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ORIGINAL ARTICLE

Gradient RP-HPLC method for the determination of potential impurities in dabigatran etexilate in bulk drug and capsule formulations

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KEYWORDS

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Isolation and characterization;
RP-HPLC;
LC-MS;
Stress study;
Related impurities

Abstract A selective RP-HPLC method for separation and determination of potential related impurities (starting materials and by-products of synthesis, and degradants) of dabigatran etexilate (DAB) drug substance has been developed and validated. The separation was accomplished on a Inertsil ODS 3V (250 mm × 4.6 mm, 5 μm) column connected to a photodiode array (PDA) detector using 20 mM ammonium formate with 0.1% of triethylamine (pH: 5.0 adjusted with formic acid) as mobile phase-A and acetonitrile as mobile phase-B, under gradient elution. Two unknown impurities found in dabigatran etexilate industrial batch stability condition at levels more than 0.1% in HPLC analysis were characterized preliminarily by ESI-MS/MS studies. The major unknown (unknown-1) was enriched and isolated by preparative LC and structure was evidenced by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry and FT-IR. Another unknown (unknown-2) found as secondary degradant and structure was proposed by ESI-MS/MS study. This method can be used for the quality control of both drug substance and drug product. The performance of the method was validated according to the ICH guide lines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, ruggedness and robustness.

1. Introduction

Dabigatran etexilate is a potent, non peptidic small molecule that specifically and reversibly inhibits both free and clot-bound thrombin (Eriksson et al., 2005; Wienen et al., 2007; Van Ryn et al., 2007). It has been approved for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation by US Food and Drug Administration in October 2010 and by European Medicines Agency (EMA) in August 2011. EMA also approved it in 2008 for prophylaxis of thromboembolism in patients undergoing total knee or

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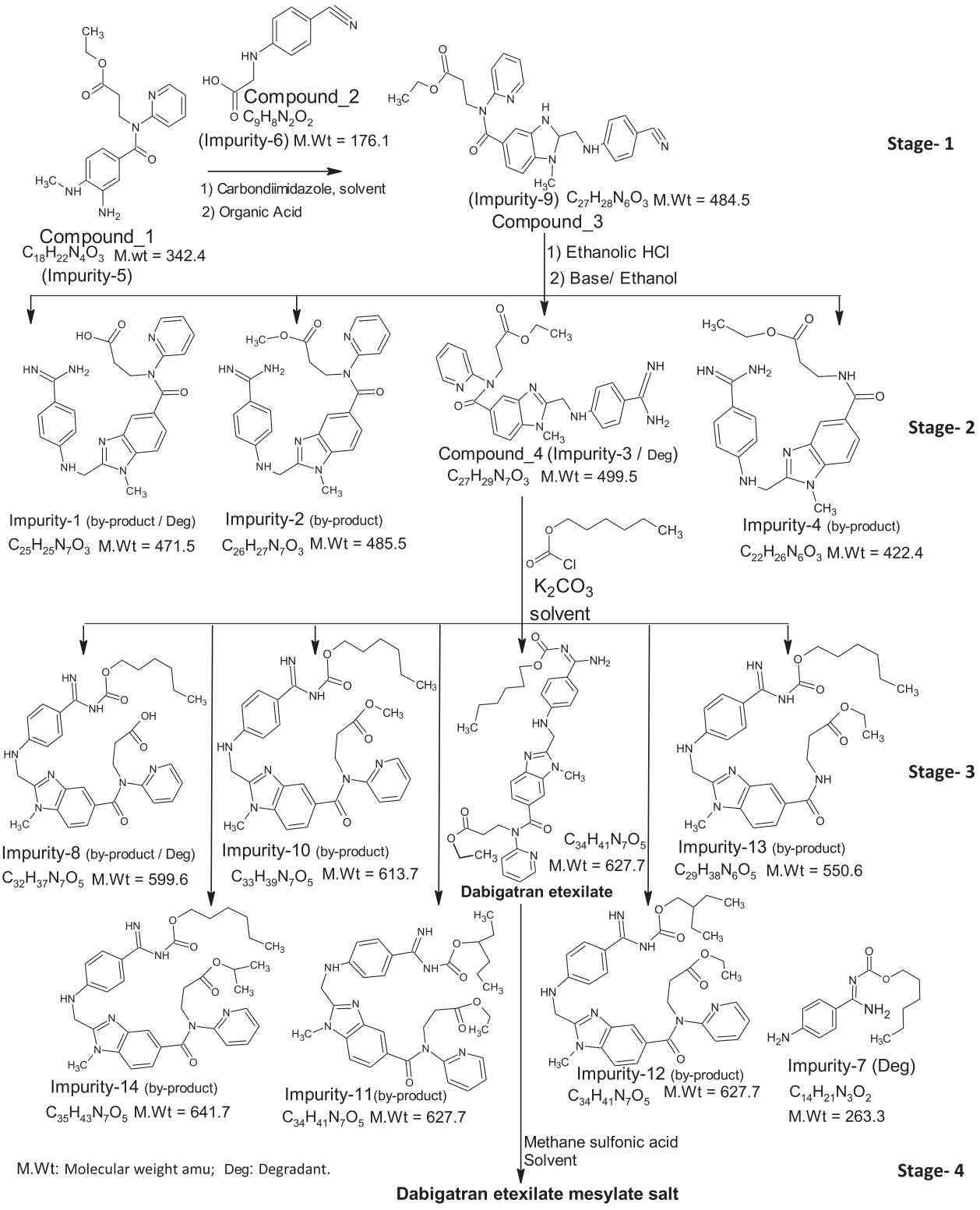

Figure 1 Potential associated impurities at different stages of DAB synthesis.

hip replacement (EMEA, 2008). Dabigatran etexilate mesylate capsule is marketed under the trade name Pradaxa in the United States, Australia and European countries. Dabigatran etexilate (DAB) is chemically designated as (ethyl N-{[2-({[4-((E)-amino{[hexyloxy)carbonyl]imino} methyl)phenyl]amino} methyl)-1-methyl-1H-benzimidazol-5-yl]carbonyl}-N-pyridin-2-yl-b-alaninate. It has an empirical formula of $C_{34}H_{41}N_7O_5$, and a molecular weight of 627.73 g mole^{-1}.

Currently available oral vitamin K antagonists such as warfarin have unpredictable pharmacokinetics and show

numerous drug and food interactions (Ansell et al., 2004), whereas unfractionated and low molecular weight heparins and fondaparinux require parenteral administration. Oral administration of dabigatran etexilate produces a predictable pharmacodynamic and has been clinically developed in various indications using fixed-dosing without the need for routine coagulation monitoring or dose adjustment. Anticoagulant treatment reduces the incidence of death and cardio embolic events (Cinteza, 2013). Since thrombin plays a key role in the formation of fibrin and is very important for blood coagulation and platelet activation, it represents a prime target for the development of anticoagulant agents for the prevention and treatment of thromboembolic disorders. A recent trial has also shown that dabigatran etexilate significantly reduces stroke risk with a better safety profile compared with warfarin in patients with atrial fibrillation (Connolly et al., 2009). Dabigatran etexilate converted to a major active metabolite known as dabigatran by non-specific plasma and liver esterase (Blech et al., 2008).

