Research Article

Drug Testing and Analysis

Received: 12 August 2009 Revised: 1 December 2009 Accepted: 4 January 2010 Published online in Wiley Interscience: 9 February 2010

(www.drugtestinganalysis.com) DOI 10.1002/dta.116

Strategy for identification and characterization of small quantities of drug degradation products using LC and LC-MS: Application to valsartan, a model drug

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The present study demonstrates the applicability of a strategy involving use of liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC-MS) techniques for the identification and characterization of minute quantities of degradation products, without their isolation from the reaction matrix in pure form. Valsartan was used as a model drug. It was subjected to forced degradation studies under the International Conference on Harmonisation (ICH) prescribed conditions of hydrolysis (acid, base and neutral), photolysis, oxidation and thermal stress. The drug showed labiality under acid/neutral hydrolytic and photolytic conditions, while it was stable to base hydrolytic, oxidative and thermal stress. Three small degradation products were formed, which were separated on a C-18 column using a gradient method. The same were characterized with the help of their fragmentation pattern and accurate masses obtained upon LC-MS/TOF analyses and online H/D exchange studies. The structures were supported by appropriate mechanistic explanation.

Keywords: valsartan; stress degradation; characterization; degradation products; LC-MS/TOF

Introduction

The parent ICH stability testing guideline requires the drugs to be subjected to stress decomposition studies followed by identification and characterization of the degradation products.[1] In parallel, the ICH guideline on impurities[2] necessitates characterization of all degradation products formed in drug products at $\geq$0.1%. Therefore, the emphasis today is on techniques that allow characterization of very low quantities of degradation products, against the conventional process of isolation and spectral analysis, which is tedious and time consuming. The hyphenated techniques are in focus for the purpose, among which LC-MS tools have been explored more strongly due to their potential to directly characterize small quantities of degradation products.[3,4]

We have also made extensive use of liquid chromatography-mass spectrometry (LC-MS) techniques in our laboratories lately for exploring the degradation behaviour of various drugs.[5,6] In due course, a strategy was finalized for unequivocal elucidation of structures of degradation products present in minute amounts (Figure 1). An endeavour of the present study was to validate this strategy by its further application to forced decomposition samples of valsartan, an angiotensin receptor antagonist. The drug acts by binding selectively and non-competitively to angiotensin II receptor type 1 and is used in the treatment of hypertension of all grades.[7]

The degradation behaviour of valsartan along with amlodipine was reported by Chitlange *et al*.[8] but none of the degradation products were characterized. At least two reports exist on the characterization of process related impurities of the drug,[9,10] but its degradation chemistry under various stress conditions is not yet reported. Hence it was of interest to study the same. The investigation involved the following steps: (1) subjecting of drug to ICH prescribed hydrolysis, oxidative, photolytic and thermal stress; (2) separation of degradation products on an HPLC column; (3) establishment of fragmentation pattern of the drug using MS/TOF, MS^n and H/D exchange studies; (4) characterization of degradation products from LC-MS/TOF and on-line H/D exchange data; and (5) justification of elucidated structures through mechanistic explanation.

Experimental

Drug and reagents

Pure valsartan was obtained as a gratis sample from Aurobindo Pharma (Hyderabad, India). Analytical reagent (AR) grade sodium hydroxide (NaOH) was purchased from Ranbaxy Laboratories (SAS Nagar, India); hydrochloric acid (HCl) from LOBA Chemie Pvt. Ltd. (Mumbai, India), and hydrogen peroxide (H_2O_2) from s.d. Fine-Chem Ltd. (Boisar, India). Buffer salts of AR grade and all other chemicals were bought from local suppliers. HPLC grade acetonitrile (ACN) was procured from J.T. Baker (Phillipsburg, NJ, USA). Ultra pure water was obtained from a bench-top purification system (ELGA, Wycombe, Bucks, UK). Deuterated water (D_2O, 99.9%) was obtained from Aldrich (St Louis, MO, USA).

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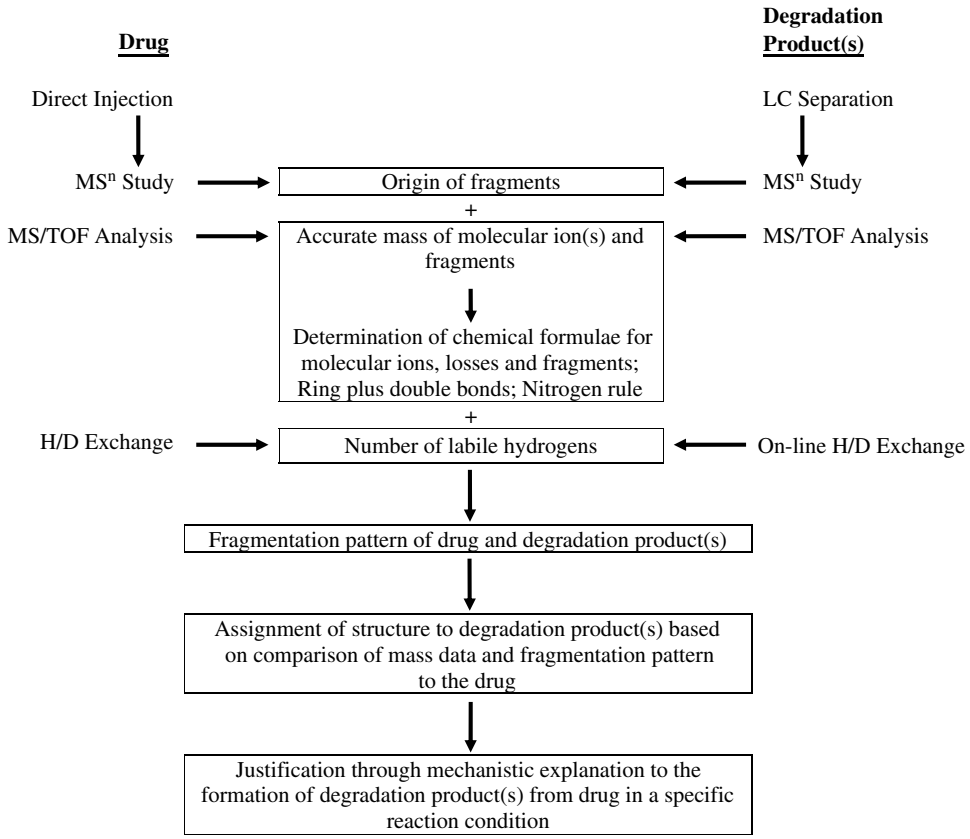


