for the protonated molecular ion. The characterized Tandem MS spectra were acquired using Nitrogen as collision gas (4 x 10^{-3} mbar) different collision energies (30–60 eV) at 4000 Q TRAP® mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada).

Results and Discussion

Selection of Chromatographic Conditions

The chromatographic conditions were optimized to develop a stability-indicating method to separate the degradation products from the drug. To obtain the best chromatographic conditions, the wavelength for detection, the column, and the mobile phase composition were adequately selected. The best chromatographic condition was achieved using disodium hydrogen phosphate buffer 40 mM containing triethylamine (pH 6.8) and acetonitrile (75:25, v/v).

The flow rate used was 1.0 ml/min. PDA detection was used to select the best wavelength for analysis as well as to verify peak purity during analysis. With this mobile phase, adequate results were obtained in terms of peak parameters (shape peak, selectivity, theoretical plates, symmetry, capacity factor, repeatability). A typical chromatogram obtained by the proposed method for VEN is shown in Figure 2A.

System suitability

The system suitability assessment for this HPLC method established instrument performance parameters such as the retention factor, *k*, theoretical plates number, *N*, and tailing factor, T_f, for VEN. The mean (n = 6) *k*,

*T*f and *N* were 2.76, 1.6 and 3201.4 plates/m, respectively, for VEN reference substance.

Linearity and Limits of Detection (LOD) and Quantitation (LOQ)

The calibration curves constructed for VEN were found to be linear in the 0.45-1.05 mg/ml range. The values of the correlation coefficients and calibration equations calculated were: r^2 = 0.9999, y = 20938304.6 + 181176.5x where x is concentration and y is the peak absolute area. This r-value indicated the linearity of the calibration curves for the method. Furthermore, ANOVA demonstrated

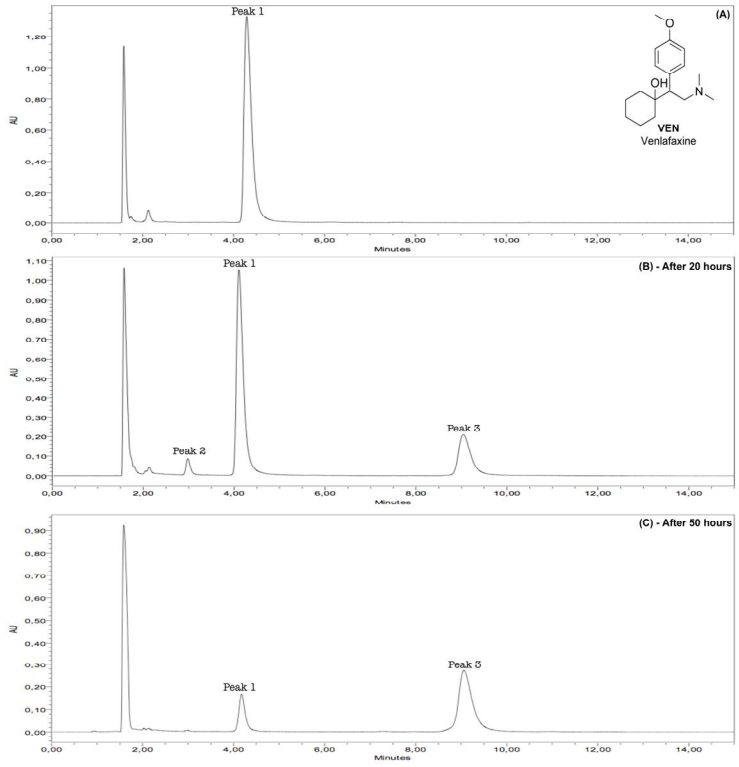

Figure 2. HPLC chromatograms of VEN (A) extended-release capsules (0.75 mg/ml), (B) after 24 h exposure at acid and (C) after 50 h exposure at acid.

significant linear regression and non-significant linearity deviation (P < 0.05).

For calculating the LOD and LOQ, the calibration equation for VEN was generated by using the mean values of the three independent calibration graphs. The values calculated for the LOD and LOQ were 0.00043 and 0.00145 mg/ml, respectively.

Precision and Accuracy

The intermediate precision was assessed by analyzing one sample of the pharmaceutical formulation on two different days by the same analyst, showed good results: 99.81 and 101.07% (Table 1). The maximum RSD value for inter-day precision was 0.55% (n = 6). Between-analysts precision was determined by calculating the RSD for the analysis of one samples of the pharmaceutical formulation by

two analysts; the maximum value was found to be 0.67% (Table 1).