Several research papers have been reported in the literature for the determination of DAB (Bernardi et al., 2013a,b; Damle and Bagwe, 2014; Geetharam et al., 2014; Pani Kumar et al., 2015; Khan et al., 2014; Reddy and Rao, 2014) and these papers were limited to the assay of alone DAB from its degradants and concurrently DAB in human plasma and other biological stuff, based on UPLC MS/MS (Delavenne et al., 2012), HPLC and LC/MS/MS (Bernardi et al., 2015; Nouman et al., 2015; Laizure et al., 2014; Li et al., 2014; Härtter et al., 2012). Monitoring and control of by-products or impurities at each stage of synthesis is required to get a final pure form of DAB as per predefined specifications. The reported related substance methods (Dare et al., 2015; Pantović et al., 2015) were not suitable to monitor stage wise by-products during the synthesis of DAB and were only restricted to only for some of related impurities and the details of unknown degradants formed under the stress conditions employed were neither discussed nor characterized. Liquid chromatography combined with multistage mass spectrometry ($LC\text{-}MS_n$) method was available to characterize the degradants of dabigatran etexilate (Amrani et al., 2015) and was not suitable for routine monitoring and quantification of impurities. To the best of our knowledge, a complete validated HPLC method for the determination of impurities at different stages of synthesis i.e. raw materials, by-products and degradants of DAB in bulk drug substances is not reported till date. Further, no monograph on DAB is published in any of the pharmacopoeia for compendial applications. During the analysis of laboratory batches from synthetic process development of DAB, the impurity-1 to impurity-15 (Figs. 1 and 7) were identified as potential monitoring impurities to ensure the quality of active pharma ingredient (API) by controlling them less than 0.1% as per ICH (ICH, Q3A, 2008). In which impurity-15 was unknown-1 and found along with unknown-2 in accelerated stability condition (3 months 40 °C/75RH) of industrial batch at about 0.26% and 0.09%. Unknown-2 was not isolated, being proved as secondary degradant.

The present manuscript describes (i) Development of a RP-HPLC method for the separation and determination of DAB potential related impurities i.e. raw materials, by-products and degradants of mentioned synthetic procedure in Figs. 1 and 7 (namely Impurities-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13 and -15) and validation as per ICH guidelines (ICH, Q1A (R2), 2003; ICH, Q2 (R1), 2005; ICH, Q7, 2000) and (ii) Enrichment, isolation and purification of major unknown-1 (impurity-15) using preparative LC and characterization by ^{1}H, ^{13}C, ESI-MS studies and FT-IR. Proposing unknown-2 as secondary degradation impurity based on forced degradation study and ESI-MS data.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile, ammonium formate and formic acid were obtained from Merck, Darmstadt, Germany. Triethylamine was purchased from J.T.baker, Mallinckrodt Inc., Phillipsburg, NJ, USA. Water used for preparing mobile phase

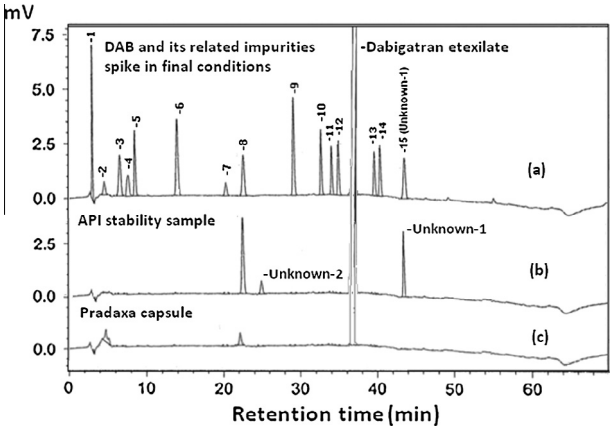

Figure 2 Typical chromatograms of (a) Dabigatran etexilate spiked with 0.15% of all impurities in final chromatographic conditions (pH 5.0), (b) bulk drug stability sample containing unknown impurities, and (c) Pradaxa capsule test sample.

Table 1 System suitability parameters.

S.No	Compound	RT(min) ($n = 6$)[a]	RRT[b] ($n = 6$)[a]	USP resolution[c] ($n = 6$)[a]	USP tailing ($n = 6$)[a]
1	Impurity-1	3.01	0.08	–	1.20
2	Impurity-2	4.80	0.13	5.3	1.11
3	Impurity-3	6.60	0.18	6.2	1.08
4	Impurity-4	7.62	0.21	2.3	1.07
5	Impurity-5	8.51	0.23	2.4	1.16
6	Impurity-6	13.93	0.38	12.8	1.05
7	Impurity-7	20.29	0.56	14.6	1.04
8	Impurity-8	22.57	0.62	4.4	1.02
9	Impurity-9	29.15	0.80	10.7	1.03
10	Impurity-10	32.68	0.90	8.3	1.07
11	Impurity-11	34.01	0.94	3.9	1.07
12	Impurity-12	34.90	0.96	2.6	1.12
13	DAB	36.37	1.00	3.2	1.07
14	Impurity-13	39.62	1.09	3.9	1.08
15	Impurity-14	40.37	1.11	2.5	1.07
16	Impurity-15	43.35	1.19	5.8	1.13

DAB = Dabigatran etexilate.
[a] Mean ± SD ($n = 6$).
[b] Relative retention times (RRT) were calculated against the retention time (RT) of dabigatran etexilate.
[c] Resolutions were calculated between two adjacent components.

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Table 2 Columns, mobile phase composition, mobile phase buffer pH and modes of elution tried during optimization process.

Trial No.	Column (250 × 4.6) mm, 5 μm	Mobile phase composition	Elution mode	Result
1	Inertsil ODS 3V	pH 5.0, 20 mM ammonium formate with 0.1% of triethylamine:ACN (60:40) (v/v)	Isocratic	Out of 15 impurities six impurities were merged
2	Inertsil ODS 3V	pH 5.0, 20 mM ammonium formate with 0.1% of triethylamine:MeOH (60:40) (v/v)	Isocratic	Out of 15 impurities six impurities were merged
3	Inertsil ODS 3V	pH 5.5, 20 mM ammonium formate with 0.1% of triethylamine:ACN	Gradient	Impurities 13 and 14 were merged
4	Inertsil ODS 3V	pH 4.5, 20 mM ammonium formate with 0.1% of triethylamine:ACN	Gradient	Impurities 11 and 12 were merged
5	Inertsil C8	pH 5.0, 20 mM ammonium formate with 0.1% of triethylamine:ACN	Gradient	Impurities 11 and 12 were merged
6	Ace-C18	pH 5.0, 20 mM ammonium formate with 0.1% of triethylamine:ACN	Gradient	Impurities 11 and 12, and impurities 14 and 15 were closely eluted
7	Zorbax XDB-C18	pH 5.0, 20 mM ammonium formate with 0.1% of triethylamine:ACN	Gradient	Impurities 4 and 5, and impurities 13 and 14 were merged
8	Zorbax XDB-C8	pH 5.0, 20 mM ammonium formate with 0.1% of triethylamine:ACN	Gradient	Impurities 10 and 11 were merged

Table 3 Comparative ^{1}H and ^{13}C NMR spectral data for DAB and unknown-1 impurity.