Figure 1. Strategy for identification and characterization of degradation product(s) using LC and LC-MS.

Apparatus and equipment

Precision water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for solution degradation studies. A Dri-Bath (Thermolyne, IA, USA) was used for solid state thermal stress study. Accelerated stability studies were carried out in humidity (KBF720, WTC Binder, Tuttlingen, Germany) and photostability (KBWF 240, WTC Binder, Tuttlingen, Germany) chambers, both set at 40 ± 1 °C/75 ± 3% RH. The photostability chamber was equipped with an illumination bank on inside top, consisting of a combination of two UV (OSRAM L18 W/73) and four white fluorescent (PHILIPS TRULITE 18 W/86) lamps, in accordance with Option 2 of the ICH guideline Q1B.[11] Both fluorescent and UV lamps were put on simultaneously. The samples were placed at a distance of 9 inches from the light bank. A calibrated lux meter (model ELM 201, Escorp, New Delhi, India) and a calibrated UV radiometer (model 206, PRC Krochmann GmbH, Berlin, Germany) were used to measure visible illumination and UV energy, respectively.

pH/Ion analyzer (MA 235, Mettler Toledo, Schwerzenbach, Switzerland) was used to check and adjust the pH of buffer solutions. Other smaller equipment used were sonicator (3210, Branson Ultrasoincs Corporation, Danbury, CT, USA), precision analytical balance (AG 135, Mettler Toledo, Schwerzenbach, Switzerland) and auto pipettes (Eppendorf, Hamburg, Germany).

The degradation behaviour of the drug was studied on a liquid chromatography (LC) system equipped with a photo-diode array detector and controlled by SP1 software ver. 6.14 (VP series, Shimadzu, Kyoto, Japan). MS^n studies were carried out on an LTQ XL MS 2.5.0 system (Thermo, San Jose, CA, USA). The same was controlled by Xcalibur (version 2.0.7 SP1) software. LC–MS/TOF results were obtained on a system in which HPLC (1100, Agilent Technologies, Waldbronn, Germany) was hyphenated to MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), using Hyphenation Star (version 3.1) and MicrOTOF Control (version 2.0) software. The calibration solution used was ES Tuning Mix solution (Agilent Technologies, Palo Alto, CA, USA), diluted to a suitable concentration with a mixture of ACN-water (95 : 5%v/v). All masses were corrected by use of internal reference ions of m/z 322.0481 ($C_6H_{19}O_6N_3P_3$), 622.0290 ($C_{12}H_{19}O_6N_3P_3F_{12}$), and 922.0098 ($C_{18}H_{19}O_6N_3P_3F_{24}$). The TOF instrument was also used for H/D exchange study on the drug, while MS^n system was employed for online H/D exchange investigations on the degradation products. In all the studies, the separations were achieved on a Luna C-18 (150 mm × 4.6 mm i.d., particle size 5 µm) column (Phenomenex, Torrance, CA, USA).

Stress studies

Acidic and alkaline hydrolysis were carried out in 1N HCl and 2N NaOH, respectively, whereas neutral hydrolysis was performed in water. All the hydrolytic studies were conducted at 80 °C. The oxidative study was carried out in 30% H_2O_2 at room temperature for 2 days. Photolytic studies on the drug in the solid and solution state were carried out by exposure to a combination of UV and fluorescent lamps in a photostability chamber set at accelerated conditions of temperature and humidity (40 °C/75% RH). A parallel blank set was kept in the dark for comparison. For thermal stress testing, the drug was sealed in glass vials and placed in the thermostatic block at 50 °C for 21 days. The optimized stressed conditions are enlisted in Table 1.

