Journal of Pharmaceutical and Biomedical Analysis 57 (2012) 39–51

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Identification, characterization and quantification of new impurities by LC–ESI/MS/MS and LC–UV methods in rivastigmine tartrate active pharmaceutical ingredient

Saji Thomas [a,*], Sanjeev Shandilya [a], Amber Bharati [a], Saroj Kumar Paul [a], Ashutosh Agarwal [a], Chandra S. Mathela [b]

[a] Jubilant Life Sciences Ltd., Analytical Research Department, R&D Centre, C-26, Sector-59, Noida, Uttar Pradesh 201 301, India
[b] Department of Chemistry, Kumaun University, D.S.B. Campus, Nainital, Uttarakhand 263 001, India

ARTICLE INFO

Article history:
Received 30 June 2011
Received in revised form 31 July 2011
Accepted 4 August 2011
Available online 11 August 2011

Keywords:
Rivastigmine
Impurity
LC/MS/MS
Characterization
Validation

ABSTRACT

Six impurities were detected at trace level in rivastigmine tartrate drug substance by a newly developed high performance liquid chromatography method. Three impurities were characterized rapidly and three impurities were found to be unknown. The unknown impurities were enriched and identified with a combination of semi-preparative HPLC and LC/MS/MS techniques. Proposed structures were further confirmed by characterization using NMR, FT-IR, and EA techniques of impurity standards. Based on the spectroscopic, spectrometric and elemental analysis data unknown impurities were characterized as 3-[1-(dimethylamino)ethyl]phenyl *N*-ethyl-*N*-methyl carbamate *N*-oxide, ethyl-methyl-carbamic acid 4-(1-dimethylamino-ethyl)-phenyl ester and ethyl-methyl-carbamic acid 2-(1-dimethylamino-ethyl)-phenyl ester. A plausible mechanism for the formation of these impurities is also proposed. The method was validated according to ICH guidelines for fourteen impurities to demonstrate specificity, precision, linearity, accuracy and stability indicating nature of the method. Regression analysis showed correlation coefficient value greater than 0.999 for rivastigmine tartrate and its impurities. Accuracy of the method was established based on the recovery obtained between 93.41 and 113.33% for all impurities.

1. Introduction

Rivastigmine tartrate (Fig. 1a) is chemically (*S*)-3-[(1-dimethylamino)ethyl]phenyl *N*-ethyl-*N*-methyl carbamate hydrogen tartrate, which was synthesized as a single enantiomer because (*S*)-enantiomer is pharmacologically more potent than (*R*)-enantiomer [1]. It is a carbamate type reversible acetyl cholinesterase inhibitor used for symptomatic treatment of mild to moderate dementia in Alzheimer's disease and idiopathic Parkinson's disease. Alzheimer's disease is a progressive, irreversible brain disorder with no known cause or cure. It attacks the brain and slowly leads to memory loss, confusion, impaired judgment, personality changes, disorientation and loss of language skills. It is estimated that there are currently about 35 million people worldwide affected by Alzheimer's disease and is expected to triple by 2025 [2,3].

Several methods that have been reported in the literature for rivastigmine tartrate, some of which include a stability indicating LC method [4], voltammetric behavior [5], a validated chiral LC method for the enantiomeric separation [6], identification and characterization of new impurities [7], stability indicating HPTLC determination in the bulk drug and in pharmaceutical dosage forms [8] and few LC–MS methods for the analysis of rivastigmine tartrate in biological samples [9,10]. Recently HPLC methods for the determination of related substances have also been reported in US pharmacopoeia and pharmaeuropa [11,12].

ICH guidelines indicate that unknown impurities at or above 0.05% in the drug substance require identification [13] depending on the maximum daily dosage. Presence of impurities in drug substance can have significant impact on the quality, safety and efficacy of drug products. 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 almost the most important issue in the modern pharmaceutical analysis [14–20] for developing process technology to manufacture high purity drug substance. In recent years liquid chromatography coupled with mass spectrometry (LC/MS/MS) has emerged as an essential and versatile tool for structural elucidation of impurities [21,22].

* Corresponding author. Tel.: +91 120 4362210; fax: +91 120 2580033.
E-mail address: saji_thomas@jubl.com (S. Thomas).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.08.014

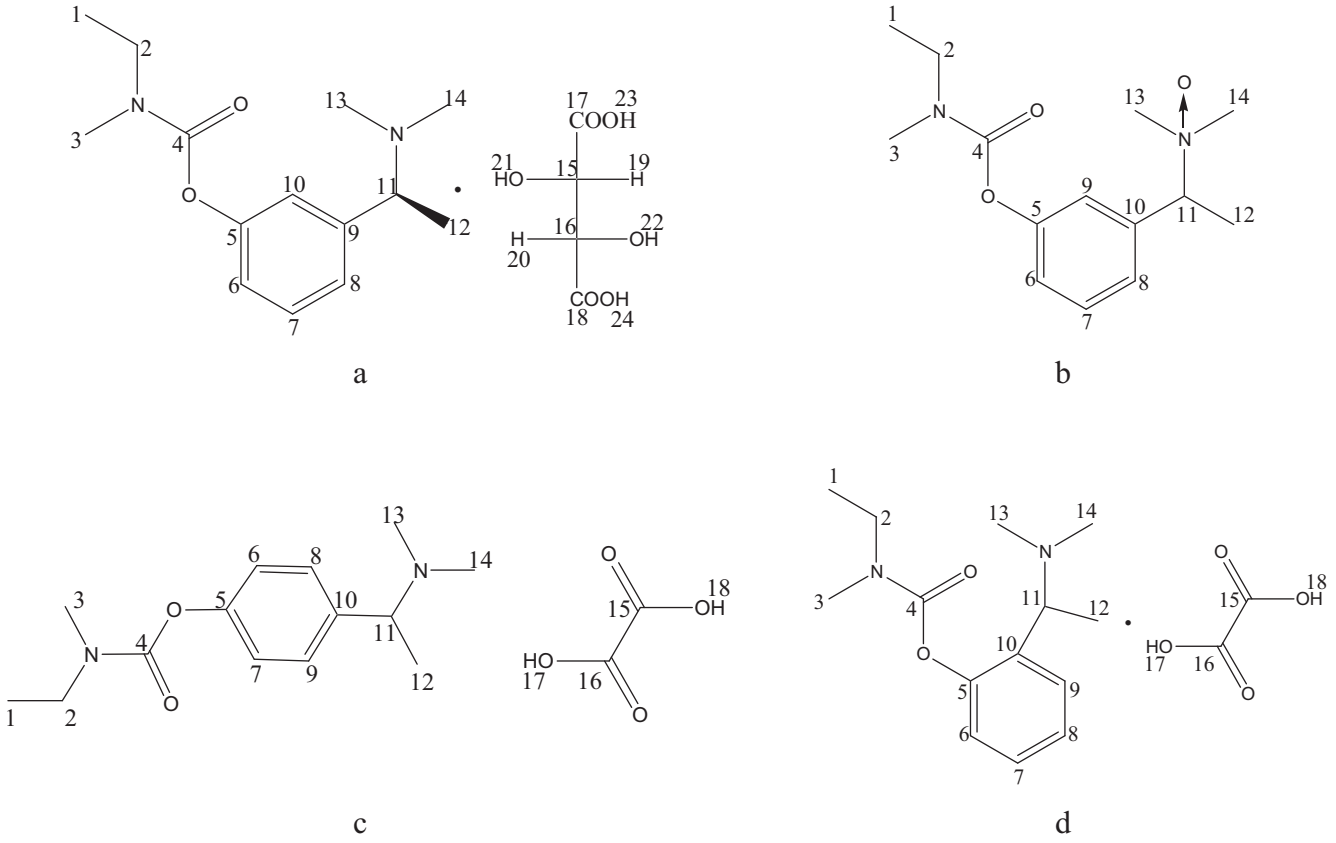


Fig. 1. (a) Rivastigmine tartrate, (b) Imp-A, (c) Imp-B, (d) Imp-C. *Numbering has been assigned only for NMR characterization.

A critical evaluation of HPLC methods [4,7,11,12] revealed that specificity and resolution of all known impurities (Table 1) were a major concern which were essential requirements for quantitative determination of impurities. Further use of very high concentration of ion pair reagent in mobile phase significantly reduces the column life resulting in expensive routine analysis. Therefore, it was strongly felt necessary to develop simple, efficient, sensitive, selective and cost effective RP-HPLC method. It should be noted that selective and sensitive analytical methods are required for quantitative analysis of impurities in drug substance.