Position	Dabigatran etexilate mesylate			Unknown-1 (Impurity-15)		
	δC (ppm)	^{1}H	δH ppm/Hz	δC (ppm)	^{1}H	δH ppm/Hz
1	13.75	3H	0.84–0.89/t, 6.0	13.95	3H	0.87–0.91/t, 6.0
2	21.87	2H	1.25–1.36/m	22.49	2H	1.30–1.40/m
3	30.18	2H	1.25–1.36/m	30.21	2H	1.30–1.40/m
4	24.68	2H	1.25–1.36/m	25.42	2H	1.30–1.40/m
5	27.82	2H	1.64–1.69/m	28.63	2H	1.64–1.73/m
6	66.95	2H	3.92–3.99/t, 60	66.20	2H	4.19–4.23/t, 6.0
7	155.74	–	–	151.03	–	–
8	163.33	–	–	170.52	–	–
9	123.35	–	–	124.15	–	–
10, 10′	121.94	2H	7.41–7.64/m	129.80	2H	7.65–7.68/m
11, 11′	111.72	2H	6.84–6.91/m	111.98	2H	6.68–6.73/m
12	153.24	–	–	152.46	–	–
13	44.29	2H	4.69/br	44.76	2H	4.55/br
14	153.31	–	–	151.60	–	–
15	131.38	–	–	130.80	–	–
16	110.04	1H	7.09–7.17/m	109.07	1H	6.97–7.0/m
17	121.30	1H	7.41–7.64/m	122.34	1H	7.14–7.54/m
18	131.38	–	–		–	–
19	112.39	1H	7.41–7.64/m	119.81	1H	7.30–7.37/m
20	137.90	–	–	137.45	–	–
21	169.86	–	–	170.73	–	–
22	153.96	–	–	156.02	–	–
23	118.50	1H	7.41–7.64/m	121.16	1H	7.65–7.68/m
24	121.30	1H	7.41–7.64/m	137.45	1H	7.30–7.37/m
25	118.50	1H	7.09–7.17/m	121.67	1H	6.68–6.73/m
26	148.62	1H	8.36–8.38/m	148.98	1H	8.41–8.43/m
27	39.66	2H	4.18–4.27/t, 7.5	40.43	2H	4.39–4.44/t, 7.5
28	32.92	2H	2.64–2.69/t, 7.5	33.31	2H	2.77–2.82/t, 7.5
29	170.88	–		171.59	–	–
30	59.90	2H	4.18–4.27/q, 7.0	60.50	2H	4.03–4.10/q,7.0
31	13.86	3H	1.08–1.13/t, 7.5	14.12	3H	1.18–1.23/t,7.5
32	30.69	3H	3.77/s	31.40	3H	3.75/s
33	–	3H	2.28/s	–	–	–
NH, NH_2	–	3H	10.05, 10.65, 11.80/br		NH	8.03, 5.58/br
OH	–	1H	6.84–6.91/m			

Refer to Fig. 7 for numbering. s, singlet; d, doublet; t, triplet; m, multiplet; br-broad.

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Table 4 Summary of forced degradation studies.

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	Imp-7	Imp-8	Imp-9	Imp-10	Imp-11	Imp-12	Imp-13	Imp-14	Imp-15	% SMU[a]	% Total unknown impurity	% Degradation	% Assay	[b]Mass balance
% Impurity formed																				
Acid	ND	ND	ND	ND	ND	ND	ND	8.16	ND	ND	ND	ND	ND	ND		0.09	0.12	8.37	90.4	98.7
Base	ND	ND	ND	ND	ND	ND	0.06	6.51	ND	ND	ND	ND	ND	ND	0.25	0.03	0.03	6.82	92.6	99.4
Peroxide	0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08	0.11	0.19	98.5	98.3
Water	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	9.29	0.06	0.08	9.43	90.1	99.5
Humidity	ND	ND	ND	ND	ND	ND	ND	0.06	ND	ND	ND	ND	ND	ND	0.5	0.02	0.13	0.71	99.1	99.3

Imp: Impurity; ND, not detected.
[a] SMU: single maximum unknown impurity.
[b] Mass balance: (%assay + % sum of all compounds + % sum of all degradants).

was purified using Millipore Milli-Q plus purification system. DAB and process related substances were gift samples from a local manufacturing company in Chennai, India.

2.2. Apparatus and conditions

HPLC: The system used for method development, forced degradation and validation study was Agilent HPLC 1200 series (Agilent Technologies, Deutschland GmbH, Waldbronn, Germany) with Ezchrom elite software equipped with G1322A degasser, G1311A quaternary pump, G1315C auto injector, G16A column oven and G1315C DAD detector.

The HPLC columns used in this study were as follows:

Inertsil ODS 3V, 250 mm × 4.6 mm, 5 μm (GL Sciences, Japan).
Inertsil C8, 250 mm × 4.6 mm, 5 μm (GL Sciences, Japan).
ACE C18, 250 mm × 4.6 mm, 5 μm (Advanced Chromatography, Scotland).
Zorbax XDB-C18, 250 mm × 4.6 mm, 5 μm (Agilent technologies Inc., USA).
Zorbax XDB-C8, 250 mm × 4.6 mm, 5 μm (Agilent technologies Inc., USA).

2.3. Chromatographic conditions

The stationary phase used was Inertsil ODS-3V, 250 mm × 4.6 mm, 5 μm particles (GL Sciences Inc., Japan). The mobile phase-A contains 20 mM of ammonium formate with 0.1% of triethylamine (pH: 5.0 adjusted with formic acid) and mobile phase-B contains acetonitrile. The flow rate was 1.0 mL min^{-1}. The developed gradient program was 0.01 min – 30% B, 5.0 min – 30% B, 15.0 min – 35% B, 30.0 min – 45% B, 45.0 min – 55% B, 54.0 min – 65.0% B, 55.0 min – 30.0% B and 60.0 min – 30.0% B. The detection was performed at 255 nm using a DAD detector. The injection volume was 10 μL. The column temperature was 30 °C.

2.4. LC-MS conditions

The LC-ESI/MS studies were carried out on Q-TOF (Agilent, USA) 4100 Quadrupole mass spectrometer, ion source voltage 5500 V, de clustering potential 40 V, entrance potential 10 V, with the nebulizer gas as nitrogen at 40 psi. The LC part consisted of Agilent 1260 series HPLC system with binary gradient pump with a degasser and an autosampler. Inertsil ODS-3V, 250 mm × 4.6 mm, 5 μm particle size was used for chromatographic separation. Mobile phase-A as 20 mM ammonium formate (pH 5.0 with formic acid) and mobile phase-B as acetonitrile was used. The flow rate was maintained at 1.0 mL min^{-1} and the injection volume is 10 μL. The gradient program was 0.01 min – 30% B, 5.0 min – 30% B, 15.0 min – 35% B, 30.0 min – 45% B, 45.0 min – 55% B, 54.0 min – 65.0% B, 55.0 min – 30.0% B and 60.0 min – 30.0% B. The column temperature was 30 °C.