Table 1. Optimized stress conditions for valsartan

Stress condition	Concentration of stressor	Exposure condition	Duration
Hydrolysis			
Acid	1 N HCl	80 °C	12 h
Neutral	H_2O	80 °C	36 h
Base	2 N NaOH	80 °C	48 h
Oxidation	30% H_2O_2	RT	2 d
Photolysis	8500 Lux. h fluorescent and 0.05 W/m^2 UV light at 40 °C/75% RH		
Acid	0.1N HCl		6 d
Neutral	H_2O		6 d
Base	0.2N NaOH		6 d
Solid	–		10 d
Thermal	–	50 °C	21 d

The stressors, choice of their concentration and preparation of samples were based on a previous publication.[12] As the drug was insoluble in water, the drug stock was prepared in ACN at a concentration of 2 mg/ml. Before the study, the stock was diluted in 50 : 50 ratios with the stressor (e.g., HCl, NaOH, water, etc.). After subjecting the samples to stress studies, they were withdrawn at suitable time intervals and diluted four times with water: ACN (50 : 50 v/v) before LC injection. Also, all the stressed samples were mixed in an equal volume and used for the final high performance liquid chromatography (HPLC) method development. The injection volume was 6 µl in all the cases.

HPLC method development and optimization

Initial studies were carried out using varied proportions of ACN (A) and potassium dihydrogen orthophosphate buffer (B). The pH of the buffer, flow rate and composition of the mobile phase were systematically varied to optimize the method. Finally, adequate separation of peaks with good resolution was obtained using A and B (pH 3.0; 0.01M) in a gradient mode (T_{min}/A : B; T_0/22 : 78; T_5/70 : 30; T_8/70 : 30; T_{11}/22 : 78; T_{16}/22 : 78). The detection wavelength was 250 nm and the flow rate was 1.0 ml/min.

MS/TOF, MS^n and H/D exchange studies on the drug

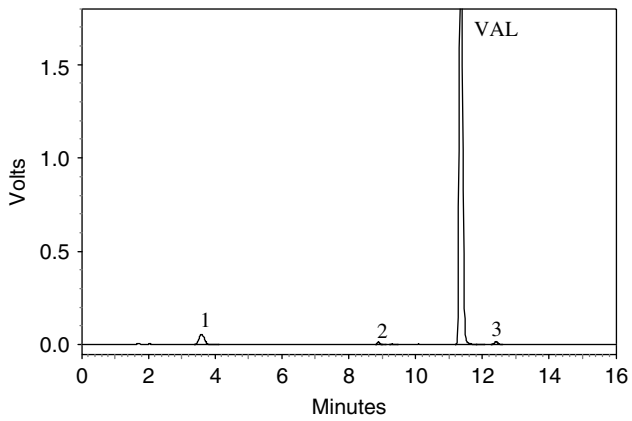
In order to establish a comprehensive fragmentation pathway of the drug, MS/TOF studies were performed in ESI positive mode in the mass range of 50 to 1500 Da. High purity nitrogen was used as a nebulizer as well as an auxiliary gas. Mass parameters were optimized, as listed in Table 2. The drug was further subjected to multistage mass studies (MS^n) in ESI positive mode. Fragmentation of various precursor ions formed in MS^n studies was achieved at different collision energies. This was followed by conduct of H/D exchange studies on the drug, wherein the drug solution was prepared in a mixture of CH_3CN and D_2O.

LC-MS/TOF studies on degradation products

The stressed samples were subjected to LC-MS/TOF studies using the developed LC method, but after replacing phosphate with ammonium acetate at same buffer concentration and pH. The identity of each degradation product was established with the help of LC-MS/TOF accurate mass values, and comparison of fragmentation profiles with the drug.

Table 2. Parameters of the developed MS/TOF methods in ESI +ve mode

Purpose		Molecular ions of drug/ degradation products	Optimum fragmentation
Source	End plate offset (V)	−500	−500
	Capillary (V)	−4500	−4500
	Nebuliser (Bar)	1.2	1.2
	Dry gas (L/min)	6.0	6.0
	Dry temperature (°C)	200	200
Transfer	Funnel 1 RF (Vpp)	150	200
	Funnel 2 RF (Vpp)	200	250
	ISCID energy (eV)	0.0	7.0
	Hexapole RF (Vpp)	280	280
Quadrupole	Ion energy	4.0	10.0
	Low Mass (m/z)	200	300
Collision Cell	Collision energy (eV/z)	7.0	20.0
	Transfer time (µs)	36.0	48.0
	Collision RF (Vpp)	300	300
	Pre pulse storage (µs)	16.0	10.0
Detector	Source (V)	−1200	−1200



Peaks	1	2	3
Stress conditions	Acid & Neutral	Photo-neutral	Photo-acid

Figure 2. Chromatogram showing separation of DPs (1–3) and valsartan (VAL) in the mixture of stressed samples.

Results and Discussion

Degradation behaviour

The drug degraded an extent of 14.1%, 2.3%, 6.8% and 13.1% under acid, neutral, photoneutral and photo-acid conditions, respectively to form one degradation product (DP) each. However, the chromatogram of the mixture of degraded samples showed only three peaks of DPs (Figure 2). The reason was the similarity

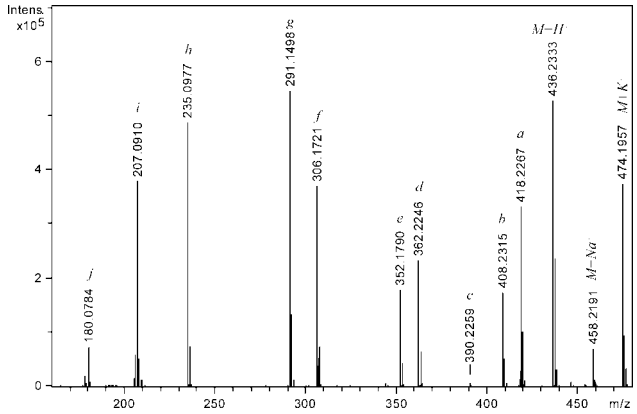


Figure 3. Line spectrum of valsartan obtained in MS/TOF study.

