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Article

LC-MS characterization of valsartan degradation products and comparison with LC-PDA

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Valsartan was submitted to forced degradation under acid hydrolysis condition as prescribed by the ICH. Degraded sample aliquots were separated via HPLC using a Hypersil ODS (C18) column (250 x 4.6 mm i.d., 5 µm). Either photodiode array (PDA) detection or mass spectrometry (MS) full scan monitoring of HPLC runs were used. HPLC-PDA failed to indicate Valsartan degradation under forced acid degradation, showing an insignificant peak area variation and that Valsartan apparently remained pure. HPLC-MS using electrospray ionization (ESI) and total ionic current (TIC) monitoring did not reveal any peak variation either, but inspection of the ESI mass spectra showed the appearance of m/z 306 and m/z 352 ions for the same retention time as that of Valsartan (m/z 436). These ions were identified as being protonated molecules of two co-eluting degradation products formed by hydrolysis. These assignments were confirmed by ESI-MS/MS with direct infusion of the degraded samples. The results showed that the use of selective HPLC-MS is essential for monitoring Valsartan degradation. Efficient HPLC separation coupled to selective and structural diagnostic MS monitoring seems therefore mandatory for comprehensive drug degradation studies, particularly for new drugs and formulations, and for method development.

Uniterms: Valsartan. Stress testing. Degradation products. Stability-indicating. Acidic hydrolysis.

Valsartana (VAL) foi submetida à degradação forçada em meio ácido conforme procedimento descrito no ICH. Os produtos de degradação (PDs) foram monitorados ao longo do tempo de degradação pela técnica de Cromatografia Líquida (LC) utilizando uma coluna Hypersil ODS (C18) (250 x 4,6 mm d.i., 5 µm). A detecção foi feita com dois detectores: espectrofotométrico (PDA) e espectrometria de massas (MS) por corrente iônica total. Ambas as técnicas falharam na identificação dos PDs obtidos ao longo do monitoramento, mostrando insignificantes variações na área do pico e permanecendo com pureza de pico ao longo de toda a eluição. Somente depois da avaliação por íon extraído (XIC), foi possível observar o aumento do íon m/z 306 e m/z 352 exatamente no mesmo tempo de retenção do íon molecular (m/z 436). Estes resultados mostram um caso simples e didático em que somente o uso de um método seletivo de LC-MS pode ser utilizado para monitorar produtos de degradação. Neste trabalho, é apresentado um caso real em que a separação por LC deve ser acoplada a métodos seletivos obtidos por MS, especialmente no estudo de PDs para novos fármacos, formulações e no desenvolvimento de métodos.

Unitermos: Valsartana. Teste de estresse. Produtos de degradação. Indicativo de estabilidade. Hidrólise ácida.

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INTRODUCTION

The identification of drug degradation products plays a crucial role in drug discovery and development (Alsante *et al.*, 2003). Understanding drug degradation pathways affords information that is critical for drug safety and potency assessment, since very fast degradation and harmful degradation products may deactivate active molecules. The parent ICH stability testing guideline (ICH, 2003) and the Brazilian Health Surveillance Agency (ANVISA) (Brasil, 2013) require that drugs be subjected to a series of stress degradation studies, followed by proper characterization of the degradation products (DP).

Degradation studies via stress testing simulate the most likely environments that the drug may be subjected to from production to storage. These stress tests are important for inference of the degradation routes of pharmaceutical compounds and inform molecule stability across different stress conditions. The ICH guidelines (ICH, 2003) were conceived for the characterization of the inherent stability of a particular product, determination of possible DP, validation of the ability of analytical methods to evaluate the chemical stability of products and finally the quantification of the total and individual impurities present in formulations.

DP are normally associated with storage, but may also be formed during formulation dosage and/or processing under the influence of temperature, humidity, and light (Liu *et al*., 2011a). HPLC-PDA and HPLC-UV are the primary stability-indicating methods used to monitor impurities/DP during stress testing and storage (Alsante *et al.*, 2003). Although these techniques seem efficient for a variety of degradation studies (Thomas *et al.*, 2012; Sharma *et al.*, 2011; Bansal *et al.*, 2008), they may fail to detect products lacking a chromophore group (Bansal *et al.*, 2008) or with co-eluting sets of degradation products which may even co-elute with the parent drug and lead to false stability results.