2.5. Preparative HPLC

A Shimadzu LC-8A Preparative LC equipped with SPD-10 AVP, UV–VIS detector (Shimadzu Corporation, Analytical

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Instruments Division, Kyoto, Japan) was used. ACE C18 (250 mm × 21.2 mm, particle size 10 μm) columns were employed for separation and isolation of impurity using ammonium formate (20 mM) as mobile phase-A and ACN as mobile phase-B with varying compositions and flow rate of 15–20 mL min^{-1} and detection was carried out at 255 nm.

2.6. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were acquired with AVANCE (Bruker, Switzerland) 300 and 400 MHz spectrometers at room temperature, 22 °C using DMSO-d6 as solvent and tetramethylsilane (TMS) as internal standard. The data acquisition and processing was carried out by XWIN-NMR software.

2.7. FT-IR spectroscopy

The FT-IR spectra were recorded as KBr pellets using PerkinElmer Spectrum-2 FT-IR Spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA).

2.8. Differential scanning calorimeter (DSC)

The DSC Q200 (TA Instruments, Waters LLC) was used for melting point determination.

2.9. Preparation of standard solutions

Working solutions containing 1000 μg mL^{-1} DAB and 100 μg mL^{-1} process related substance stocks were prepared for the determination of related substances. Mixed and individual stock solutions (100 μg mL^{-1}) of the impurities (denoted as impurity-1 to impurity-15) were diluted to get a concentration about 1.5 μg mL^{-1} in diluent, which is in the ratio of 70:30 (mobile phase-A:acetonitrile). The specification limit considered for validation studies was 0.15% for each impurity. Pradaxa (150 mg) capsule powder solution equivalent to 1000 μg mL^{-1} of the drug was prepared by dissolving in the diluent and was filtered through a 0.45 μm nylon membrane filter. The resulting solution was analyzed by HPLC for the estimation of related substances.

3. Results and discussions

3.1. Synthetic process and identification of impurities

Method development was initiated by using crude samples at various stages of synthetic process. A brief synthetic scheme along with origination of by-products during the synthesis is detailed in Fig. 1, where condensation of key starting materials compound_1 and compound_2 gives a salt form of compound_3, which is subsequently reacted to ethanolic hydrochloric acid, ethanol and base to give the salt of ethyl 3-{[(2-{[(4-carbamimidoylphenyl)amino]methyl}-1-methyl-1H-benzimidazol-5-yl)carbonyl](pyridin-2-yl)amino}propanoate (compound_4). The reaction between compound_4 and n-hexyl chloroformate, in the presence of potassium carbonate and solvent gives dabigatran etexilate, finally treating dabigatran etexilate with methane sulfonic acid in the presence of solvent to give dabigatran etexilate mesylate salt. The mesylate salt (1:1) of dabigatran etexilate is known to be therapeutically useful and is commercially marketed.

As a common practice, efforts should be made to identify and characterize all unknown impurities in the drug substance due to the ever increasing demand from regulatory agencies to manufacture high purity drug substances. Impurity profiling of drugs is the most important issue in modern pharmaceutical analysis (Rao and Nagaraju, 2003; Görög, 2000; Ahuja, 1998) for developing process technology to manufacture high purity drug substance. Hence, the method should be capable of separating all potential related impurities, including starting materials, by-products and degradants.

3.2. Optimization of chromatographic conditions

Drug impurity profiling, i.e., identification, structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations is one of the most important fields of activities in modern pharmaceutical analysis (Bartos and Gorog, 2008). The main objective of this chromatographic method is

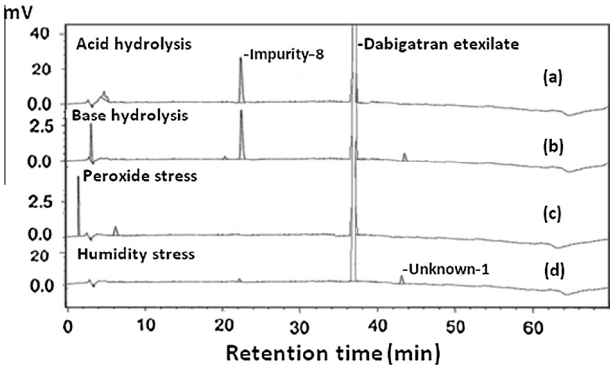


Figure 3 Typical chromatograms of forced degradation of dabigatran etexilate at control condition (a) acid hydrolysis, (b) base hydrolysis, (c) peroxide stress, and (d) humidity stress.

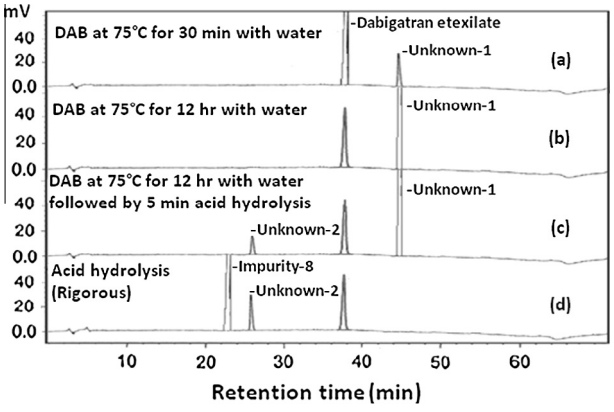
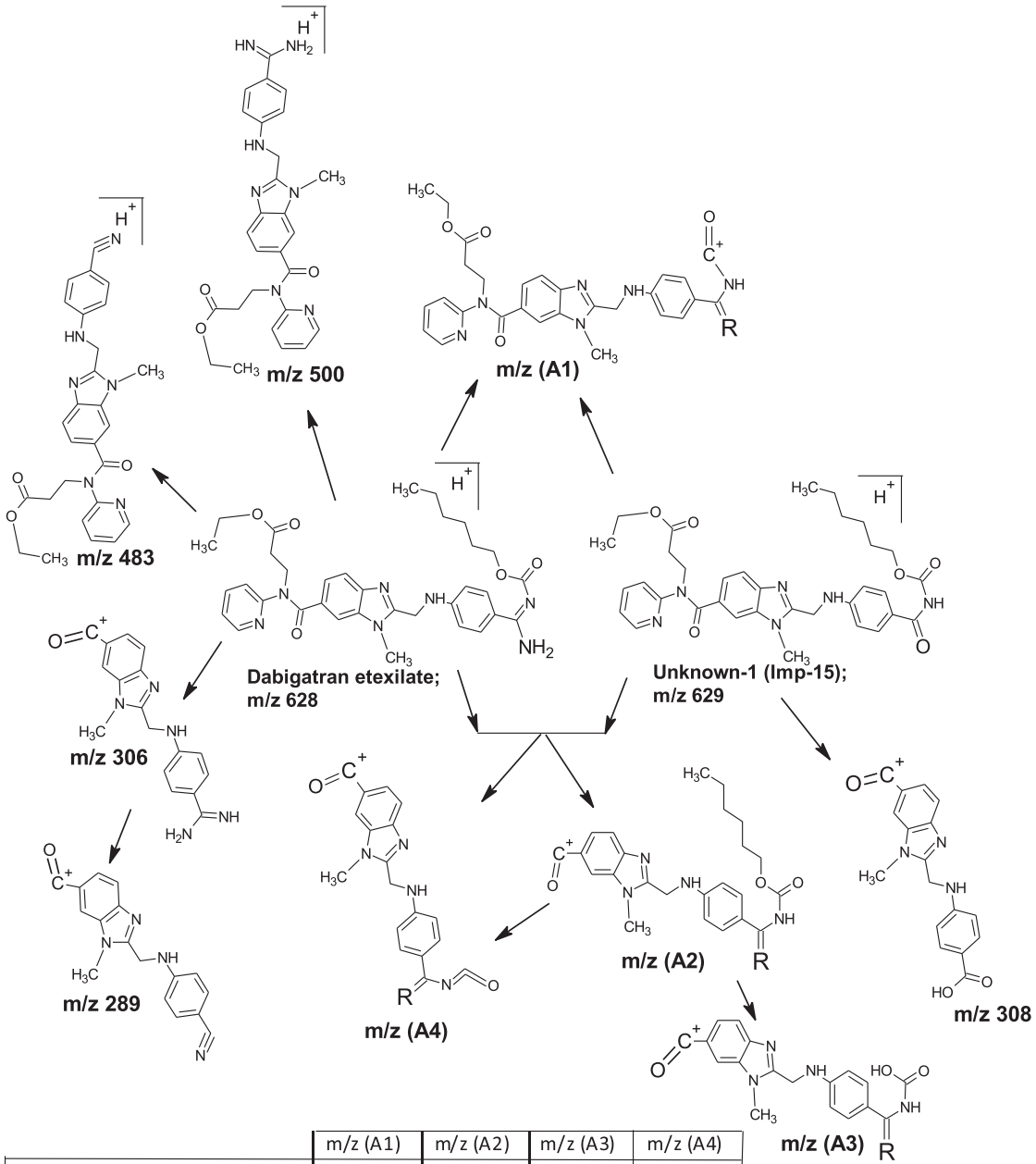