Table 4. MS^n fragmentation of valsartan

MS^n	Precursor ion	Product ions
MS^2	436	418, 408, 362, 352, 291, 235, 207
MS^3	418	390, 362, 306, 235, 207
	408	362, 291
	352	306, 235, 207
MS^4	390	362, 347, 306, 235, 207
	362	347[a], 291[a]
	306	235, 207, 180
MS^5	235	207[a], 180

[a] Fragments had low intensity, so could not be captured for further MS^n.

of DPs formed in acid and neutral conditions. The drug was stable under all other stress conditions, including hydrolysis in alkali; oxidation; exposure of alkali solutions and solid drug to light, and dry heating at 80 °C.

Mass fragmentation behaviour of the drug

Figure 3 shows line spectrum of the drug obtained from MS/TOF studies. In total, ten fragments (labelled 'a–j') were formed from the drug ($M+H^+$), apart from Na and K adducts. The accurate mass of each fragment was used to determine the most probable molecular formula (Table 3), taking the help of elemental composition calculator. Subsequently, MS^n studies were performed on the drug to determine the origin of each fragment (Table 4). This was followed by proposition of tentative structures to each, also taking into account data from H/D exchange studies, and calculated values of ring plus double bonds (RDBs).

The fragmentation behaviour of valsartan, correlated to data in Tables 3 and 4, is outlined in Figure 4. The figure shows existence and involvement of three possible protonated forms of the precursor [m/z 436] in the drug's fragmentation pathway. The precursor with protonation at the carboxylic group produced a daughter ion of m/z 418, whereas those charged at amide nitrogen and tetrazole nitrogen reduced to ions of m/z 408 and 352, respectively. The ion of m/z 418 further fragmented in MS^3 step into an ion of m/z 390, which on MS^4 resulted in two parallel daughter fragments of m/z 362 and 306. The ion of m/z 362, on further MS^4 and MS^5 analyses fragmented to m/z 347 → 291, while the one of m/z 306 followed the pathway m/z 235 → 207 → 180.

LC-MS/TOF and on-line H/D exchange studies on degradation products

Subsequent to establishment of mass fragmentation pattern for the drug, the DPs were also subjected to LC-MS/TOF analyses to elucidate their structures. The mass spectra obtained thereof are shown in Figure 5. Evidently, Na and K adducts were observed in case of DP-2 and DP-3, as in the drug (Figure 3). The data of accurate masses, possible molecular formulae, major fragments and on-line H/D exchange for the three DPs are enlisted in Table 5.

Characterization of degradation products

The DPs were characterized through systematic amalgamation of HRMS, mass fragmentation and on-line H/D exchange data.

Table 3. Interpretation of MS/TOF, H-D exchange and MS^n data of fragments of valsartan

Peak No.	MS/TOF data	Best possible molecular formulae	Exact mass of most probable structure	Error in ppm	RDB	Possible parent fragment	Difference from parent ion	Possible molecular formulae for Loss		H/D Exchange data	No. of labile hydrogens	
								L1	L2		$M+H^+$	M^+
$M+H^+$	436.2333	$C_{24}H_{30}N_5O_3$	436.2343	−2.2	12.5	–				439	3	2
a	418.2267	$C_{24}H_{28}N_5O_2$	418.2238	6.9	13.5	$M+H^+$	18.0066	H_2O	–	419	1	0
b	408.2315	$C_{24}H_{30}N_3O_3$	408.2282	8.0	11.5	$M+H^+$	28.0018	N_2	–	411	3	2
c	390.2259	$C_{23}H_{28}N_5O$	390.2288	−7.4	12.5	a	28.0008	N_2	CO	391	1	0
d	362.2246	$C_{23}H_{28}N_3O$	362.2227	5.2	11.5	b, c		CH_2O_2, N_2	–	363	1	0
e	352.1790	$C_{19}H_{22}N_5O_2$	352.1768	6.2	11.5	$M+H^+$	84.0543	$C_3H_6N_3$	C_5H_8O	356	4	3
f	306.1721	$C_{18}H_{20}N_5$	306.1713	2.6	11.5	c, e		C_5H_8O, CH_2O_2	$C_3H_6N_3$,	308	2	1
G	291.1498	$C_{19}H_{19}N_2O$	291.1492	2.0	11.5	♦				292	1	0
H	235.0977	$C_{14}H_{11}N_4$	235.0978	−0.4	11.5	f	71.0743	C_4H_9N	–	236	1	0
I	207.0910	$C_{14}H_{11}N_2$	207.0917	−3.3	10.5	h	28.0067	N_2	–	208	1	0
J	180.0784	$C_{13}H_{10}N$	180.0808	−13.3	9.5	♦				181	1	0

♦ MS^n study could not be achieved.