HPLC-UV and HPLC-PAD are two largely used stability-indicating methods, but Liquid Chromatography Coupled to Mass Spectrometry (LC-MS) has become the gold standard technique in drug degradation monitoring due to its high DP sensitivity and selectivity as a result of the detailed structural information it provides (Zhang and Pramanik, 2010; Jocic *et al.*, 2009; Bhardwaj, Singh, 2008; Liu *et al.*, 2011b; Pan *et al.*, 2011). At present, direct MS analysis approaches using soft ionization methods applied to drug pills have been reported and may also be suitable for some drugs (Amaral *et al.*, 2011).

Valsartan (VAL) is a potent and highly selective oral drug largely used for the treatment of hypertension due to its effect as an active antagonist at the angiotensin II AT1-receptor (Krishanaiah *et al*., 2010) and because its solid-state characterization and compatibility have been established (Julio *et al.*, 2013). In the present study, we report on false Valsartan stability results observed in a degradation study under acid stress condition using HPLC-PDA and the actual instability obtained by HPLC-MS monitoring.

MATERIAL AND METHODS

Samples and reagents

Valsartan (VAL) was supplied by Dr. Reddys, India (batch AFEH002828) and used without further purification. MS grade Acetonitrile (ACN) from Sigma-Aldrich (São Paulo, Brazil), analytical grade acetic acid from Mallinckrodt Baker and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Water was purified using a Millipore Direct-Q 3UV water purification system with pump (Millipore, Molsheim, France). A VAL stock solution of 1 $mg.mL^{-1}$ was prepared in acetonitrile.

Acid stress condition

Studies of VAL degradation by acidic hydrolysis were carried out in 1 M HCl. For that, ca. 10 mg VAL were weighed and quantitatively transferred to a 50-mL two-neck round bottom flask and stirred with a magnetic bar with 50 mL of 1 M HCl. The acidic mixture was kept in the dark and under reflux at 358 K for 3 h. Samples were taken at 30-min intervals, neutralized and diluted with ammonium acetate buffer to 17 $\mu g.mL^{-1}$ prior to HPLC analysis.

High Performance Liquid Chromatography (HPLC)

HPLC separations were performed using a Hypersil ODS (C18) column (250 x 4.6 mm i.d., 5 µm) at room temperature in both HPLC-PDA and HPLC-MS apparatuses. Isocratic elution was carried out at 1 $mL.min^{-1}$with a mobile phase containing acetonitrile:water (45:55 v/v, 1.0% formic acid). The injection volume was 20 µL in both cases. The HPLC-PDA system was made up of an on-line degasser (DGU-20S5), a liquid chromatography apparatus (LC-20AT), a communication bus module (CBM-20A), an auto sampler (SIL-20A), a column oven (CTO-10ASVP), a photodiode array (PDA) detector (SPD-M20A) with scan range of 190-800 nm and LC Solutions software version 1.21 SP1 (Shimadzu, Kyoto, Japan).

The HPLC-MS system was made up of an Alliance® HPLC separation module (Waters 2695) coupled to an electrospray (ESI) source and a QTrap 2000 mass spectrometer (Applied Biosystems/ABSciex). The ESI source temperature was 623 K and the transfer voltage was 4500 V. The ESI was operated in positive ion mode, viz. ESI (+).

ESI (+)- MS/MS analysis

To study the fragmentation of $[VAL+H]^+$, a Q-TOF mass spectrometer (Micromass, Manchester, U.K.) was used for ESI(+)MS/MS analysis. Spectra were collected in the m/z range from 50 to 500. A formic acid aqueous solution was added to the sample mixture up to a total volume of 1,000 µL and a final concentration of 0.1% (v/v). Direct infusion was performed at a flow rate of 10 µL min^{-1} via a syringe pump (Harvard Apparatus). High purity nitrogen at 1.5 L min^{-1} was used as a nebulizer as well as an auxiliary gas. The capillary and cone voltages were set at 3.0 kV and 35 V, respectively. Both the curved desolvation line (CDL) and heat block temperatures were maintained at 373 K. Collision-induced dissociation (CID) of the selected precursor ion was carried out with argon as the collision gas at a pressure optimized to produce extensive fragmentation of the ion under investigation.