The objective of the current study was to identify and characterize six impurities detected in low level during a HPLC investigation of rivastigmine tartrate lab sample. Three impurities were found to be reported previously [9,11] and three impurities were unknown. Impurities were enriched by semi-preparative HPLC and identified by liquid chromatography–tandem mass spectrometry using an electrospray ionization source and Q-trap mass analyser. The structure of the unknown impurities in rivastigmine tartrate was additionally confirmed by characterization using impurity reference standards with the help of NMR, IR, and EA techniques. This paper describes in detail about method development, identification and characterization of unknown impurities, degradation study and identification of degradants. Method was validated as per ICH guidelines [24]. A plausible mechanism for the formation of new impurities was proposed. To the best of our knowledge, these impurities have not been reported previously.

2. Experimental

2.1. Materials and reagents

Sample of rivastigmine tartrate API (batch No. RVS-crude) and standards of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11 were obtained from Chemical Research and Development Department, Jubilant Life Sciences Limited (Noida, India). Deionized water was prepared using a Milli-Q plus water purification system from Millipore (Bedford, MA, USA). HPLC grade methanol, acetonitrile, dipotasssium hydrogen phosphate, ammonium formate, ammonium bicarbonate, analytical reagent grade ammonia solution and ortho phosphoric acid were purchased from Merck India Limited (Mumbai, India). Dimethyl sulphoxide-d6 (for NMR) was purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). Potassium bromide FT-IR grade was purchased from Merck KGaA (Darmstadt, Germany).

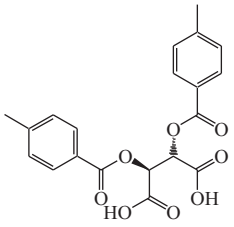
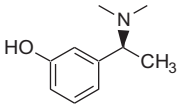
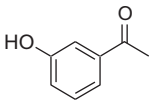
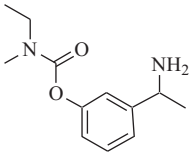
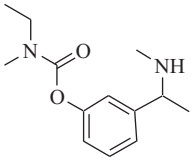
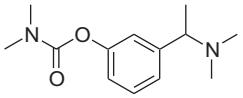
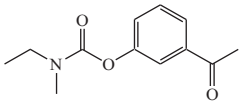
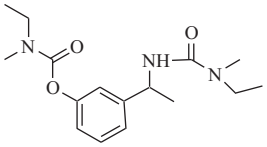
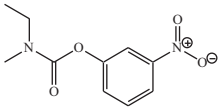
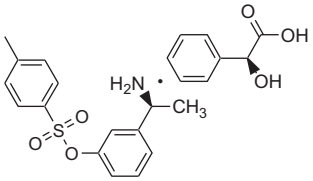
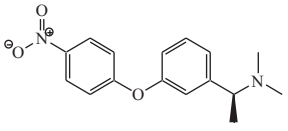
2.2. High performance liquid chromatography

Samples were analysed on a Waters alliance 2690 separation module equipped with 2487 UV detector (Waters Corporation, Milford, MA, USA) using an Xterra RP-18, (250 mm × 4.6 mm, 5 μm, Waters Corporation, Milford, MA, USA). A mobile phase consisted of A, 10 mM dipotassium hydrogen phosphate adjusted to pH 7.6 ± 0.05 with orthophosphoric acid–acetonitrile (90:10, v/v) and B, acetonitrile–methanol (60:40, v/v) with a timed gradient mode *T* (min)/%B: 0/5, 3/10, 10/20, 20/35, 55/55 60/5 70/5. A flow rate of 1.0 mL/min was used throughout the analysis. The injection volume was 10 μL for a sample concentration of 0.5 mg/mL prepared in diluent (mobile phase A-methanol, 40:60, v/v). Detector wavelength was fixed at 210 nm and the column temperature was maintained at 40 °C.

2.3. Semi-preparative HPLC

The impurities were enriched from rivastigmine tartrate lab sample, using a Shimadzu semi-preparative HPLC system consisted

Table 1
List of known impurities in rivastigmine.

S. no.	Name	IUPAC Name	Structure
1	Imp-1	(+)-Di-(*p*-toluoyl)-D-tartaric acid	
2	Imp-2	3-(1-Dimethylamino-ethyl)-phenol	
3	Imp-3	1-(3-Hydroxyphenyl)ethanone	
4	Imp-4	3-[1-Aminoethyl]phenyl *N*-ethyl-*N*-methyl carbamate	
5	Imp-5	3-[1-(Methyamino)ethyl]phenyl *N*-ethyl-*N*-methyl carbamate	
6	Imp-6	Dimethyl-carbamic acid 3-(1-dimethylamino-ethyl)-phenyl ester	
7	Imp-7	Ethyl-methyl-carbamic acid 3-acetyl-phenyl ester	
8	Imp-8	3-[1-(3-Ethyl-3-methyl-ureido)ethyl]phenyl *N*-ethyl-*N*-methyl carbamate	
9	Imp-9	3-Nitrophenyl ethyl(methyl)carbamate	
10	Imp-10	3-[(1*S*)-1-Aminoethyl]phenyl 4-methylbenzenesulphonate (*S*)-mandelate	
11	Imp-11	(*S*)-*N*,*N*-Dimethyl-1-[3-(4-nitrophenoxy)phenyl]ethanamine	

of LC-8A binary gradient pump, a SPD-10AVP UV detector, SIL-10AP auto sampler and FRC-10A fraction collector (Shimadzu Corporation, Kyoto, Japan). An Inertsil ODS-3 column (GL Sciences Inc., Tokyo, Japan) (250 mm × 2.1 cm, particle size 10 μm) was used for semi-preparative isolation. A LC isocratic method consisted of a mixture of 0.1 mM ammonium formate–acetonitrile (75:25, v/v) was used as mobile phase at a flow rate of 20 mL/min. A sample concentration of 150 mg/mL was prepared using methanol as diluent. The injection volume was 1.0 mL and the detection was monitored at 210 nm. The collected fractions were combined and concentrated to about 10 mL by evaporation under high vacuum using a buchi rotavapor (Buchi Labortechnik AG, Flawil, Switzerland).

2.4. Liquid chromatography–tandem mass spectrometry (LC/MS/MS)

The MS and MS/MS studies were performed on 3200 Q-trap mass spectrometer (AB Sciex, Foster City, CA, USA). The instrument was operated in enhanced product ion mode in positive polarity mode with the following settings: collision energy of 40 V, collision energy spread 10 V and declustering potential 10 V. Nitrogen was used as curtain gas at a pressure of 12 psi, and as collision associated dissociation (CAD) gas. Zero air was used as nebulizer gas and heater gas at pressure of 50 psi. The ion spray voltage was 5500 V. The HPLC consisted of LC-20AD binary gradient pump, a SPD-10AVP UV detector, SIL-10HTC auto sampler and a column oven CTO-10ASVP (Shimadzu Corporation, Kyoto, Japan). An Xterra RP-18 (250 mm × 4.6 mm, 5 μm) was used for chromatographic separation. A mobile phase consisting of A, 20 mM ammonium bicarbonate adjusted to pH 7.6 ± 0.05 with ammonia and B, acetonitrile–methanol (60:40, v/v) in gradient mode; *T* (min)/%B: 0/5, 3/10, 20/20, 30/35, 60/65 65/5 70/5. Column temperature was maintained at 50 °C and the flow rate was 1.0 mL/min.

2.5. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 399.957 MHz and 100.432 MHz respectively, using a Bruker AVANCE 400 MHz spectrometer (Bruker, Fallanden, Switzerland) equipped with a 5 mm BBO probe and a *z*-gradient shim system. The ^{1}H spectra were recorded with 1 s pulse repetition time using 30° flip angle, while ^{13}C spectra were recorded with power gated decoupling using 30° flip angle with repetition time of 2 s. Samples were dissolved in dimethyl sulphoxide-d6. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to DMSO-d6 (2.50 ppm). All spectra were recorded with sample spinning.

2.6. FT-IR spectroscopy

The IR spectrum was recorded in the solid state as KBr powder dispersion using Nicolet FT-IR model AVTAR 370 (Thermo Electron Scientific Instruments, Madison, WI, USA) with a DTGBS KBr detector. Data were collected between 400 and 4000 cm^{-1}, with a resolution of 4.0 cm^{-1}. A total of 16 scans were obtained and processed using the OMNIC software version 6.0.

2.7. Elemental analysis

Elemental analysis (C, H, N, and S) was carried out using an elemental analyser model Vario EL III with TCD detector (Elementar Analysensysteme GmbH, Hanau, Germany). Samples were weighed in a tin boat, to which tungsten oxide was added and neatly packed. The sample in tin boat was loaded in an auto sampler tray and was dropped into the combustion tube automatically at a temperature of 1200 °C. Complete combustion of sample was ensured with a special oxygen jet injection.