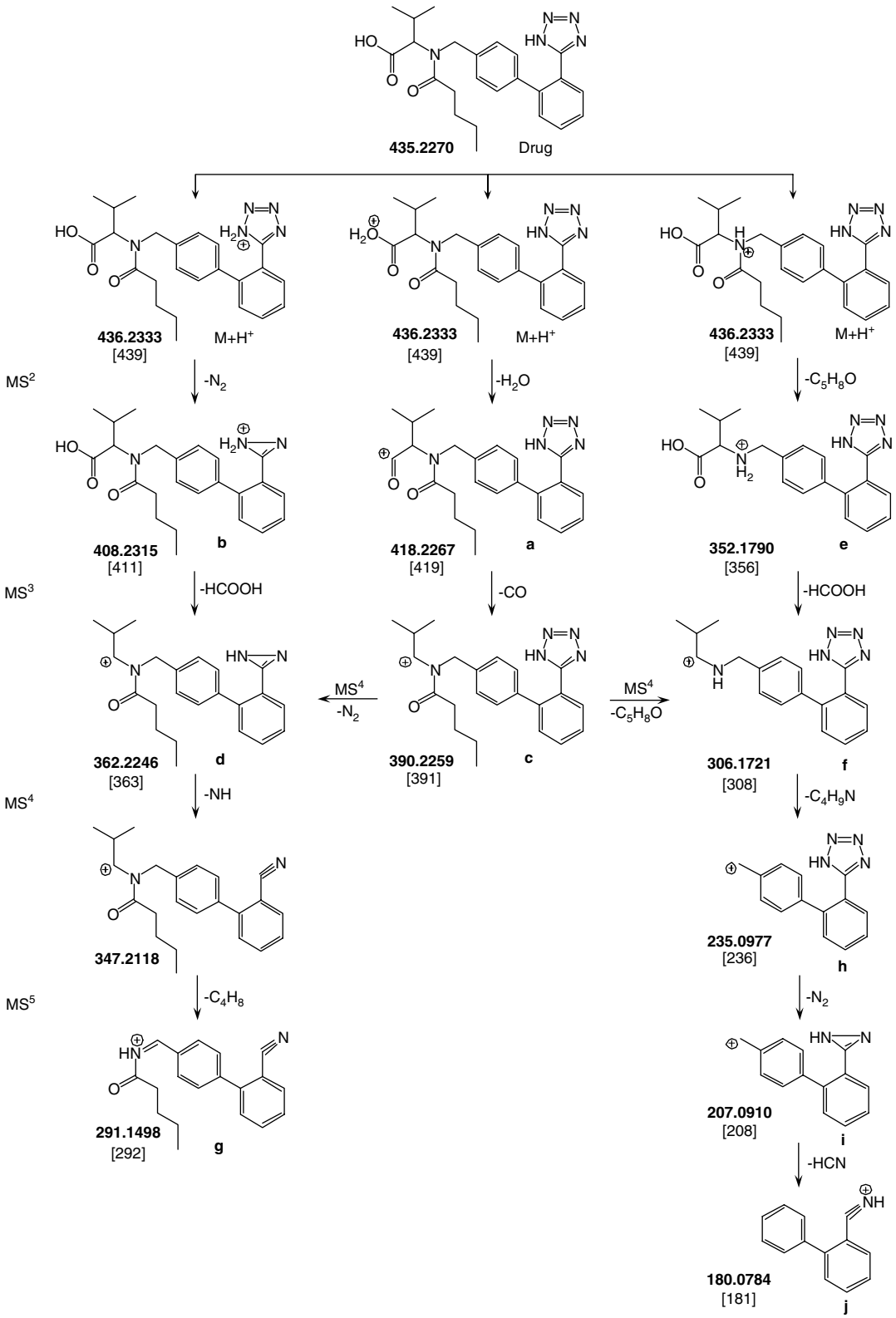


Figure 4. Fragmentation pathway of valsartan. The value of obtained accurate mass is shown below each structure, along with the mass obtained in H/D exchange study (in square brackets). $M+H^+$ denotes the protonated form of precursor; while a–j represent fragments in the line spectrum shown in Fig. 3.