RESULTS AND DISCUSSION

Degradation product monitoring

Forced acid degradation of VAL was monitored by HPLC-PDA. Figure 1 shows the representative HPLC-PDA chromatograms of VAL samples under acid stress condition taken at 30-min intervals.

The retention time of VAL was 10.7 min, which was confirmed by measurement of the drug standard solution. No extra peak indicative of degradation was detected during exposure to 1 M HCl. Chromatographic data were also evaluated by asymmetric factor, theoretical plates and purity plot to identify the possible co-elution of degradation products. As shown in Figure 2, the VAL purity peak matches that of the VAL standard, showing no degradation over time. These results would induce the conclusion of high stability of VAL under exposure to 1 M HCl up to 180 min. However, the asymmetric factor and theoretical plates varied slightly over time under acid stress condition, as shown in Table 1, indicating a possible co-elution of degradation products.

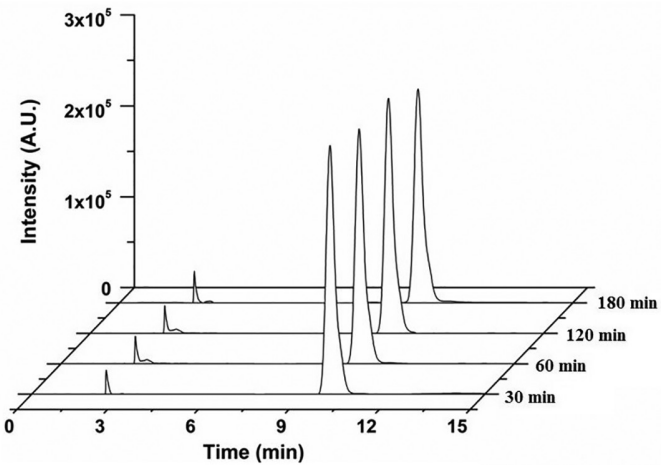

FIGURE 1 - HPLC-PDA chromatogram for forced acid degradation of VAL in 1 M HCl under different stress conditions.

To confirm the HPLC-PDA results, the same samples were also analyzed by HPLC-MS. Figure 3 shows HPLC chromatograms obtained using ESI(+) and total ion current (TIC) monitoring, which sums up the intensity of all ions across the selected m/z range. Note that HPLC-MS with TIC monitoring indicated no degradation either, likewise the HPLC-PDA results. A significance *t*-test was performed to compare the peak areas of the VAL standard

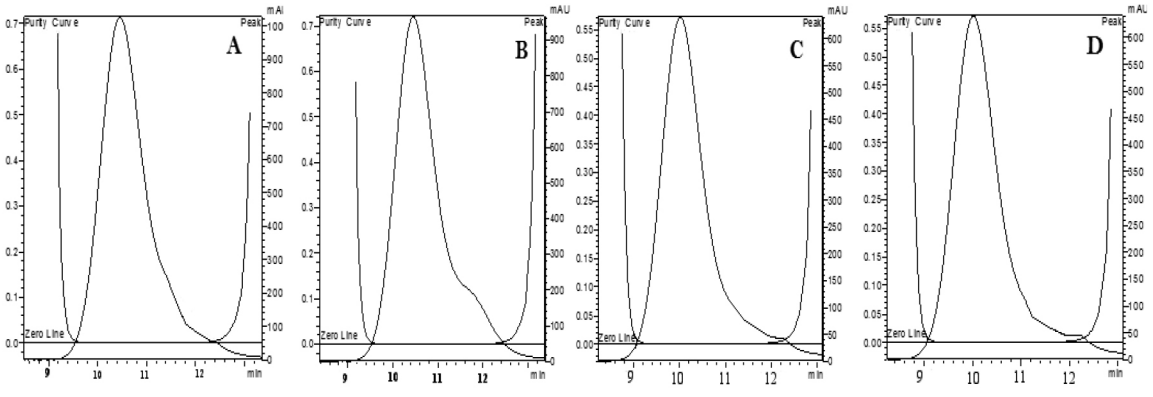

FIGURE 2 - Peak purity plot for forced acid degradation of VAL in 1 M at 30 min (A), 60 min (B), 120 min (C) and 180 min (D).