2.8. Photo stability

Photo stability studies were carried out using a photo stability chamber model TP 0000090G (Thermo Lab Equipments Pvt. Ltd, Mumbai, India). 500 mg each sample was kept in two separate LOD bottles were used for study. One bottle was covered with lid and then with aluminum foils (dark control). Another bottle (photolytic exposed sample) was covered with lid and kept into the photolytic chamber to get a minimum exposure of 1.2 million lux hours for light and 200 watt hours/square meter for ultraviolet region.

2.9. Preparation of stock solutions for method validation

A test preparation of 1500 μg/mL of rivastigmine tartrate API sample was prepared by dissolving the appropriate amount in diluent (buffer solution and methanol 80:120, v/v). A stock solution of impurities was prepared by dissolving 5 mg each of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and 5 mg of rivastigmine tartrate in 10 mL of diluent. From this solution 4.5 mL was diluted to 100 mL with diluent. A standard solution containing 2.25 μg/mL of each impurities and rivastigmine tartrate was prepared from the stock solution for checking solution stability and robustness parameters.

3. Results and discussion

3.1. Method development

Initially rivastigmine sample was analysed by reported methods. A critical evaluation of chromatographic data revealed that in few methods [4,7,11], impurities were either merged with each other, or with the main peak. Peak broadening and close elution of impurities was also major concern [12] about the specificity of the method. All the methods employed have almost similar chromatographic conditions in isocratic mode. The main objective of the method development was to achieve efficient separation of closely eluting impurities in gradient mode. Several experiments were conducted to optimize the chromatographic conditions as described in Section 2.2. Ratio of acetonitrile–methanol and column temperature was optimized in order to achieve the desired resolution of closely eluting impurities using simple chromatographic conditions.

MS-compatible method development was found to be extremely difficult due to co-elution of Imp-C along with rivastigmine peak. Series of experiments were conducted to optimize the chromatographic conditions as described in Section 2.4. Column temperature at 50 °C and acetonitrile–methanol ratio was found to be very critical for achieving the resolution between Imp-C and rivastigmine.

3.2. Detection of impurities by newly developed HPLC method

Rivastigmine tartrate API lab sample prepared by known synthetic route [23] was analysed by HPLC method as described in Section 2.2. The analysis revealed the presence of six impurities. All known impurities were spiked with rivastigmine sample and RRTs were compared. Three impurities at RRTs 0.35, 0.65, 0.72 were found to be known and marked as Imp-2, Imp-5 and Imp-6 respectively. The unknown impurities at RRTs at 0.46, 0.92 and 0.95 were marked as Imp-A, Imp-B and Imp-C respectively. A typical chromatogram of rivastigmine tartrate lab sample highlighting the retention time of these impurities was showed in Fig. 2.

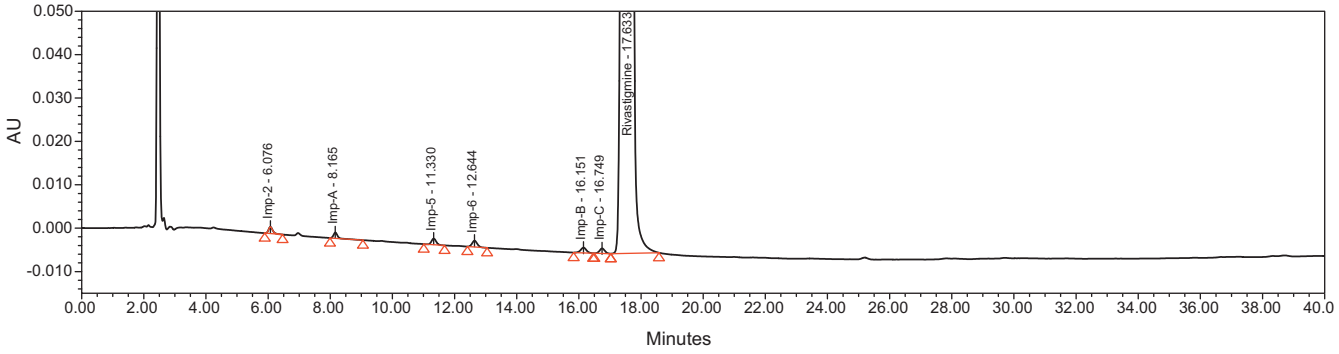


Fig. 2. Typical chromatogram of rivastigmine tartrate crude sample in new method.

3.3. Enrichment of impurities by semi-preparative HPLC

Attempts were initially made to identify the trace level impurities by directly analysing the sample using LC/MS/MS experiment. Due to poor MS response for impurities, semi-preparative HPLC was employed to enrich the impurity from rivastigmine tartrate sample in order to acquire better LC/MS/MS data. Rivastigmine tartrate sample containing 0.02–0.05% of impurities (area normalization method) was subjected to semi-preparative isolation using conditions as described in Section 2.3. Fractions were collected using fraction collector between retention time 5.0 min and 19 min. All the fractions were combined concentrated to 10 mL and was used for LC/MS analysis. The isolated fraction was found to contain more than 0.4% of each impurities.

3.4. Identification of impurities by LC/MS/MS

To further investigate these impurities, a HPLC method, as described in Section 2.4 was used. Mass spectral data showed protonated molecular ion peaks at *m/z* 166, *m/z* 267, *m/z* 237, *m/z* 237, *m/z* 251 and *m/z* 251 for Imp-2, Imp-A, Imp-5, Imp-6, Imp-B and Imp-C respectively. On the basis of RRT with respect to rivastigmine tartrate and mass spectral data, Imp-2 having molecular ion peak at *m/z* 166 was identified as 3-(1-dimethylamino-ethyl)-phenol (RVS-III). Imp-5 having molecular ion peak at *m/z* 237 was identified as 3-[1-aminoethyl]phenyl *N*-ethyl-*N*-methyl carbamate, Imp-6 having molecular ion peak at *m/z* 237 was identified as dimethylcarbamic acid 3-(1-dimethylamino-ethyl)-phenyl ester. The mass spectral data obtained for Imp-A, Imp-B and Imp-C did not match with any of the known impurities (Table 1). Therefore, a comprehensive investigation was carried out for the identification and characterization of these impurities.

Prior to characterization of impurities of rivastigmine, it is logical to understand the LC–MS/MS data of the parent drug molecule. The MS^2 spectrum obtained for rivastigmine parent ion at *m/z* 251 (Fig. 4a), showed two prominent peaks at *m/z* 206 and *m/z* 86 (Fig. 4b). The formation of product ion at *m/z* 206 can be attributed to the neutral loss of dimethyl amine ($NH(CH_3)_2$, 45 amu) from the parent ion. The product ion at *m/z* 206 was further subjected to MS^3 analysis, which gave daughter ions at *m/z* 178, *m/z* 149 and *m/z* 86 (Fig. 4c). Formation of these product ions can be rationalized by considering the fragmentation pattern as depicted in Fig. 5a. The proposed fragmentation mechanism was further supported by previously reported results [9,10].

The protonated molecular ion of Imp-A was obtained at *m/z* 267 (Fig. 4d), which is 16 amu higher than that of rivastigmine molecule. MS^2 analysis showed fragments at *m/z* 206 and *m/z* 86 (Fig. 4e) similar to that of rivastigmine. In addition, the MS^3 analysis of the precursor ion of *m/z* 206 gave fragments at *m/z* 178, *m/z* 149 and *m/z* 86 (Fig. 4f) matching with that of rivastigmine. A critical inspection of molecular ion along with the fragmentation pattern in both MS^2 and MS^3 spectra indicated that Imp-A contains an extra oxygen atom and the most plausible structure for Imp-A can be proposed as rivastigmine *N*-oxide. A plausible mass fragmentation profile for Imp-A based on multistage mass spectrometry is depicted in Fig. 5b.

Imp-B showed protonated molecular ion peak at *m/z* 251 (Fig. 4g). The MS^2 analysis of Imp-B showed daughter ions at *m/z* 206 and *m/z* 86 (Fig. 4h) similar to that of rivastigmine indicating it to be a regio-isomeric impurity. The MS^3 spectrum of the precursor ion at *m/z* 206 gave prominent fragments at *m/z* 162 and *m/z* 86 (Fig. 4i) and the two prominent fragment ions at *m/z* 178 and *m/z* 149 observed in rivastigmine molecule were absent. The formation of daughter ion at *m/z* 162 is due to the neutral loss of carbon dioxide (CO_2). The plausible pathway for the formation of the daughter ions is shown in Fig. 5c.