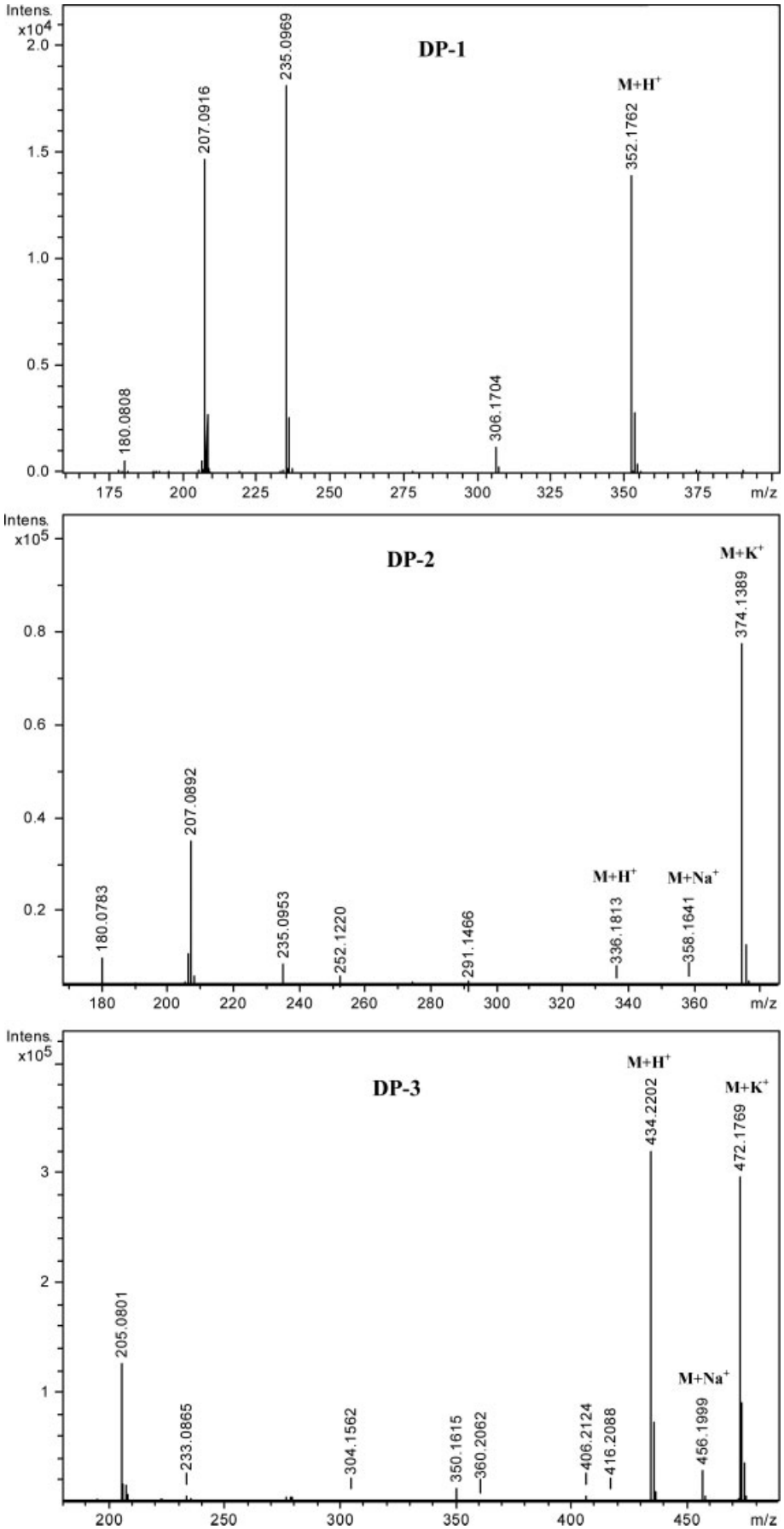


Figure 5. Line spectra of degradation products DP-1 to DP-3.

Table 5. MS/TOF and on-line H/D exchange data of DPs (1–3) along with their molecular formulae and major fragments

DPs	Accurate mass	Molecular formulae (exact mass; RDB; error in ppm)	Major fragments (chemical formulae; error in ppm)	Mass after H/D exchange in ESI +ve mode	Number of labile hydrogens
1	352.1762	$C_{19}H_{22}N_5O_2^+$ (352.1768; 11.5; −1.7)	306.1704 ($C_{18}H_{20}N_5^+$; −2.9), 235.0969 ($C_{14}H_{11}N_4^+$; −3.8), 207.0916 ($C_{14}H_{11}N_2^+$; −0.4), 180.0808 ($C_{13}H_{10}N^+$; 0.0)	356	3
2	336.1813	$C_{19}H_{22}N_5O^+$ (336.1819; 11.5; −1.7)	291.1466 ($C_{19}H_{19}N_2O^+$; −8.93), 252.1220 ($C_{14}H_{14}N_5^+$; −9.5), 235.0953 ($C_{14}H_{11}N_4^+$; −36.5), 207.0892 ($C_{14}H_{11}N_2^+$; −12.0), 180.0783 ($C_{13}H_{10}N^+$; −13.9),	339	2
3	434.2202	$C_{24}H_{28}N_5O_3^+$ (434.2187; 13.5; 3.4)	416.2088 ($C_{24}H_{26}N_5O_2^+$; 1.6), 406.2124 ($C_{24}H_{28}N_3O_3^+$; −0.2), 360.2062 ($C_{23}H_{26}N_3O^+$; 2.2), 350.1615 ($C_{19}H_{20}N_5O_2^+$; 0.85), 304.1562 ($C_{18}H_{18}N_5^+$; 1.6), 205.0801 ($C_{14}H_9N_2^+$; 19.9)	436	1

DP-1

The HRMS value of DP-1 was 352.1762 (Figure 5, Table 5). Its major fragments had m/z of 306, 235, 207 and 180. All these fragments were also formed from the product ion of m/z 352.1768 in case of the drug (Figure 4). This clearly meant that DP-1 had the same structure as the drug fragment of equal mass. The same was supported by the presence of three labile hydrogens (as determined by H/D exchange study) and very small error of −1.7 ppm between exact and observed masses. The proposed structure of DP-1 is shown in Figure 6. Incidentally, this DP has also been reported as an impurity by Sampath *et al*.[10]

DP-2

The precursor of DP-2 had accurate m/z of 336.1813, while the fragments had masses of m/z 291, 252, 235, 207 and 180 (Figure 5). The latter were similar to those present in the drug (Figure 3), except ion of m/z of 252, which was typical for the product. This helped in proposition of the structure of DP-2 (Figure 6). The calculated error between exact and observed masses of the proposed structure was just -1.7 ppm, while H/D exchange studies showed that there were two labile hydrogens, which conformed to two –NH, one each of tetrazole and amide in the proposed structure.