Figure 4 Typical chromatograms of (a) water stress of dabigatran etexilate, (b) rigorous water stress of dabigatran etexilate, (c) rigorous water hydrolysis followed by acid hydrolysis for 5 min of dabigatran etexilate, and (d) rigorous acid hydrolysis of dabigatran etexilate.

to get the separation of DAB from all the process related impurities, by-products and degradants. The knowledge of drug properties such as log *P* and pK_a was important, as most of the pH-related changes in retention occur at pH values within 1.5 units of the pK_a value and log *P* gives an indication about hydrophobicity or polarity nature of the molecule. The selectivity of a separation also depends upon the chemistry of the analyte i.e. the impurity-4 was depyridynyl form of impurity-3 and impurity-11 and 12 were chain isomers of hexyl group present on impurity-10 and impurity-13 and impurity-14 were differed by one methyl group. The main purpose of the method development was to optimize selectivity of these critical closely eluting pair of compounds by altering mobile and stationary phases.

The blend containing 1000 μg mL^{-1} of DAB and 1.5 μg mL^{-1} of each impurity was prepared and used for method optimization. To start the method optimization the effect of pH (from 4.0 to 6.0) of ammonium formate buffer was studied to understand the elution and separation of impurities. To improve peak shapes 0.1%TEA was added as an additive to the buffer. Preliminary experiments on method development were carried out on Inertsil ODS 3V column using pH 5.5–20 mM ammonium formate with 0.1% of triethylamine:ACN (60:40) (v/v) in isocratic mode. Six impurities were merged and the same kind of separation was observed by changing organic modifier as methanol. It was found that gradient elution resulted better separation than isocratic mode of elution on this column with acetonitrile as organic modifier but



	m/z (A1)	m/z (A2)	m/z (A3)	m/z (A4)
For 'DAB' m/z 628 **R = NH**	m/z 526	m/z 434	m/z 350	m/z 332
For 'Unknown-1' m/z 629 **R = O**	m/z 527	m/z 435	m/z 351	m/z 333

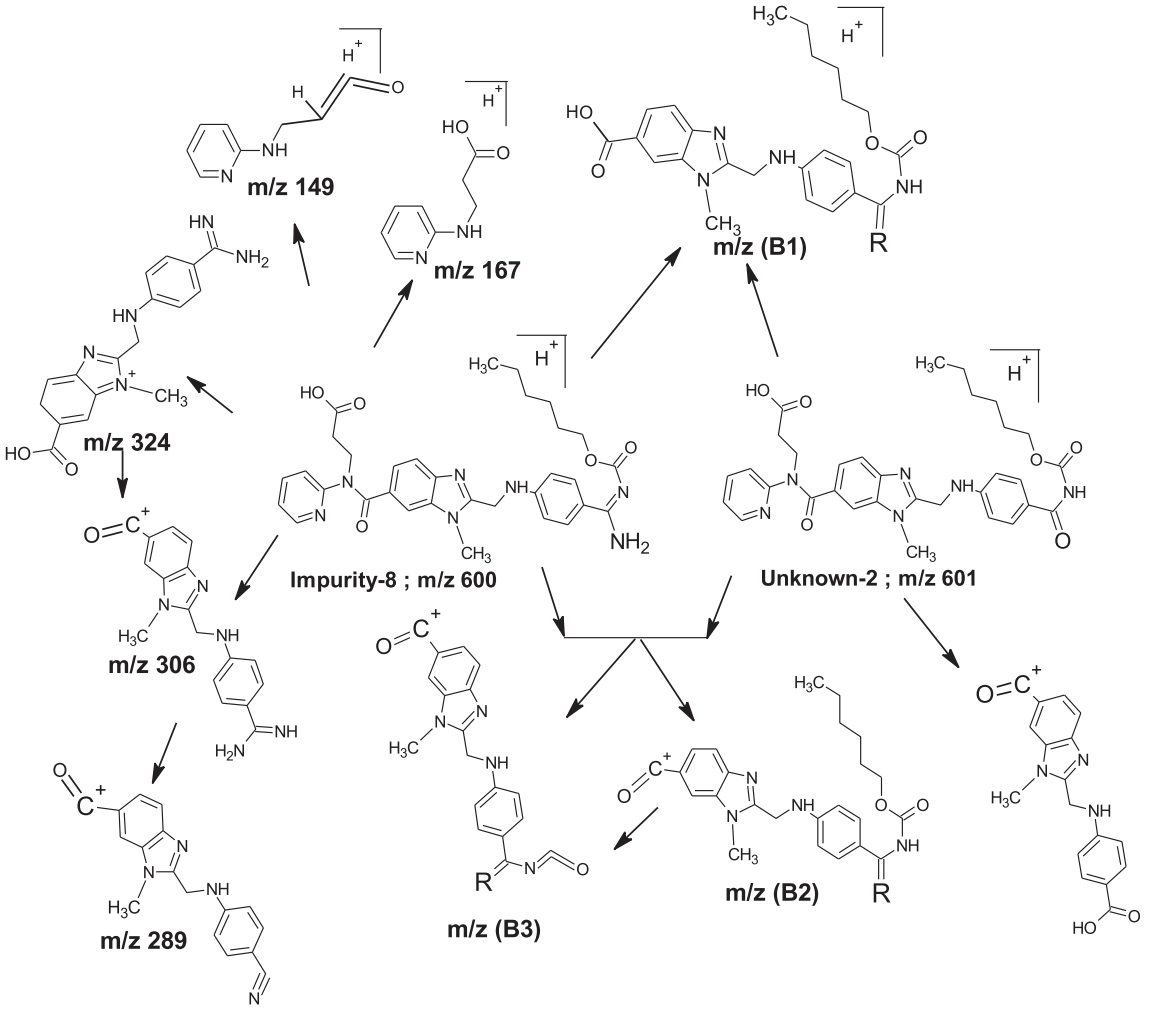
Figure 5 ESI-MS/MS fragmentation patterns for dabigatran etexilate and unknown-1 impurity.

only the critical pair's viz. impurity-11 and impurity-12, impurity-13 and impurity-14 separation was not observed. Attempts were made with gradient elution to separate the critical pairs changing different column chemistries but no selectivity was observed. The best results were obtained using the chromatographic conditions given in Section 2.3 and the typical chromatogram is shown in Fig. 2(a). The method development trials are shown in Table 2.