In the accuracy test three concentrations were evaluated (0.60, 0.75 and 0.90 mg/ml) and mean recoveries were 99.73, 100.11, and 100.25% with a mean value of 100.03% and RSD of 0.27% (Table 2). These results show that the method was accurate within the desired range.

Table 1. Inter-day and between-analysts precision data of the method

Inter-day			Between- analysts		
Day	Recovery* (%)	RSD(%)†	Analysts	Recovery* (%)	RSD(%)†
1	99.81	0.55	A	100.90	0.67
2	101.07	0.39	B	100.56	0.66

* Mean of six replicates

† RSD = Relative standard deviation

Table 2. Accuracy data of the method

Added Concentration (mg/ml)	Mean Concentration found* (mg/ml)	RSD (%)†	Accuracy (%)
0.60	0.6007	0.62	100.11
0.75	0.7480	0.37	99.73
0.90	0.9022	1.41	100.25

* Mean of six replicates

† RSD = Relative standard deviation

Robustness

The results and experimental range of the selected variables evaluated in the robustness assessment are shown in Table 3. Variations of flow rate (0.9, 1.0 and 1.1 ml/min), mobile phase pH (6.7, 6.8 and 6.9), oven temperature (38, 40 and 42 °C), buffer concentration (74, 75 and 76%) and different lots of chromatographic column (XTerra C8 column, lot 01: 011634337129 07 and lot 02: 011734357117 10), resulted in no significant changes (RSD < 2.0%) regarding quantitation of VEN, showing that this method is robust for small variations (Table 3). The values for these parameters were satisfactory in accordance with the literature (ICH, 2005; USP, 2007). None of these alterations caused a significant effect on the determination of this drug in extended-release capsules, indicating robustness or stability of the method.

Degradation Behavior

VEN did not degrade under basic and oxidative conditions as the chromatograms of the basic media and hydrogen peroxide treated samples showed only the peak of the pure drug

(retention time 4.32 min) (Makhija and Vavia, 2002; Asafu-Adjaye *et al*., 2007). However, the

Table 3. Evaluation of robustness for the venlafaxine HPLC assay (n = 6)

Robustness	Tested conditions	Substance analyzed	R_t [a]	k [b]	T [c]	N [d]
Flow (ml/min)	0.9	Reference	5.29	3.25	1.64	2614.2
	0.9	Sample	5.30	3.26	1.63	2612.2
	1.0	Reference	4.80	2.85	1.67	2354.6
	1.0	Sample	4.74	2.81	1.70	2497.7
	1.1	Reference	4.33	2.48	1.63	2749.9
	1.1	Sample	4.33	2.48	1.63	2704.2
pH	6.7	Reference	4.32	2.47	1.85	2315.4
	6.7	Sample	4.31	2.46	1.86	2237.4
	6.8	Reference	4.80	2.85	1.67	2354.6
	6.8	Sample	4.74	2.81	1.70	2597.7
	6.9	Reference	4.66	2.74	1.64	2337.6
	6.9	Sample	4.67	2.75	1.58	2354.2
	38.0	Reference	4.83	2.88	1.46	3993.7
Oven temperature of the column (°C)	38.0	Sample	4.83	2.88	1.47	4154.5
	40.0	Reference	4.80	2.85	1.67	2354.6
	40.0	Sample	4.74	2.81	1.70	2797.7
	42.0	Reference	4.83	2.88	1.40	3677.3
	42.0	Sample	4.83	2.88	1.42	4007.1
Proportion of mobile phase (v/v)[e]	74:26	Reference	4.49	2.60	1.64	2597.3
	74:26	Sample	4.48	2.60	1.62	2534.6
	75:25	Reference	4.80	2.85	1.67	2354.6
	75:25	Sample	4.74	2.81	1.70	2597.7
	76:24	Reference	5.08	3.08	1.64	2494.3
	76:24	Sample	5.08	3.08	1.63	2479.3
Column[f]	Column 01	Reference	4.74	2.81	1.60	3304.7
	Column 01	Sample	4.76	2.82	1.58	3462.2
	Column 02	Reference	4.80	2.85	1.67	2354.6
	Column 02	Sample	4.74	2.81	1.70	2697.7

[a] R_t retention time

[b] k retention factor

[c] T tailing factor

[d] N theoretical plate number

[e] Mobile fase: Acetonitrile:0.04M disodium hydrogen phosphate buffer of pH 6.8 (25:75, v/v)

[f] Lot columns XTerra: column 01: 011634337129 07; column 02: 011734357117 10

forced degradation studies in acidic media at 60 °C for 24 h produced two additional eluting peaks: one with a shorter retention time than the VEN peak and other late eluting peak (RT 3.12 min and RT 9.34, respectively) (Fig. 2B). This is contrary to findings by Asafu-Adjaye and co-workers[17] who reported that acid degradation studies at 70 °C for 1 h of VEN did produce just the late eluting peak and also to findings reported by Makhija and Vavia (Makhija and Vavia, 2002) who described just the presence of the shorter additional eluting peak, using different acid and chromatographic conditions. Furthermore, the acid degraded sample 60 °C for 50 h showed just one additional peak, the late eluting peak (Fig. 2C). The two additional eluting peaks detected by the LC-UV method were further identified by ESI-MS/MS, in order provide structural characterization of the degradants.