DP-3

In MS/TOF studies, the precursor of DP-3 showed m/z of 434.2202, along with fragments of m/z 416, 406, 360, 350, 304, 233 and 205 (Figure 5). Incidentally, all these were two mass units less than the corresponding masses for the drug (m/z 436, 418, 408, 362, 352, 306, 235 and 207). The best structure that could be postulated was a cyclized product of the drug (Figure 6), formed on removal of hydrogen from tetrazole and biphenyl rings. Such cyclization phenomenon under photolytic conditions has been reported earlier for candesartan cilexetil.[6] Online H/D exchange studies supported the supposition, which showed that there was only single labile hydrogen in DP-3, instead of two in the drug. The same was justified from the fact that the formation of ring between tetrazole and biphenyl rings resulted in utilization of labile –NH of tetrazole, leaving only single hydrogen of carboxyl for replacement. Further support was provided by the parallelism of fragmentation pattern of DP-3 (Figure 6) with that of the drug (Figure 4).

Mechanism of formation of degradation products

Figure 7 outlines the mechanisms of formation of DP-1 to DP-3. As shown, DP-1 was generated by simple amide hydrolysis under acidic and neutral conditions.[13] The drug underwent *N*-dealkylation in photoneutral condition, mediated by a free radical mechanism[14] to form DP-2. In photo-acidic condition, there was generation of two aryl radicals, which further rearranged to cyclise with the tetrazole ring to form DP-3.[15]

Conclusions

The degradation behaviour of valsartan was explored under stress degradation conditions prescribed by ICH guidelines. The drug underwent hydrolysis and photodegradation in acid and neutral conditions. To characterize the three degradation products, the strategy outlined in Figure 1 was employed. First a complete fragmentation pathway of the drug was established using MS^n and MS/TOF data. The DPs were then subjected to LC-MS/TOF analysis and structures were proposed based on their accurate mass and H/D exchange data, and also through comparison of fragmentation pathway of each DP to that of the drug. Further support was provided by mechanistic explanation to the origin of each DP.

The study highlights that minutiae in drug products can be characterized successfully by the use of proposed strategy involving LC and LC-MS techniques.