The full scan spectra of impurity-C showed its protonated molecular ion at *m/z* 251 (Fig. 4j). The MS^2 analysis of impurity-B showed daughter ion peak at *m/z* 206 and *m/z* 86 (Fig. 4k). The product ions at *m/z* 206 can be attributed to the neutral loss of dimethyl amine ($NH(CH_3)_2$, 45 amu). The MS^3 analysis of the precursor ion of *m/z* 206 gave fragments at *m/z* 178, *m/z* 149 and *m/z* 86 (Fig. 4l) matching with that of rivastigmine. Loss of similar neutral moieties both from rivastigmine and Imp-C indicated the presence of same functional groups in both molecules. Based on LC/MS/MS analysis this impurity was suspected to be the *o*-isomer of rivastigmine (Fig. 1d). A plausible fragmentation pattern showing the formation of these daughter ions is depicted in Fig. 5d.

3.5. Structural elucidation of rivastigmine Imp-A, Imp-B, Imp-C

Based on the proposed structure of impurities by LC/MS/MS, Imp-A, Imp-B and Imp-C were independently synthesized and used for further structural confirmation by NMR, FT-IR and EA techniques. Synthesized impurities were analysed by HPLC method as described in Section 2.2 and the purity was found to be 97.5%, 98.2% and 96.1% for Imp-A, Imp-B and Imp-C respectively.

3.5.1. Structural elucidation of rivastigmine and Imp-A

The ^1H NMR spectral data of rivastigmine tartrate showed a singlet for six protons at δ2.649 corresponding to *N,N*-dimethyl group. Four aromatic protons showed their characteristic signals ranging from δ 7.172 to 7.414. The chemical shift values at δ 44.12 and δ 122.69, 129.97, 126.17 and 135.09 in the ^{13}C– NMR spectrum further confirms for the *N,N*-dimethyl and aromatic nucleus respectively. ^1H and ^{13}C NMR values and assignments are listed in Tables 2 and 3 respectively.

The IR spectrum showed a broad signal characteristic of the hydroxyl group at 3457.23. Further, a sharp peak at 1715.98 for C=O stretching (carbamate), 1590.82 for aromatic nucleus (C=C)

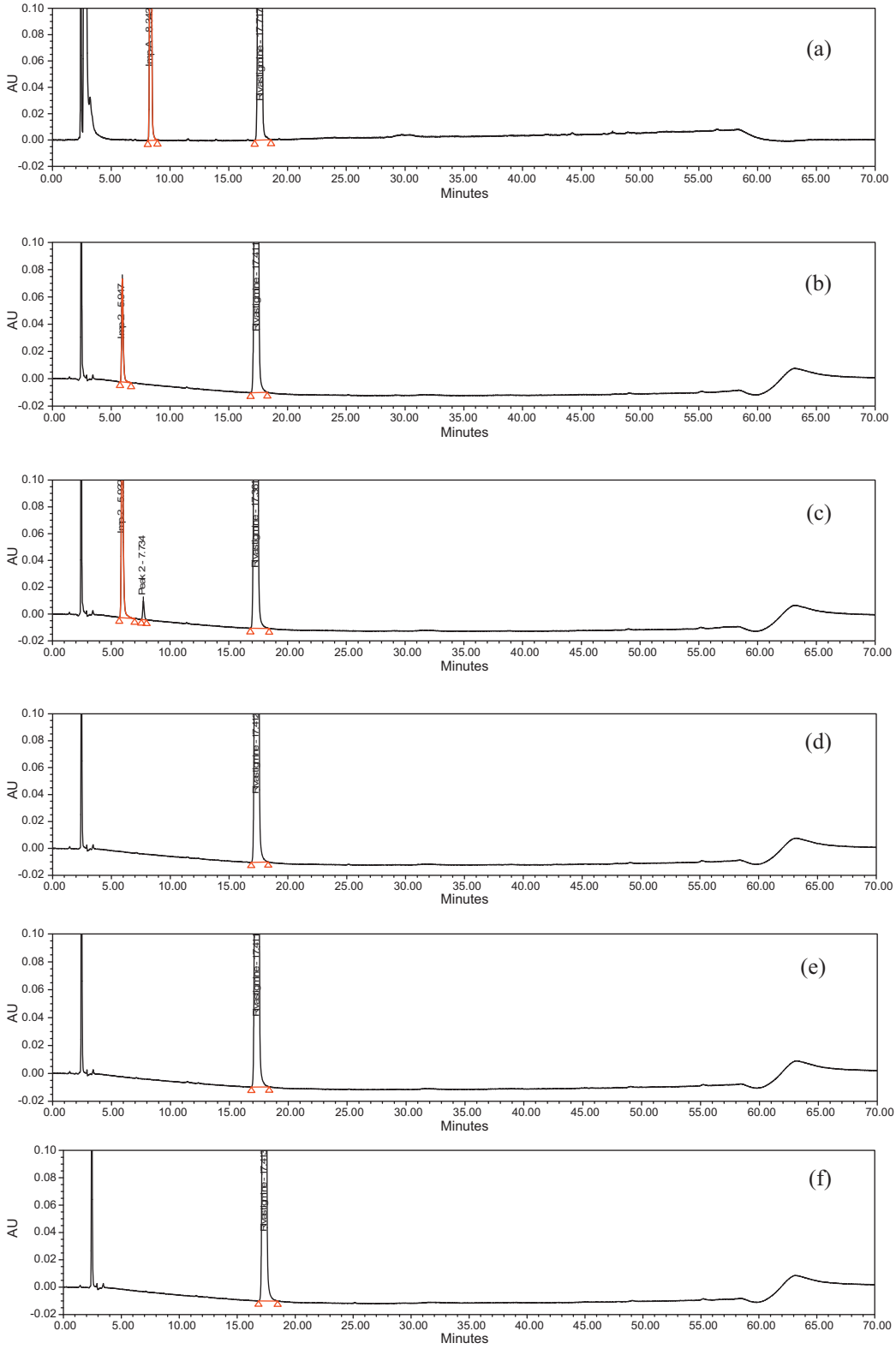


Fig. 3. Typical chromatogram of rivastigmine tartrate under stress conditions: (a) oxidative degradation, (b) acid hydrolysis, (c) base hydrolysis, (d) water hydrolysis, (e) thermal degradation, (f) degradation in light and UV.

and the *N,N*-dimethyl stretching frequency at 2696.17 were quite informative for the structural assignment.

The ^{1}H NMR spectral data of Imp-A showed a broad singlet at δ 7.414 for one proton along with multiplets for three protons coupled to one another in aromatic region which is an indication for the meta disubstituted aromatic nucleus. Further, the downfield value of chemical shift at δ 3.012 (6H, s; *N,N*-dimethyl) in Imp-A as compared to rivastigmine indicated it to be around a more electronegative atom (probability of *N*-oxide). In ^{13}C NMR spectrum, the chemical shift values for *N*-dimethyl at δ 45.15 and 45.21