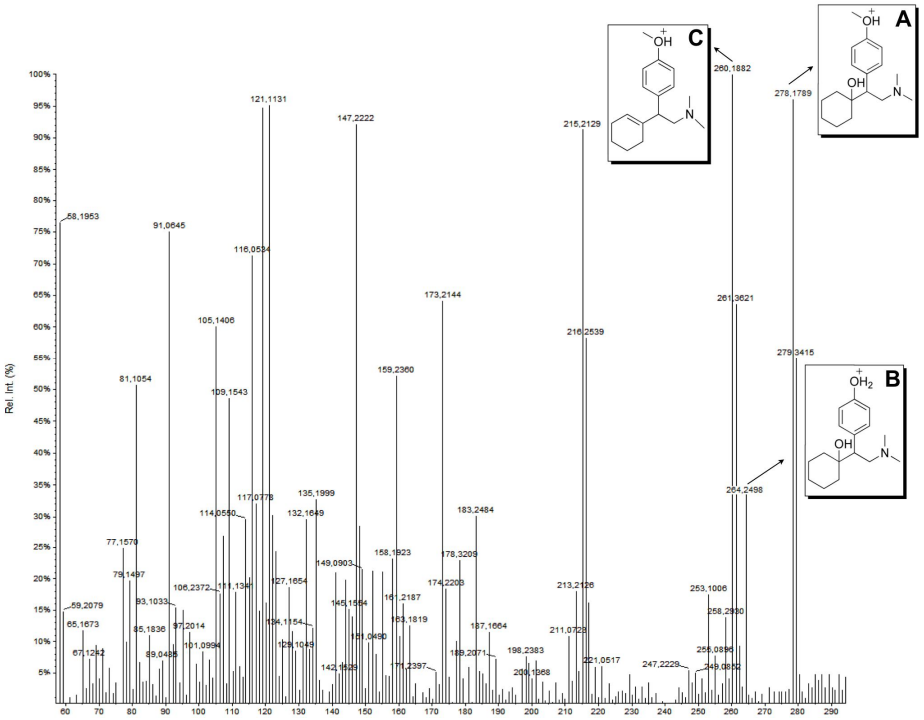

Figure 3. ESI (+) full-scan ion spectra of: **(A)** venlafaxine (VEN); **(B)** desvenlafaxine; **(C)** dehydration product of VEN.

Identification of degradation products using ESI-MS/MS

Using 50% (v/v) MeCN containing 0.1% (v/v) formic acid and a potential of 30-50V at the sample cone, the molecular mass of each degradant was obtained. ESI-MS/MS studies were carried out employing suitable collision energies. The LC-UV chromatograms and the ESI-MS/MS spectra for the drug and degradation products are displayed in Figures 2 and 3, respectively.

The *m/z* values of the drug and degradation products at full-scam mode were observed at 278.1, 264.2 and 260.1 (Fig. 3), corresponding to peaks 1, 2 and 3 (Fig. 2), respectively. The MS/MS analysis of signal corresponding to parent drug at *m/z* 278.1 resulted in four major fragments at *m/z* 147.1, 120.8, 91.0 and 58.2, was attributable to VEN.

Combining the information obtained from chromatographic profiles (Fig. 2) with MS data (Fig. 3), product-ion (Fig. 4) and the precursor-ion (Fig. 5) experiments, the molecular structures were rationalized for two degradation products. Moreover, Figure 5 shows that the degradation products followed a very similar fragmentation pattern to the drug.

Figure 4A shows the fragment ions present in the MS/MS spectrum and the product-ion furnished the main fragmentation that characterized the dehydration product of venlafaxine *m/z* 260.1 $[M+H]^+$, with a longer retention time than VEN.

Figure 4B shows the product-ion for desvenlafaxine $[M+H]^+$, *m/z* 264.2, resulted in four major fragments at *m/z* 106.9, 120.9, 220.4 and 58.09, with a shorter retention time than VEN.