TABLE I - Asymmetry factor and theoretical plates of VAL at different times under acid stress condition

Time/min	0	30	60	120	180
A_s [a]	1.3	1.4	1.2	1.5	1.8
N [b]	1410	1385	1210	1180	1071

[a] Asymmetry factor; [b] Theoretical plates

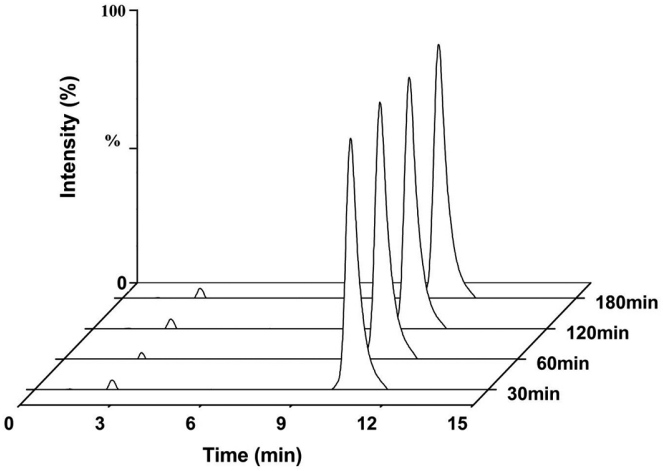

FIGURE 3 - HPLC-MS chromatograms using ESI(+) and TIC for the acid stress degraded samples of VAL.

(n=3) and those in the chromatograms shown in Figure 1 and Figure 2. The degree of freedom is 10; therefore, the critical value is t_{10}=2.23 (P=0.05). The value obtained of t=1.89 is smaller than the critical value, thus, the variation in the VAL peak area is not significant when detected by PDA and MS.

Fortunately, however, the full ESI(+)-MS data was inspected, and as Figure 4 shows, at the VAL chromatographic peak at 10.7 min, the raising of two major ions of m/z 306 and 352 was detected, together with the ions from the parent drug, that is, m/z 436 for $[M+H]^+$ and m/z 458 for $[M+Na]^+$.

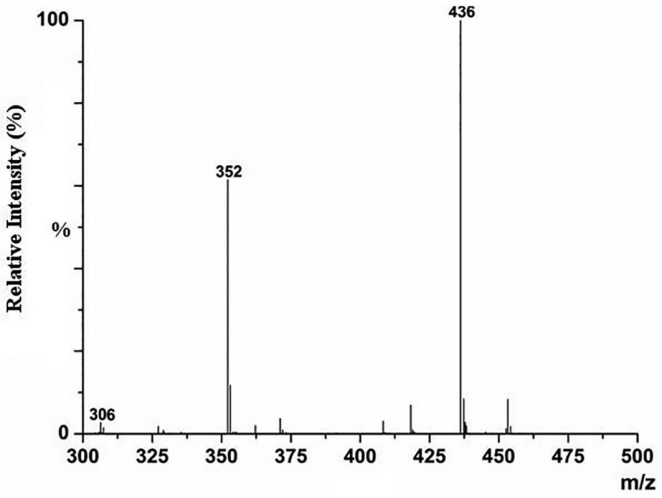

FIGURE 4 - Typical ESI(+) full mass spectrum for the VAL peak at RT = 10.7 min.

The ESI(+)-MS data unequivocally indicates that VAL indeed degraded to co-eluting products. The ions of m/z 306 and m/z 352, as well as that of VAL (m/z 436), were therefore recorded in the extracted ion chromatograms (XIC) (Figure 5). The XIC for the other minor ions observed in Figure 4 were also recorded and monitored as a function of the degradation time, but their profiles did not match those of the DP and were attributed to either VAL ion gas phase fragments or impurities.