The affinities of all impurities toward different commercially available columns were also evaluated to get the best resolution between critical pair of impurities. Inertsil ODS 3V column at pH 5.0 was selected to get the good resolution between the critical pairs, which was due to its high surface area, more hydrophobicity, and high reproducibility compared with other commercially available columns. In this optimized method all the peaks were symmetrical and show the tailing factor $\leqslant 1.3$ and the resolution $\geqslant 2.0$ from blend chromatogram of system suitability. System suitability parameters are described in Table 1.

3.3. Analysis of bulk drugs stability samples

During the analysis of active pharma ingredient (API) stability sample, three impurities were observed, of which one was imp-8 and other two were unknown. Unknown were eluted around retention time 43.3 min (unknown-1) and 24.4 min (unknown-2) (Fig. 2(b)). However, they did not match with any of the process related substances by comparing retention times. The compounds imp-8, unknown-1(imp-15) and unknown-2 were present in 0.19%, 0.26% and 0.09% respectively and remaining impurities were not detected. Attempts were made to evaluate these unknown impurities in stability sample using LC-MS/MS analysis using the conditions given in Section 2.5. The obtained fragmentation pattern for unknown-1 and unknown-2 was comparable and structures were derived with the help of known DAB and impurity-8 mass spectrums interpretation. Two unknown impurities comparison with DAB and impurity-8 detailed mass fragmentation shown in Figs. 5 and 6.



	m/z (B1)	m/z (B2)	m/z (B3)
For 'Impurity-8' m/z 600 R = NH	m/z 452	m/z 434	m/z 332
For 'Unknown-2' m/z 601 R = O	m/z 453	m/z 435	m/z 333

Figure 6 ESI-MS/MS fragmentation patterns for Impurity-8 and unknown-2 impurity.

3.4. Degradation studies

Degradation study gives information about origin of degradant impurities and also useful to check the stability-indicating power of developed method. The forced degradation condition was attempted to get degradation not less than 10% (in order to get a mass balance between purity and assay). Solution state forced degradation of DAB was carried out by treating the 1.0 mg/ml solutions with 0.1 M HCl for 5 min at 75 °C, 0.25 M NaOH for 30 min at 75 °C and 3% H_2O_2 for 1 h at room temperature. Solid state forced degradation (thermal, photolytic and humidity) of the drug was conducted by exposing DAB to (1) heat at 105 °C for 120 h, (2) white fluorescent light of 1.2×10^6 lux followed by UV light of 200 W h/m^2 and (3) 90% R.H. at 25 °C for 120 h. All these solutions are analyzed by the developed method conditions mentioned in Sections 2.3 and 2.4. One degradant which was observed in bulk drug stability sample i.e. unknown-1 was detected at 0.6% level in humidity condition and no forced degradation was observed in heat and exposure with light. Peak purity test was carried out on the stressed samples of DAB by using DAD detector. Assay studies were carried out for stress samples against qualified reference standard and the mass balance (% assay + % degradation) is calculated (Table 4). Stress chromatograms are illustrated in Fig. 3(a)–(d).

3.5. Enrichment of unknown-1 and unknown-2 impurities

Degradation in controlled conditions (i.e. ⩽10%) gives a conclusion that unknown-1 was increasing in humidity stress. Based on mass spectral data and degradation studies attempts were made to enrich the impurity using forced degradation with water. The unknown-1 impurity increased about 9% in water stress at 75 °C for 30 min and about 88% when extending the stress time to 12 h (Fig. 4(a) and (b)). Intentional addition of 0.1 N HCl to 88% degraded water stress sample and holding for 5 min give a rise of unknown-2 impurity