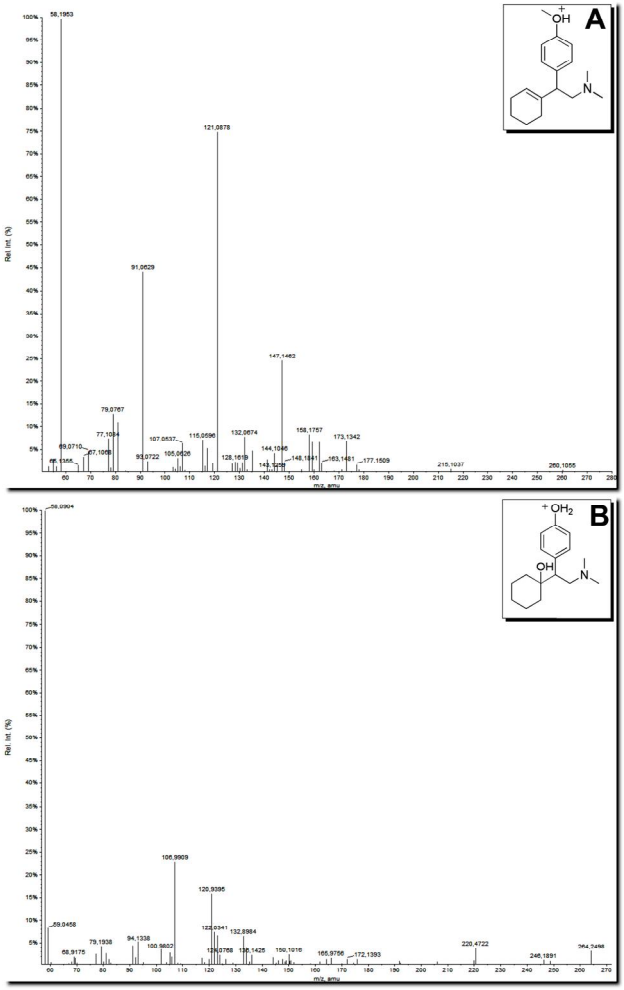

Figure 4. Collision-induced dissociation (CID) mass spectrum of the electronspray generated $[M+H]^+$ ions peaks from venlafaxine degradants. Product-ion spectrum for the $[M+H]^+$ ions (**A**) *m/z* 260.1 of dehydration product of VEN and **(B)** *m/z* 264.2 for desvenlafaxine.

A general fragmentation pattern for the drug and the degradation products could be proposed at Figures 3-5. It is postulated that fragment of *m/z* 58.1 results from elimination

in common structure proposed here, whereas the precursor-ion for this common fragment came from VEN and its degradant products, as depicted in Figure 5.

Conclusions

In conclusion, the acid degradation studies of VEN showed two degradation products, which were characterized by ESI-MS/MS as the desvenlafaxine and the dehydration product of venlafaxine. Moreover, one of the degradation products corresponds to the active metabolite of VEN, which is also been commercialized as antidepressant. The HPLC method developed and validated for analysis of VEN in extended-release capsules was considered simple, sensitive, specific, precise, accurate and reproducible.

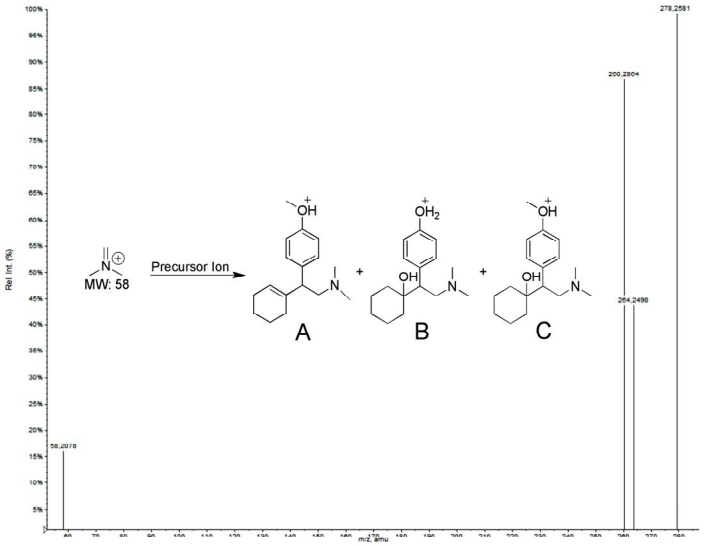

Figure 5. Precursor-ion for *m/z* 58.1 resulting from three major pattern ions, *m/z* 264.2 (dehydration product, A), 260.1 (desvenlafaxine, B) and 278.1 (venlafaxine, C).

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