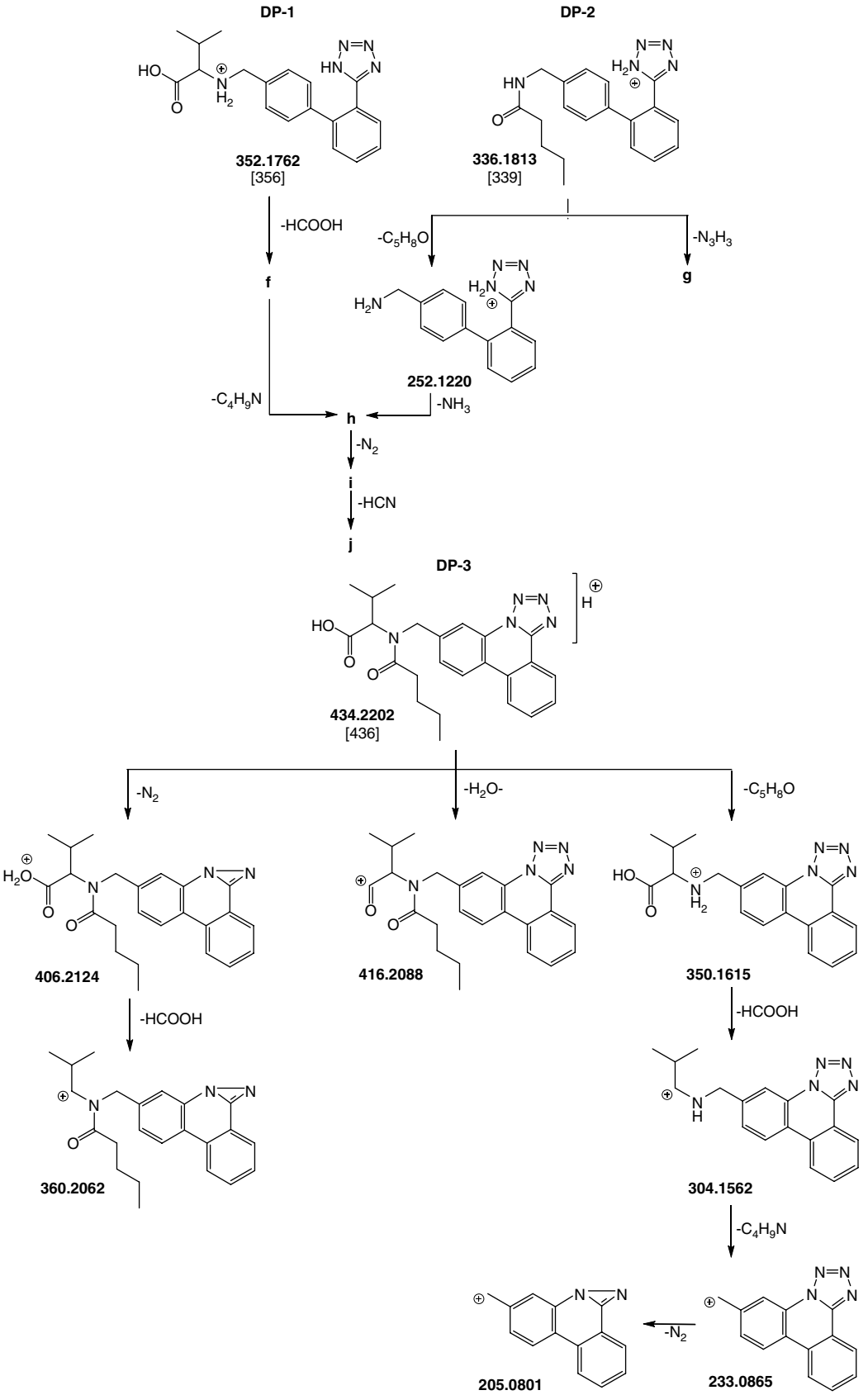


Figure 6. Fragmentation pattern of DPs of valsartan. The value of obtained accurate mass is shown below each structure, along with the mass obtained in online H/D exchange study (in square brackets). The fragments f–j were similar to those of the drug in Fig. 3 and their structures are shown in Fig. 4.

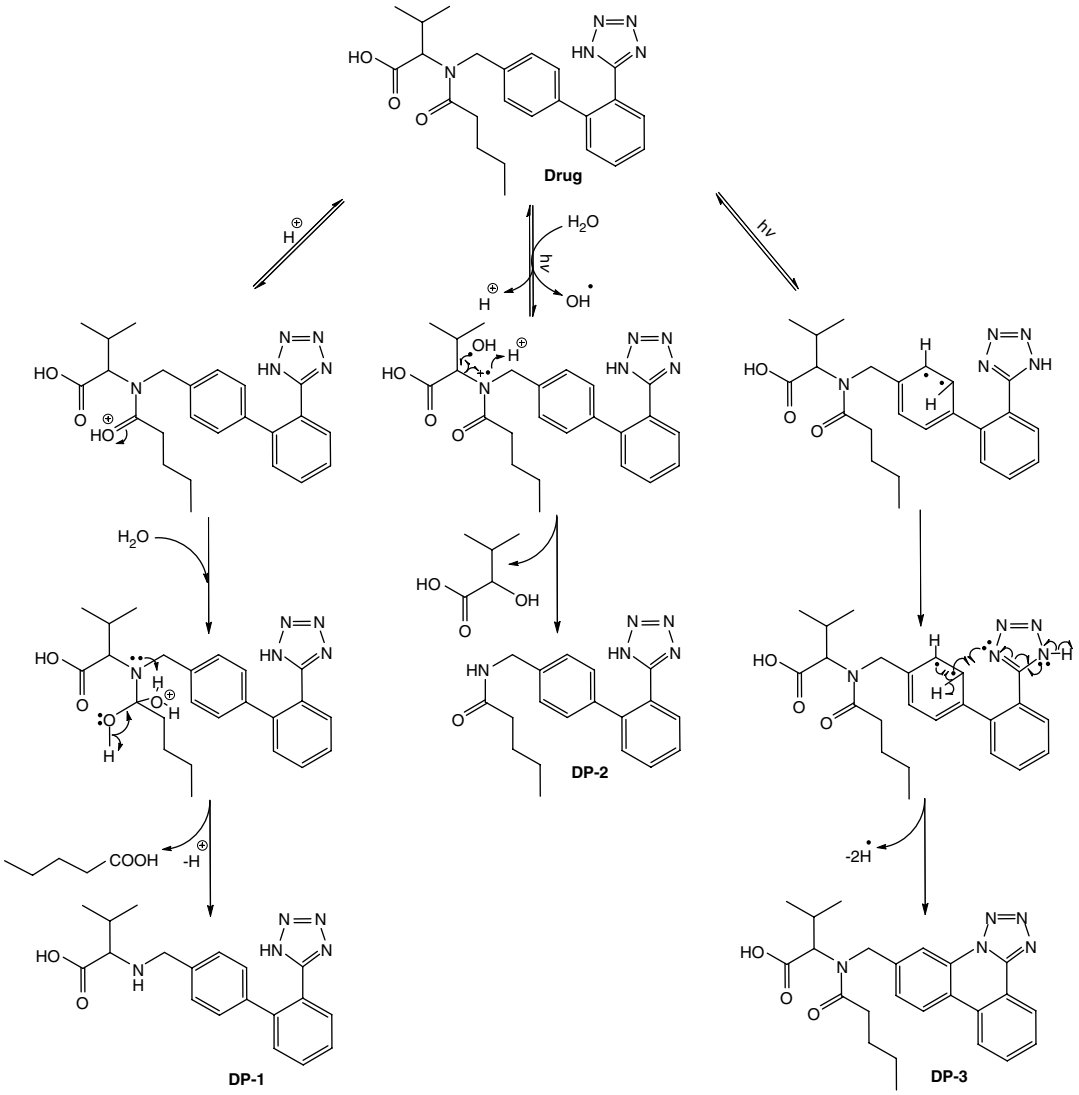


Figure 7. Mechanisms of formation of DPs of valsartan.

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