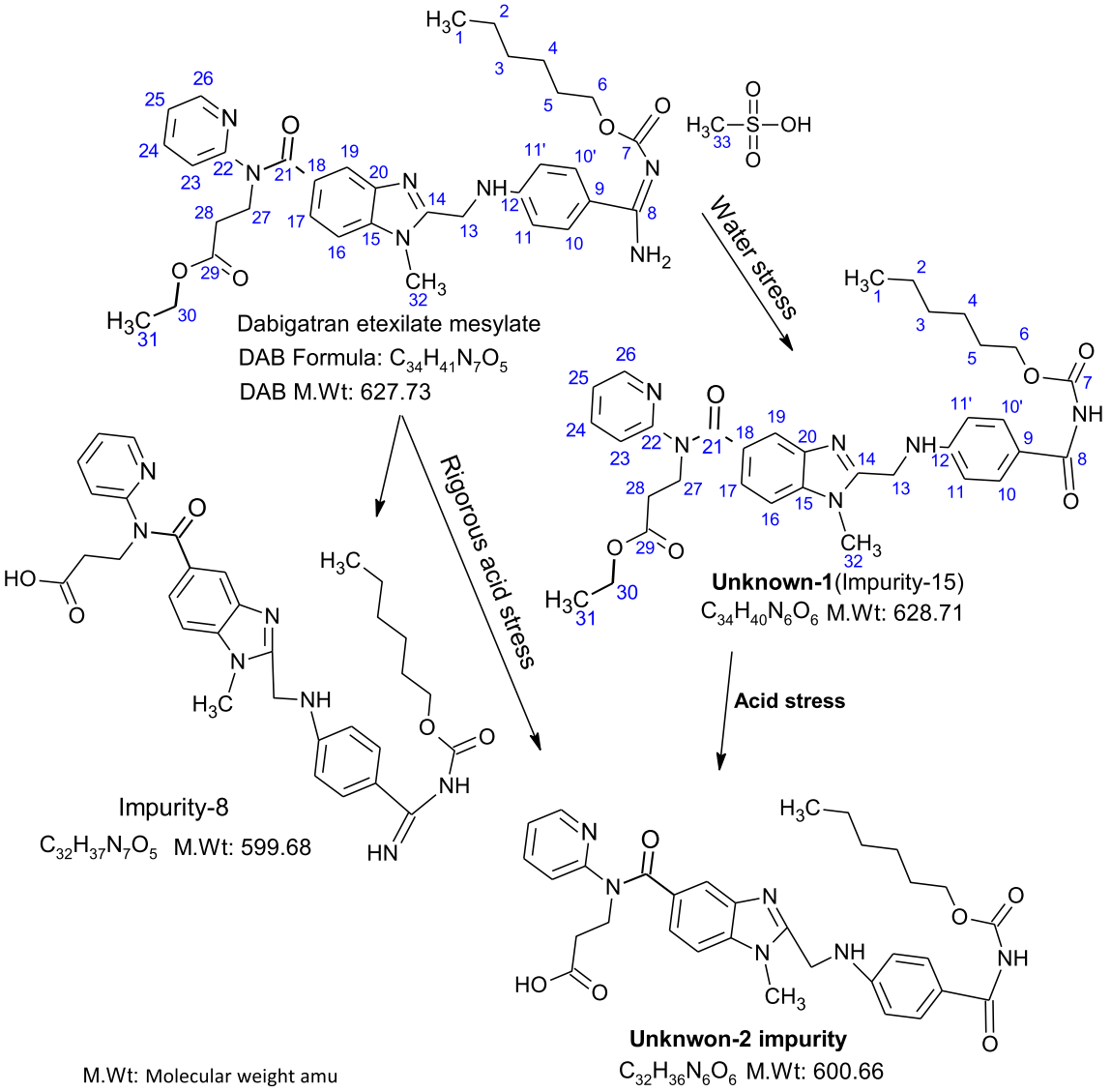


Figure 7 Proposed degradation pathway for unknown-1 and unknown-2 impurity.

(Fig. 4(c)). The unknown-2 impurity formation was also observed in intentional rigorous stress of DAB using 1 M HCl (Fig 4(d)). The stress solutions were injected into LC-MS/MS analysis to generate unknown impurities (unknown-1 and 2) mass spectral data and these were matching with unknown impurity mass spectrums of bulk drug stability sample.

3.6. Isolation of unknown-1 impurity using preparative LC

In order to isolate the unknown-1 impurity from water stress of DAB, the isolation conditions for preparative LC used were as mentioned in Section 2.5. Approximately 0.2 g of sample was injected on to column every time and this loading was continued until the sufficient amount of unknown-1 was isolated (~20 injections). The fractions containing >95% of unknown-1 were collected, concentrated using rotary evaporator at 30 °C under vacuum and finally lyophilized to obtain solid product.

3.7. Degradation pathway for unknown-1 and unknown-2

During the experiment-enrichment of unknown impurities, it was observed that intentional addition of acid to water stress of dabigatran etexilate gives rise of unknown-2 impurity (Fig. 4(c)). Based on stress study and mass spectrum fragmentation data the degradation pathway for unknown-1 and unknown-2 is proposed as shown in Fig. 7.

3.8. Structural elucidation of unknown-1

In the positive mode ESI-MS analysis of the unknown-1 exhibited a protonated molecular ion peak *m/z* 629. The difference of mass between impurity and DAB is 1 amu, indicating deletion and addition on DAB. During degradation studies this was major impurity observed in water hydrolysis of DAB. The imine group (—C═NH) on DAB hydrolysis to carbonyl (—C═O) leads to increase of 1 amu mass for impurity. This was evidenced by major product ions observed from ESI-MS/MS of DAB *m/z* 526,500, 434, 350, 332 and 306 and of unknown-1(impurity-15) were at *m/z* 527,435, 351,333 and 308 (Fig. 5). The ^{1}H, ^{13}C NMR and FT-IR spectral data of DAB and unknown-1 impurity are given in Table 3 and Fig. 8 respectively. ^{1}H NMR spectral data of unknown-1 showed chemical shift similarity with DAB in all positions except the signals corresponding to —NH_2 in DAB was absent in impurity. From ^{13}C spectral data the change in chemical shift values at eighth position of carbon gives an evidence of hydrolysis of imine group (—C═NH) on DAB to a carbonyl (—C═O). From this spectral information the structure was confirmed as ethyl 3-{[(2-{[(4-{[({hexyloxy}carbonyl)amino]carbonyl}phenyl)amino]methyl}-1-methyl-1H-benzimidazol-5-yl) carbonyl](pyridin-2-yl)amino}propanoate. Determined the melting point by DSC for unknown-1 and DAB, the results are 185 °C and 178 °C respectively.

3.9. Structural elucidation of unknown-2

The impurity unknown-2 exhibited a protonated molecular ion peak at *m/z* 601 in positive ion ESI-MS analysis; the difference of mass between DAB and impurity was 27 amu and difference

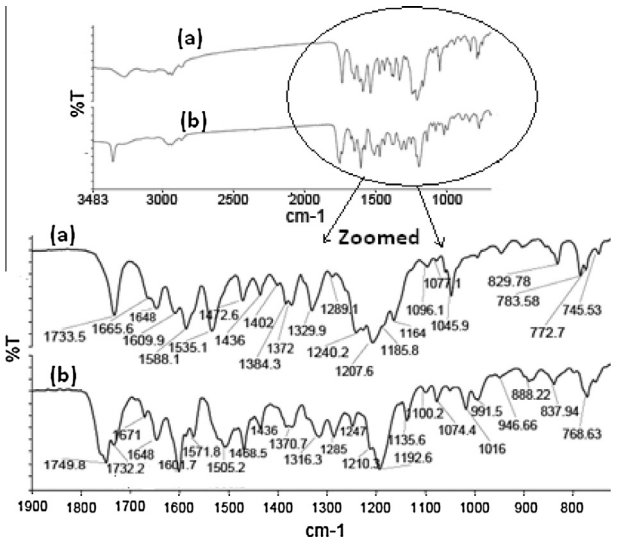


Figure 8 FT-IR spectrums of (a) dabigatran etexilate and (b) unknown-1 (Impurity-15).

of mass between unknown-1 and this impurity was 28 amu, indicating absence of —CH_2CH_3 i.e. ester hydrolysis of corresponding unknown-1 (Fig. 7). Hydrolysis (ester hydrolysis) of DAB (*m/z* 628) mainly results in the formation of a compound impurity-8 (*m/z* 600). Similarly the hydrolysis of unknown-1 (*m/z* 629) results in a formation of compound unknown-2 having *m/z* 601. This was evidenced by comparing ESI-MS/MS major product ions of impurity-8 *m/z* 452, 434 and 332 and of unknown-2 were at *m/z* 453, 435 and 333 (Fig. 6). From this mass spectrum fragmentation pattern comparison, the Unknown-2 structure was predicted as 3-{[(2-{[(4-{[({hexyloxy}carbonyl)amino]carbonyl}phenyl)amino]methyl}-1-methyl-1H-benzimidazo1-5-yl) carbonyl] (pyridin-2-yl)amino} propanoic acid.

3.10. Method validation

3.10.1. Precision

The repeatability of the method was examined by sixfold analysis of 1000 μg mL^{-1} of DAB by spiking with impurities −1 to 15 at the level of 0.15% i.e. 1.5 μg mL^{-1}. The %RSD for the area of impurities was well within 2.6%. Interday precision was evaluated using different analyst and a different instrument in the same laboratory, and the %RSD for the area of impurity-1 to impurity-15 was well within 2.4%, confirming good precision of the method. The %RSD values are presented in Table 5.