Note the continuous increase in the DP relative abundance as a function of the exposure time with a corresponding and proportional decrease in the VAL concentration. Therefore, the selective HPLC-MS data provided actual evidence of extensive degradation of VAL under acid stress condition, whereas HPLC-PAD incorrectly indicated full stability. Figure 6 compares HPLC-MS monitoring via TIC and XIC of VAL degradation under acid stress condition. The ions of m/z 306 and m/z 352 were thus attributed to the arising of VAL

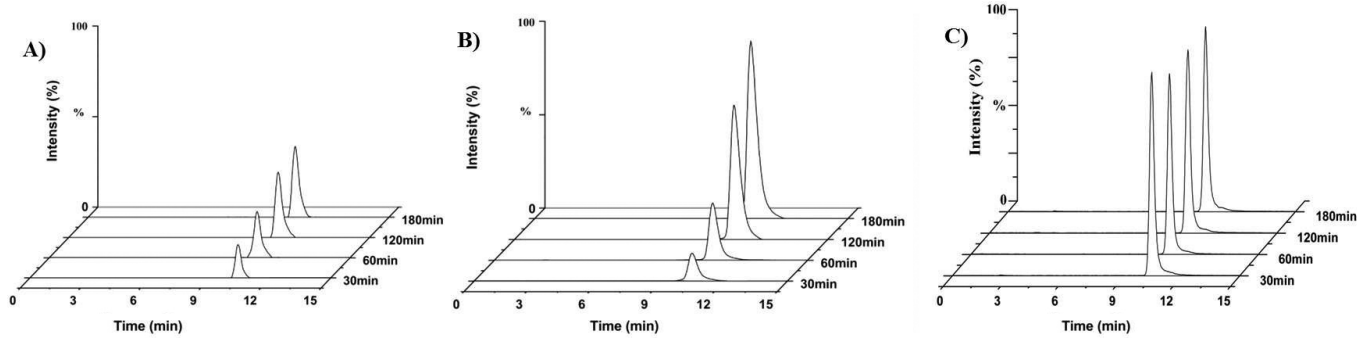

FIGURE 5 - HPLC-MS chromatograms using XIC monitoring for VAL degradation products: m/z 352 (A), m/z 306 (B) and $[VAL+H]^+$ m/z 436 (C) in 1 M HCl for different exposure times.

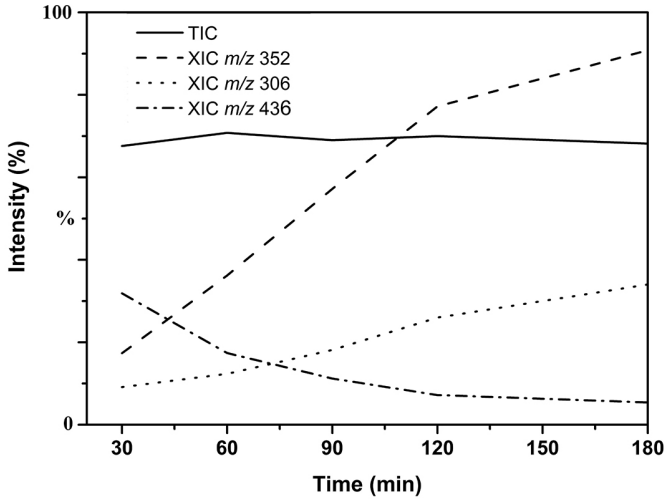

FIGURE 6 - VAL TIC and XIC HPLC-MS monitoring under acid stress degradation.

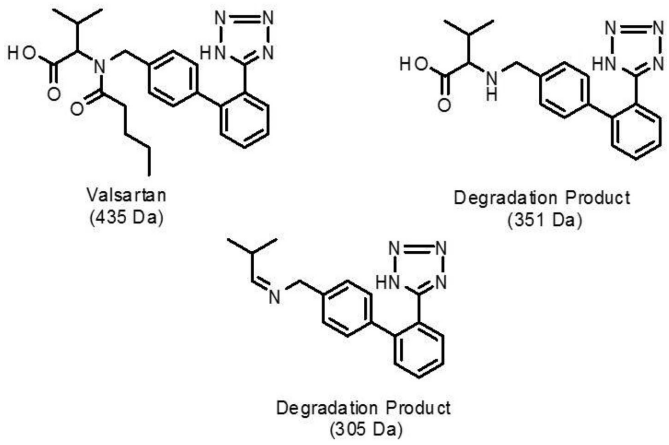

FIGURE 7 - VAL Degradation products as indicated by HPLC-MS monitoring.