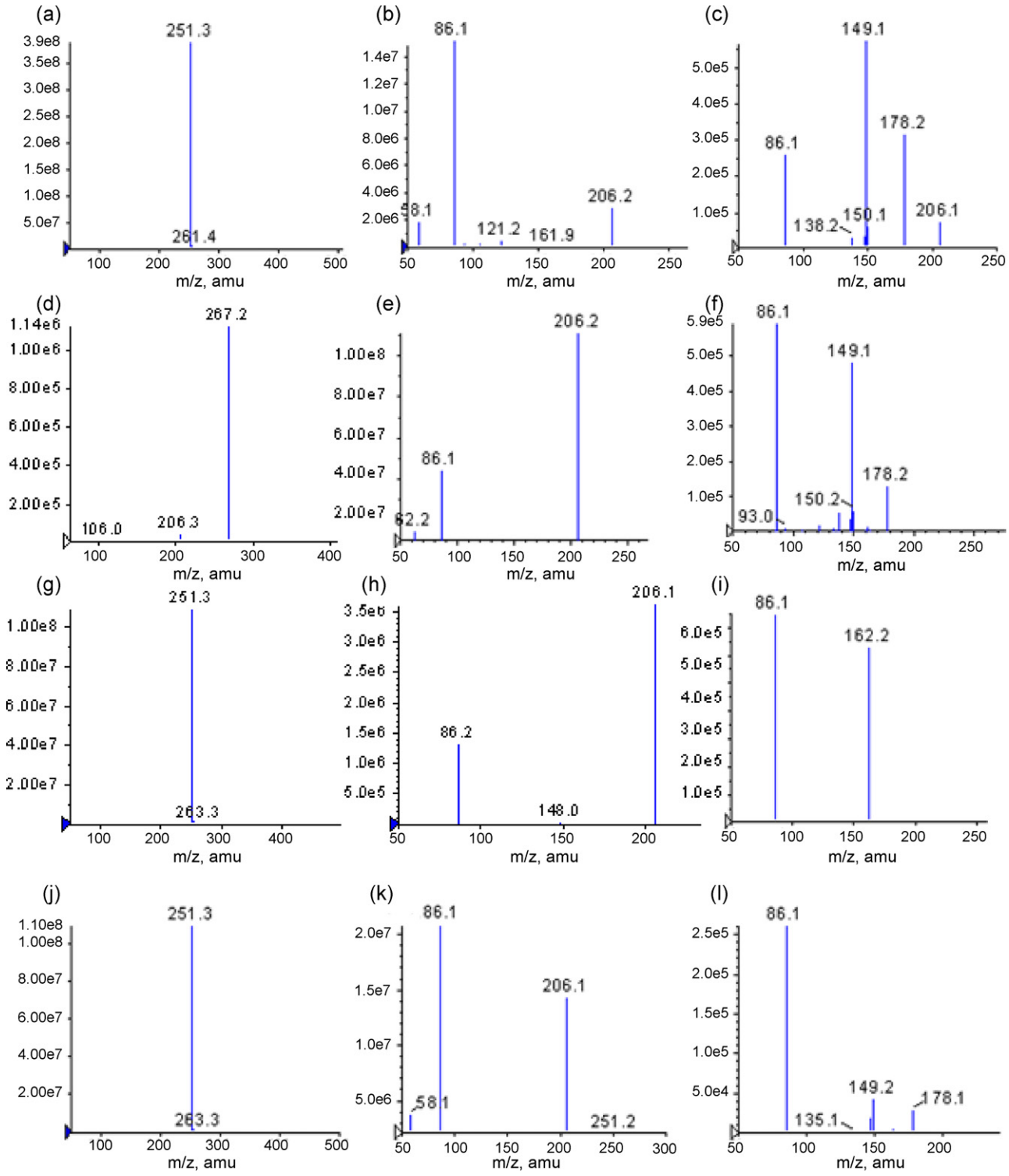


Fig. 4. (a) MS spectrum of rivastigmine, (b) MS^2 spectrum of rivastigmine, (c) MS^3 spectrum of rivastigmine, (d) MS spectrum of Imp-A, (e) MS^2 spectrum of Imp-A, (f) MS^3 spectrum of Imp-A, (g) MS spectrum of Imp-B, (h) MS^2 spectrum of Imp-B, (i) MS^3 spectrum of Imp-B, (j) MS spectrum of Imp-C, (k) MS^2 spectrum of Imp-C, (l) MS^3 spectrum of Imp-C.

and four aromatic carbon signals ranging from δ 124.12 to 128.40 further supports these assignments. ^{1}H and ^{13}C NMR values and assignments are listed in Tables 2 and 3 respectively.

The IR spectral data showed characteristic aromatic C–H stretching at 3271.07, 1704.67 for carbamate, 1611.11 and 1592.19 for aromatic skeleton and 2815.92 for *N*-methyl stretching.

CHN data obtained for Imp-A showed carbon 63.36%, nitrogen 10.68%, and hydrogen 8.57% against theoretical values of carbon 63.13%, nitrogen 10.52%, and hydrogen 8.33%. NMR and CHN data confirms the proposed structure based on LC/MS/MS data.

3.5.2. *Structural elucidation of Imp-B*

The ^{1}H NMR of Imp-C showed a singlet for six protons at δ 2.565 corresponding to *N,N*-dimethyl group. The four aromatic protons are symmetrically distributed as two equivalent sets as evident by their chemical shift and coupling constant values viz. δ 7.182–7.203

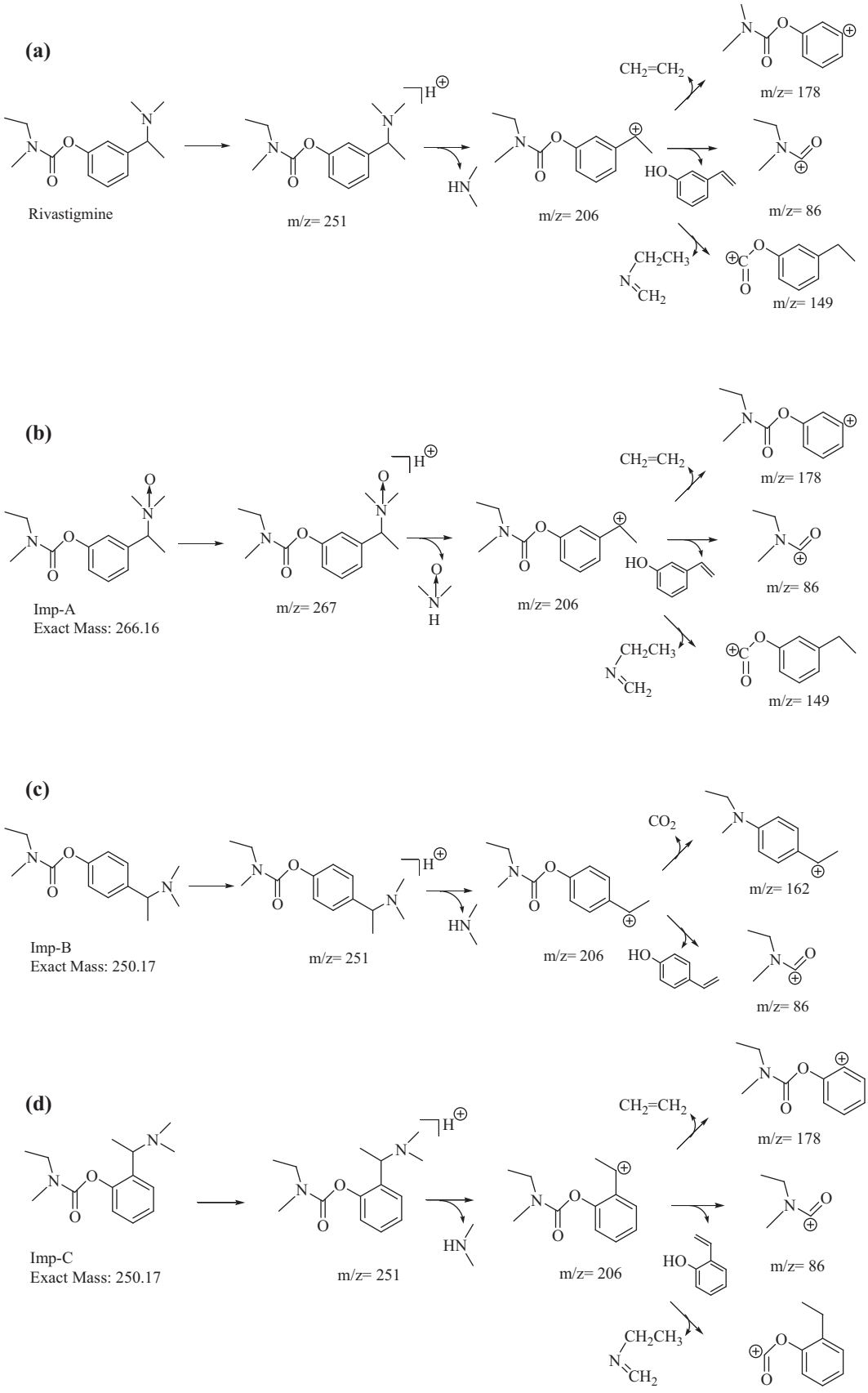


Fig. 5. Plausible fragmentation pathway for (a) rivastigmine, (b) Imp-A, (c) Imp-B, (d) Imp-C.

Table 2
Comparative ^{1}H NMR assignments for rivastigmine tartrate and its impurities.