3.10.2. Accuracy

Accuracy expresses the closeness of the agreement between the true value and the value obtained. Accuracy of the method was evaluated ranging from LOQ to 150% of target impurity concentration 0.15 μg mL^{-1} (in triplicate) in bulk drug sample and in dosage form. The percentage recovery of impurities was varied from 95.8% to 99.2%. The LC chromatogram of spiked sample at 0.15% level of all fifteen impurities in DAB sample is shown in Fig. 2(a). The % recovery values of impurities are presented in Table 6.

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Table 5 Validation summary for DAB and its impurities.

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	Imp-7	Imp-8	Imp-9	Imp-10	Imp-11	Imp-12	DAB	Imp-13	Imp-14	Imp-15
Regression equation																
Slope (b)	34,402	8239	29,611	16,697	32,897	38,660	8359	26,135	39,782	23,560	19,895	26,256	24,715	20,297	23,886	37,727
Intercept (a)	1067	−322	58	−147	30	−83	148	−197	−195	−625	287	−393	−82	−169	89	−490
R value	0.9996	0.9996	0.9999	0.9998	0.9999	0.9999	0.9998	0.9999	0.9997	0.9998	0.9999	0.9999	0.9999	0.9998	0.9997	0.9999
R^2 value	0.9993	0.9992	0.9999	0.9997	0.9999	0.9999	0.9996	0.9999	0.9995	0.9996	0.9999	0.9998	0.9997	0.9996	0.9993	0.9998
LOD (μg/mL)	0.11	0.122	0.047	0.072	0.039	0.047	0.084	0.033	0.094	0.079	0.043	0.056	0.07	0.078	0.107	0.054
LOQ (μg/mL)	0.334	0.37	0.143	0.218	0.119	0.143	0.253	0.1	0.283	0.24	0.131	0.169	0.211	0.237	0.324	0.164
Precision at LOQ $n = 6$ (%RSD)	5.1	6.6	3.4	7.2	4.2	5.3	7.9	3.9	3.2	4.4	8.3	4.6	4.4	3.4	4.2	4.1
Precision $n = 6$ (%RSD)	1.12	1.92	0.65	2.6	0.92	0.53	2.2	1.21	1.36	0.52	2.1	0.63	0.75	0.81	1.21	1.16
Intermediate $n = 6$ precision (%RSD)	0.63	1.6	1.03	1.8	1.24	0.63	2.4	0.65	0.89	0.65	2.6	0.76	0.52	0.93	0.96	0.78

Table 6 Evaluation of accuracy.

Amount spiked[a]	%Recovery[b]															
	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	Imp-7	Imp-8	Imp-9	Imp-10	Imp-11	Imp-12	DAB	Imp-13	Imp-14	Imp-15
LOQ	95.8 ± 2.8	94.5 ± 4.4	95.6 ± 3.2	96.8 ± 4.2	97.3 ± 2.2	96.4 ± 2.6	98.2 ± 4.6	97.4 ± 2.1	94.5 ± 2.5	96.5 ± 1.2	95.8 ± 3.4	97.8 ± 2.1	96.3 ± 2.4	97.4 ± 2.5	98.2 ± 2.8	96.4 ± 2.1
50%	97.3 ± 1.2	98.2 ± 0.6	97.2 ± 0.8	98.3 ± 1.1	96.8 ± 1.8	98.6 ± 1.4	97.2 ± 2.1	96.4 ± 1.1	97.1 ± 1.3	96.5 ± 0.6	98.2 ± 0.9	97.1 ± 0.6	98.2 ± 0.7	97.3 ± 1.1	97.4 ± 0.9	97.8 ± 1.1
100%	98.1 ± 0.6	96.4 ± 1.2	98.3 ± 0.9	95.2 ± 1.2	97.2 ± 0.9	94.5 ± 1.5	96.3 ± 1.1	98.2 ± 0.8	97.9 ± 0.6	96.2 ± 1.0	95.8 ± 0.6	98.2 ± 0.4	97.3 ± 0.7	98.4 ± 0.2	99.1 ± 0.3	98.9 ± 0.6
150%	97.8 ± 0.9	98.2 ± 0.8	98.4 ± 1.1	97.5 ± 0.6	98.1 ± 0.4	98.9 ± 1.1	97.9 ± 1.6	99.2 ± 0.4	98.6 ± 1.1	99.1 ± 0.9	97.1 ± 0.8	98.5 ± 1.1	98.6 ± 0.9	95.4 ± 1.1	97.3 ± 0.3	99.0 ± 0.8

[a] Amount of fifteen impurities spiked with respect to 0.15% specification level individually to 1.0 mg/ml of dabigatran etexilate.
[b] Mean %RSD for three determinations.

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3.10.3. LOD and LOQ

The LOD and LOQ were determined for DAB and each of the impurities based on the standard deviation (SD) of the response and slope (S) of the regression line as per ICH guideline according to the formulas given below:

$$LOD = 3.3 \times SD/S,\ LOQ = 10 \times SD/S$$

The residual sum of squares obtained from linearity regression data was used as SD.

The concentrations of LODs and LOQs for DAB and its impurities were found to be in the range of 0.033–0.122 μg mL^{-1} and 0.1–0.370 μg mL^{-1} respectively. The concentrations of LOQs were verified for precision by the analysis of solutions having DAB and its impurities at these levels in six replicates and are found below 10% RSD (Table 5).

3.10.4. Linearity

Linearity solutions were prepared from impurity stock solution at six different concentration levels ranging from LOQ to 150% of target concentration i.e. 2.25 μg mL^{-1}. The calibration curve was drawn by plotting impurity area versus the concentration .The correlation coefficient obtained was greater than 0.999 for all the impurities (Table 5).

3.10.5. Solution stability and mobile phase stability

The variability in the estimation of DAB impurities was within ±10% during solution stability and mobile phase experiments when performed using the method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions, sample solutions and the mobile phase were stable up to 48 h for related substances analysis.

3.10.6. Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature and composition of organic solvent), all analytes were adequately resolved and elution order remains unchanged. The effect of pH on resolution of impurities was also studied by varying ±0.2 pH units (at 4.8 and 5.2 buffer pH instead of 5.0). The resolution between critical pairs, i.e. for impurity-10, impurity-11 and impurity-12 and for impurity-13 and impurity-14 was greater than 2.0 and tailing factor for DAB and its impurities was less than 1.2. The variability in the estimation of impurities was within ±10%. There is no significant change observed by deliberate changes, which confirms the robustness of the method.

4. Conclusion

A gradient RP-HPLC method was developed and validated for quantitative determination of impurities of dabigatran etexilate drug substance. The method has higher sensitivity toward the determination of impurities and is found to be specific, sensitive, precise, linear, accurate and robust. Two unknown impurities were identified by LC-MS/MS. The isolated unknown-1 impurity was characterized using ^{1}H, ^{13}C, FT-IR and mass spectrometry. The unknown-2 was found as secondary degradant; hence, attempts were not made to isolate this impurity but its degradation pathway and structure were proposed based on mass spectral data. The developed method is stability-indicating and can be used for assessing the stability of bulk samples, dosage forms and also for monitoring the synthetic procedures of dabigatran etexilate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2015.09.006.

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