DP protonated molecules by acid hydrolysis and Figure 7 shows the proposed product structures.

ESI-MS/MS

Interestingly, the MS study of the fragmentation pattern of gaseous drugs in protonated, deprotonated and ionized forms has been found to match or provide insights on their degradation mechanisms and products (Amaral *et al.*, 2011). The fragmentation patterns of gaseous molecules reveal the weaker molecule bonds; hence, some of the fragment ions may eventually match degradation products, particularly when the behavior of a protonated molecule is compared to that expected under acid stress condition. Figure 8 shows the ESI (+)-MS/MS of $[VAL+H]^+$ of m/z 436, as well as of the protonated molecules of its two major products of m/z 352 and m/z

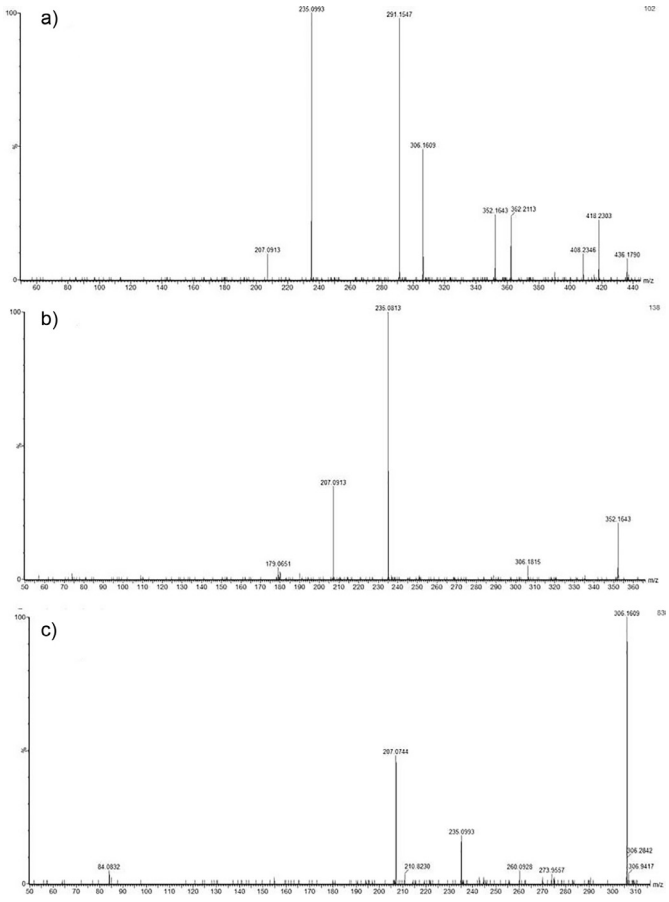

FIGURE 8 - ESI(+)-MSMS for the ions of a) *m/z* 436, b) 352 and c) 306 obtained via direct infusion of VAL samples after 180 min of exposure to acid stress condition.

306. Note that the fragments of $[VAL+H]^+$ include the protonated molecules of m/z 352 and 306 of the detected DP (Scheme 1) (Mehta, Shah, Singh, 2010). The ESI (+)-MS/MS data for VAL and its DP may therefore include products that are likely to form under longer acid degradation.

CONCLUSION

HPLC-MS monitoring has shown that VAL degradation products under acid stress condition co-elute with the parent drug, resulting in misleading stability results when monitoring is performed through a less selective detection method such as HPLC-PAD. The high structural selectivity of HPLC-MS seems therefore required for comprehensive and effective studies of VAL degradation products and their formulations. Actually, HPLC-MS seems to be essential for drug degradation studies, particularly for new drugs and formulations, as well as for method development. Drug stability methods

developed through HPLC-UV or HPLC-PAD and so far exclusively monitored by either of these methods should therefore be co-validated by HPLC-MS in search for potential co-eluting degradation products.

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