Position[a]	Rivastigmine tartrate	Imp-A δ ppm multiplicity	Imp-B δ ppm multiplicity	Imp-C δ ppm multiplicity
1	1.149–1.250 t	1.159–1.216, 1.251–1.303 t	1.083–1.117, 1.162–1.196 t	1.160–1.247, 1.311–1.354 s
2	3.368–3.472 q (7.0 Hz)	3.394–3.447, 3.489–3.566 q (6.8 Hz, 7.2 Hz)	3.284–3.335, 3.392–3.442 q	3.405–3.505, 3.571–3.633 m
3	2.960, 3.060 s	3.172 s	2.898, 3.018s	3.036, 3.197s
4	–	–	–	–
5	–	–	–	–
6	7.172–7.159 m	7.197–7.269 m	7.182–7.203 d (8.4 Hz)	7.202–7.244, 7.403–7.441m
7	7.375–7.414 m	7.439–7.486 m	7.519–7.540 d (8.4 Hz)	7.500–7.543 m
8	7.287–7.304 d (8.2 Hz)	7.439–7.486 m	7.182–7.203 d (8.4 Hz)	7.202–7.244, 7.403–7.441 m
9	–	7.414 Br s	7.519–7.540 d (8.4 Hz)	7.709–7.733 m
10	7.207 s	–	–	–
11	4.485 s	4.508–4.560 q (6.8 Hz)	4.332–4.382 q (6.8 Hz)	4.593–4.699 m
12	1.672–1.688 m	1.774–1.792 d (7.2 Hz)	1.575–1.592 d (6.8 Hz)	1.727–1.744 d
13	2.649 s	3.0123.127 d	2.565 s	2.804 s
14	2.649 s	3.012–3.127 d	2.565 s	2.804 s
15	–	–	–	–
16	–	–	–	–
17	–	–	–	–
18	–	–	–	–
19	4.366–4.383	–	–	–
20	4.366–4.383	–	–	–

s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are shown in bracket.
[a] Refer structure formula of rivastigmine tartrate and impurities in Fig. 1 for numbering.

(2H, 8.4 Hz) & δ 7.519–7.540 (2H, 8.4 Hz). The chemical shift values at δ 40.13, δ 122.09 and 129.83 in the ^{13}C– NMR spectrum further supports for the *N,N*-dimethyl and para substituted pattern respectively.

The IR spectral data showed absorption for O–H stretching at 3224.62, 1718.89 and 1734.84 for carbamate C=O stretching, 1609.70 for aromatic skeleton and 2974.13, 2852.41 for asymmetric and symmetric C–H aliphatic stretchings. These absorption values are in support for the assigned structure.

CHN data obtained for Imp-B showed carbon 53.76%, nitrogen 7.15% and hydrogen 7.08% against theoretical values of carbon 53.99%, nitrogen 7.0%, and hydrogen 7.05%. CHN data confirms the proposed structure based on LC/MS/MS data. Based on the above data the most plausible structure for Imp-B can be assigned as *p*-isomer of rivastigmine (Fig. 1c).

3.5.3. Structural elucidation of Imp-C

The ^{1}H NMR of Imp-C showed a singlet for six protons at δ 2.804 corresponding to *N,N*-dimethyl group. The four aromatic protons are distributed asymmetrically as each of them exhibited different chemical shift values indicating the structure to be ortho substituted. In ^{13}C NMR spectrum, the chemical shift values for *N*-dimethyl at δ 41.61 & 41.72 and four aromatic carbon signals ranging from δ 124.61 to 129.53 further supports these assignments. ^{1}H and ^{13}C NMR values and assignments were listed in Tables 2 and 3 respectively.

The IR spectral data showed a broad absorption peak at 3407.98 for O–H stretching, 1719.27 for C=O stretching of carbamate, 1604.39 for aromatic C=C skeleton and 2675.73 for *N*-methyl stretching. These absorption values were in support for the assigned structure.

CHN data obtained for Imp-C showed carbon 53.88%, nitrogen 7.12% and hydrogen 7.11% against theoretical values of carbon 53.99%, nitrogen 7.0%, and hydrogen 7.05%. Based on these assignments the most plausible structure for Imp-C can be assigned as *o*-isomer of rivastigmine (Fig. 1d).

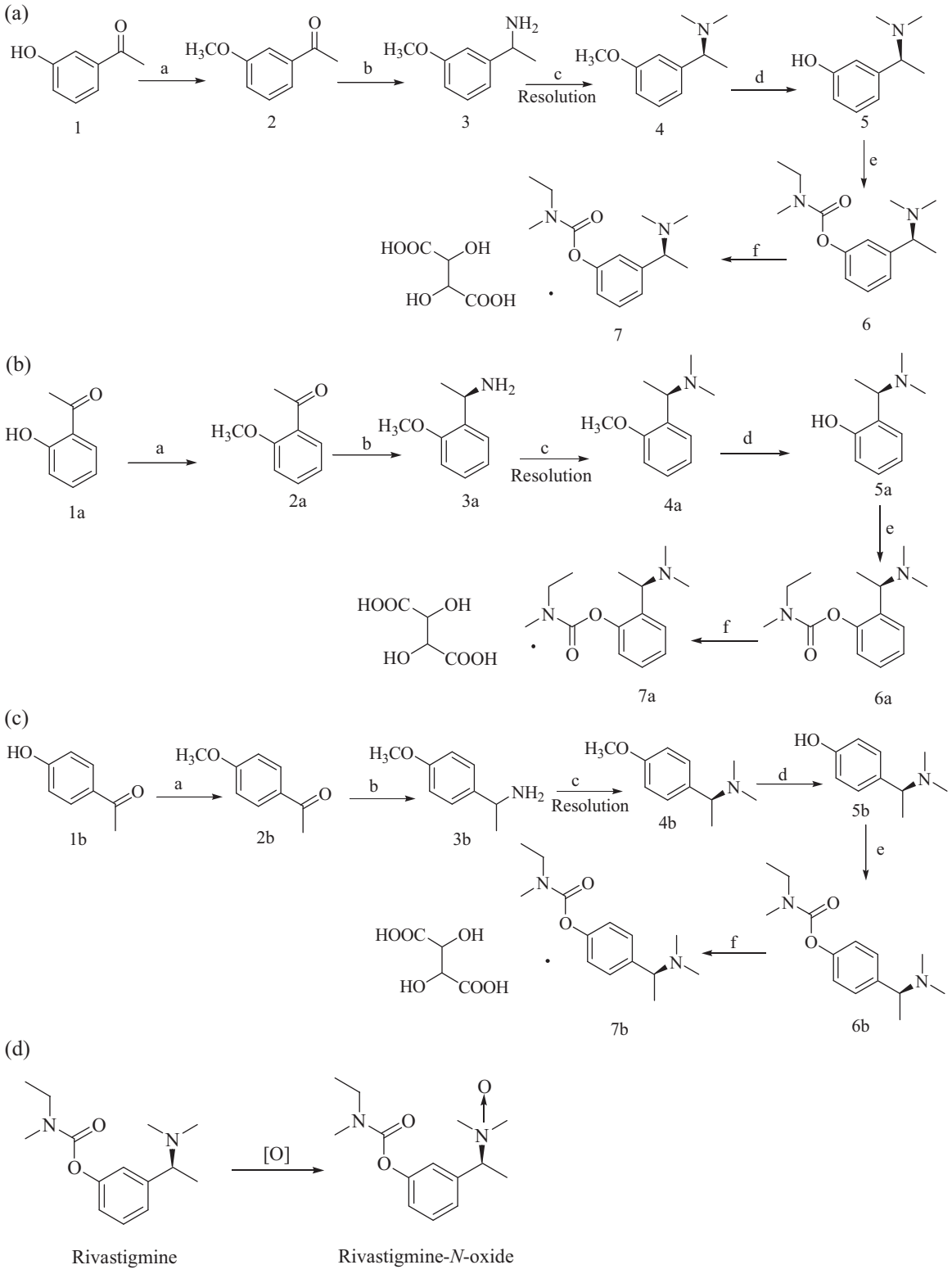
3.6. Formation of Imp-A, Imp-B and Imp-C

Rivastigmine tartrate was oxidized under mild to drastic conditions to form rivastigmine *N*-oxide. The synthetic scheme of plausible formation of Imp-A is depicted in Fig. 6d. During the synthesis of rivastigmine, 3-hydroxy acetophenone was treated with dimethyl sulphate to get RVS-I intermediate. RVS-I on further reac-

Table 3
Comparative ^{13}C NMR assignments for rivastigmine tartrate and its impurities.

Position	Rivastigmine tartrate	Imp-A	Imp-B	Imp-C
1	12.41, 13.21	12.60, 13.40	12.21, 13.02	12.65, 13.70
2	40.22	55.61	43.48	45.38, 45.54
3	33.87, 34.25	34.27, 34.55	33.57, 33.89	34.56, 34.76
4	154.08, 154.25	156.15	153.35	155.72, 155.79
5	151.72	152.79	151.71	151.25
6	122.69	124.12	122.09	124.61, 124.79
7	129.97	130.52	122.09	128.96, 129.25
8	126.17	128.40	129.83	127.76, 127.83
9	135.00, 135.09	124.89	129.83	129.47, 129.53
10	123.11	138.74	132.64	131.98, 132.02
11	64.92	58.01	63.72	60.51, 60.63
12	16.45	15.79	16.47	17.23, 17.33
13	44.12	45.15, 45.21	40.13	41.61, 41.72
14	44.12	45.15, 45.21	40.13	41.61, 41.72
15	72.62	–	164.68	168.24
16	72.62	–	164.68	168.24
17	176.31	–	–	–
18	176.31	–	–	–

[a] Refer structure formula of rivastigmine tartrate and impurities in Fig. 1 for numbering.



Reagents and conditions (a) dimethyl sulphate, K_2CO_3 (b) ammonium formate (c) toluene, ethyl acetate, HCHO,HCOOH (d) 48 % aq. HBr (e) *N*-ethyl-*N* -methylcarbamoyl chloride,acetone,K_2CO_3 (f) L-(+)-tartaric acid

Fig. 6. (a) Synthetic scheme of rivastigmine, (b) plausible pathway for the formation of Imp-C, (c) plausible pathway for the formation of Imp-B, (d) plausible pathway for the formation of Imp-A.

tion with ammonium formate yielded RVS-II intermediate. RVS-II was treated with L(+) mandelic acid to isolate the pure enantiomer which was further treated with formic acid and formaldehyde mixture to get RVS-III intermediate. RVS-III intermediate was further treated with *N*-ethyl-*N*-methyl carbamoyl chloride to get rivastigmine base. Rivastigmine base on further treatment with L-(+)-tartaric acid yielded rivastigmine tartrate (Fig. 6a). The presence of 4-hydroxy acetophenone (positional isomeric impurity) and 2-hydroxy acetophenone (positional isomeric impurity) in 3-hydroxy acetophenone was found to contribute the formation of Imp-B and Imp-C respectively. The synthetic scheme of plausible formation of Imp-B and Imp-C is depicted in Fig. 6b and c respectively.

4. Method validation

The newly developed method was validated for precision, linearity, accuracy, sensitivity, robustness and system suitability according to ICH guidelines. Validation study was carried out for Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11. The system suitability and selectivity were checked by injecting 750 μg/mL of rivstigmine tartrate solution containing 0.15% of all impurities monitored throughout the validation. Method validation results are summarized in Table 4.

4.1. Specificity

Specificity is the ability of the method to unequivocally assess the analyte response in the presence of its potential impurities. Specificity was established by injecting rivastigmine tartrate co-spiked with potential impurities where in no interference was observed. Forced degradation studies were also performed on rivastigmine tartrate to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study included acid hydrolysis (1N HCl, 80 °C, for 3 h), base hydrolysis (1 N NaOH, 50 °C, for 1 h), thermal degradation (105 °C, for 24 h), oxidation (30% H_2O_2, 80 °C, for 45 min), photolytic (1.2 million lux hours and 200 watt hours/square meter) and water hydrolysis (80 °C, for 3 h). Samples were withdrawn at appropriate times and subjected to LC analysis after suitable dilution (300 μg/mL) to evaluate the ability of the proposed method to separate rivastigmine from its degradation products.

4.2. Results of forced degradation and identification of impurity by LC/MS/MS

During stress studies, it was found that rivastigmine tartrate was susceptible to oxidative, acidic and alkaline conditions contrary to the earlier reported findings of no degradation in oxidative and acidic conditions [4]. The molecule was found to be very stable in thermal, photolytic and water hydrolysis (Fig. 3). In acidic and alkaline hydrolysis, major impurity formed at RRT 0.15 was identified as Imp-2. In oxidative degradation, impurity observed at RRT 0.47 was not matching with any of the previously reported impurities (Table 1). To further investigate this impurity, LC–MS method as described in Section 2.4 was used. Mass spectral data showed protonated molecular ion peak at *m/z* 267 which was 16 amu higher than rivastigmine protonated molecular mass of 251, indicating the presence of oxygen atom in the molecule. MS^2 and MS^3 data of this impurity were similar to that of Imp-A, hence the degradant impurity was confirmed as rivastigmine *N*-oxide (Fig. 1b).

Peak purity of stressed samples of rivastigmine tartrate was checked using a 2998 photo diode array detector (Waters Corporation, Milford, MA, USA). Data obtained from degradation studies

Table 4
System suitability, DL, QL, linearity and precision, accuracy.

Parameter	Imp-1	Imp-2	Imp-A	Imp-3	Imp-4	Imp-5	Imp-6	Imp-B	Imp-C	Rivastigmine	Imp-7	Imp-8	Imp-9	1mp-10	Imp-11
System suitability															
R_s	–	4.02	9.98	6.86	4.14	3.70	4.53	11.84	1.60	2.86	3.65	7.78	8.49	6.75	32.66
N	15 099	13 313	28 122	31 248	32 164	32 698	32 165	46 780	55 008	42 232	96 828	115 382	132 321	124 688	116 172
T	1.07	1.31	1.09	1.23	1.08	1.14	1.10	1.08	1.04	1.26	1.01	1.01	1.01	1.05	1.04
Linearity															
r	0.9999	0.9999	0.9998	0.9999	0.9998	0.9998	0.9998	0.9997	0.9998	0.9997	0.9999	0.9998	0.9999	0.9998	0.9996
Slope	457 341	330 584	362 947	938 102	437 035	443 243	452 389	279 220	278 935	277 644	1 086 759	502 526	1 028 415	263 083	465 954
Intercept	−111	−711	335	−448	46	144	−12	−358	−127	350	−193	607	−404	−489	−2226
Detection limit	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%
Quantitation limit	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%	0.03%
Precision % RSD ($n = 6$)	1.42	1.63	4.17	1.70	3.93	1.12	3.99	4.72	2.47	3.09	1.66	2.38	2.56	3.43	2.54
Accuracy at QL level ($n = 3$)															
Amount added (%)	0.032	0.030	0.030	0.030	0.030	0.030	0.031	0.032	0.031	0.030	0.031	0.029	0.033	0.030	0.032
Amount recovered (%)	0.034	0.034	0.032	0.032	0.035	0.033	0.034	0.035	0.032	0.031	0.032	0.031	0.032	0.027	0.035
% Recovery	106.25	113.33	106.67	106.67	110.67	110.00	109.68	109.38	103.23	103.33	103.23	106.90	96.97	94.34	109.38
Accuracy at 100% level ($n = 3$)															
Amount added (%)	0.152	0.150	0.148	0.149	0.149	0.150	0.152	0.159	0.151	0.150	0.150	0.144	0.155	0.150	0.149
Amount recovered (%)	0.153	0.159	0.156	0.157	0.159	0.160	0.154	0.164	0.162	0.152	0.154	0.153	0.147	0.155	0.153
% Recovery	100.66	106.00	105.41	105.37	106.71	106.67	101.32	103.14	107.28	101.33	102.67	106.25	94.84	103.33	102.68
Accuracy at 150% level ($n = 3$)															
Amount added (%)	0.226	0.226	0.223	0.224	0.224	0.227	0.230	0.240	0.228	0.225	0.229	0.217	0.221	0.226	0.222
Amount recovered (%)	0.220	0.237	0.233	0.235	0.235	0.241	0.231	0.245	0.241	0.226	0.218	0.230	0.202	0.236	0.228
% Recovery	97.35	104.87	104.48	104.91	104.91	106.17	100.43	102.08	105.70	100.44	95.2	105.99	93.4	104.42	102.7

n, number of determinations; R_s, USP resolution; *N*, number of theoretical plates; *T*, USP tailing factor; *r*, correlation coefficient; RSD, relative standard deviation.

and peak purity tests confirmed that the rivastigmine tartrate peak was homogenous and pure in all the analysed stress samples. The developed LC method was found to be specific in the presence of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and degradation products confirmed the stability indicating power of the newly developed method.

4.3. Sensitivity

Sensitivity was determined by establishing the detection limit (DL) and quantitation limit (QL) for Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and rivastigmine tartrate by injecting a series of dilute solutions with known concentrations. The precision study was also carried out at the QL level by injecting six replicate injections of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and the % RSD for the areas of each impurity was calculated. The limit of detection and the limit of quantitation were about 0.01% and 0.03% of analyte concentration, i.e. 1500 μg/mL respectively. The relative standard deviation for QL concentration for all impurities was below 5%.

4.4. Precision

The precision of the related substances method was checked by injecting six individual preparations of 1500 μg/mL rivastigmine tartrate spiked with 0.15% each Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11. Percentage RSD for peak areas of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6 Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11 was calculated. Precision study was also determined by performing the same procedures on a different day (inter-day precision). The intermediate precision (ruggedness) of the method was also evaluated by a different analyst and different instruments in the same laboratory. % RSD of areas of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11 was within 5.0, confirming good precision at low level of the developed analytical method.

4.5. Linearity and range

A linearity test solution for related substance method was prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at six concentration levels. From QL to 150% of the permitted maximum level to the impurity (i.e. 0.03%, 0.075%, 0.1125%, 0.15%, 0.1875% and 0.225%) was subjected to linear regression analysis with the least squares method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The residuals and sum of the residual squares were calculated from the corresponding predicted responses. Upper and lower levels of range were also established. Linear calibration plot for the related substance method was obtained over the calibration ranges tested, i.e. QL to 0.225% for Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6 Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11. The correlation coefficient obtained was greater than 0.999 for all impurities. The result showed an excellent correlation between the peak and concentration of all impurities. Standard deviation of peak area was significantly low and RSD was below 5.0%. The range of the method had on QL to 0.225% of the analyte concentration (1500 μg/mL).

4.6. Accuracy

The accuracy of the method was evaluated in triplicate at three concentration levels, i.e. QL, 100% level (0.15% of the drug substance 1500 μg/mL) and 150% level (0.225% of the drug substance 1500 μg/mL). At each concentration, three sets were prepared and injected in triplicate. The percentage of recovery was calculated at each level. The study was carried out in triplicate at QL, 0.15%, and 0.225% of the analyte concentration (1500 μg/mL). The percentage recovery of all impurities in drug substance ranged from 93.41 to 113.33% (Table 4). Chromatogram of rivastigmine tartrate spiked with fourteen impurities was depicted in Fig. 7.

4.7. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution

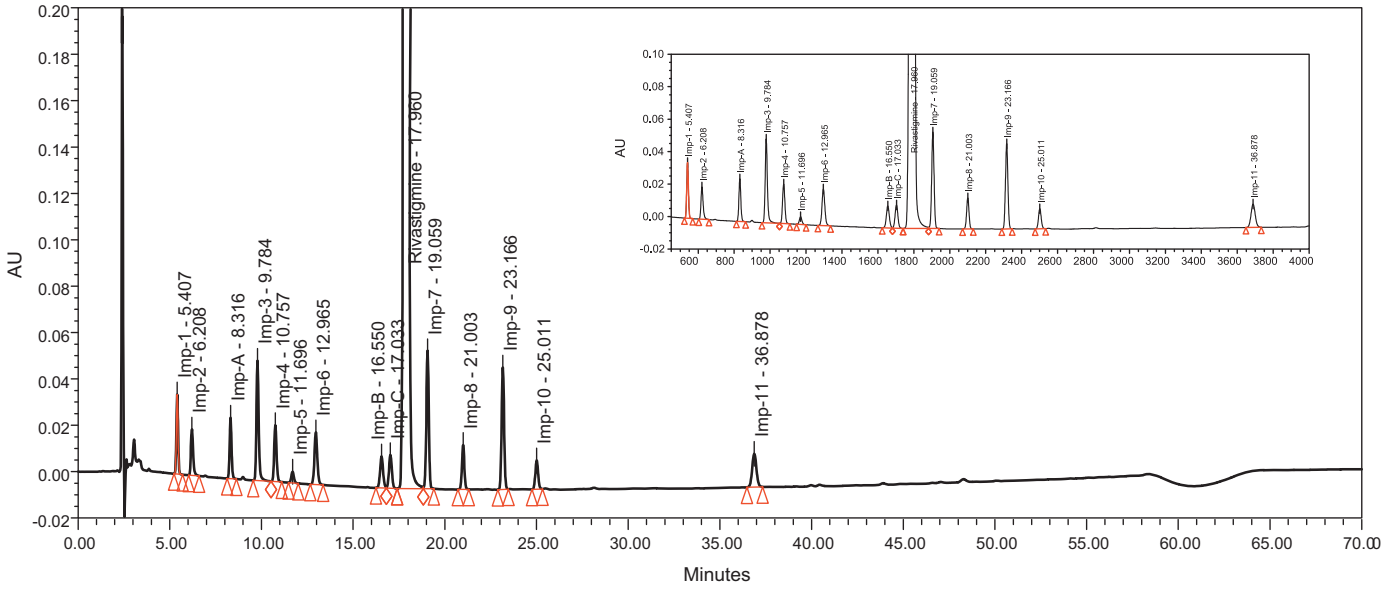


Fig. 7. Typical chromatogram of rivastigmine tartrate spiked with 14 impurities in new method.

between rivastigmine tartrate and Imp-7 was evaluated. The flow rate was studied at 1.0 ± 0.1 mL/min. The effect of column temperature was studied at 40 ± 2 °C. The effect of pH on resolution of impurities was evaluated at pH 7.60 ± 0.1. Close observation of analysis results of deliberately changed chromatographic conditions revealed that resolution between Imp-7 and rivastigmine was greater than 3.5, illustrating the robustness of the method.

4.8. Solution stability

The solution stability of rivastigmine tartrate and its related impurities was carried out by leaving both spiked and unspiked sample solutions in tightly capped HPLC vials at 25 °C for 25 h in an auto sampler. Contents of Imp-A, Imp-B, Imp-C, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6 Imp-7, Imp-8, Imp-9, Imp-10 and Imp-11 were determined at every 5 h against freshly prepared standard solution. The solution stability experimental data confirmed that sample solutions were stable up to 25 h.

5. Conclusion

In this study three unknown impurities detected using a newly developed HPLC method were enriched and identified with a combination of semi-preparative HPLC and LC/MS/MS techniques. Proposed structures were further confirmed by structural elucidation using NMR, FT-IR, MS and EA techniques of impurity standards. Based on the spectroscopic, spectrometric and elemental analyses three unknown impurities were characterized. Imp-A was characterized as 3-[1-(dimethylamino)ethyl]phenyl *N*-ethyl-*N*-methyl carbamate *N*-oxide (rivastigmine *N*-oxide). Imp-B was characterized as ethyl-methyl-carbamic acid 4-(1-dimethylamino-ethyl)-phenyl ester (rivastigmine *p*-isomer) and Imp-C was characterized as ethyl-methyl-carbamic acid 2-(1-dimethylamino-ethyl)-phenyl ester (rivastigmine *o*-isomer). Major oxidative degradant impurity was identified as rivastigmine *N*-oxide. The LC–UV method was validated as per ICH guidelines. The theoretical plates obtained for rivastigmine peak for the system suitability solution was more than 40 000 and the tailing factor was less than 1.3 indicating exceptionally high column efficiency of the newly developed method. Newly developed HPLC method was found to be simple, sensitive, selective, cost effective and stability indicating. Detection limit for impurities was found to be as low as 0.01% indicating high sensitivity of the validated method and can be conveniently used for routine and stability studies. Formation of newly characterized impurities (Imp-A, Imp-B, Imp-C) can be eliminated in the drug substance by use of inert atmosphere and control of isomeric impurities in 3-hydroxy acetophenone.

Acknowledgements

The authors are thankful to the management of Jubilant Life Sciences Limited for providing necessary facilities. Authors would like to thank Dr. Hawaldar Maurya, Dr. Sujoy Biswas, Mr. Dinesh Vishwakarma, Dr. Subhash Chandra Joshi, Ms. Samreen Siddiqui and Mr. Pawan Kumar for their co-operation in carrying out this work. One of the authors Mr. Saji Thomas would also like to thank Dr. Dharamvir for continuous encouragement throughout the course of work.

References

[1] J.E.F. Reynolds (Ed.), Martindale, The Complete Drug Reference, 36th ed., Royal Pharmaceutical Society of Great Britain, 2009, pp. 369–370.
[2] N.R. Cutler, J.J. Sramek, Review of the next generation of Alzheimer's disease therapeutics: challenges for drug development, Prog. Neurophyschopharmacol. Biol. Psychiatry 25 (2001) 27–57.
[3] J.M.O. Casademont, B. Rodriguuez-Santiago, P. Videma, R. Blesa, F. Cardellach, Cholinesterase inhibitor rivastigmine enhances the mitochondrial electron transport chain in lymphocytes of patients with Alzheimer's disease, J. Neurosci. 206 (2003) 23–26.
[4] B.M. Rao, M.K. Srinivasu, K.P. Kumar, N. Bhradwaj, R. Ravi, P.K. Mohakhud, G.O. Reddy, P.R. Kumar, Stability indicating LC method for rivastigmine hydrogen tartrate, J. Pharm. Biomed. Anal. 37 (2005) 57–63.
[5] S. Dermiş, Voltammetric behaviour of rivastigmine hydrogen tartrate and its determination in capsule dosage form, J. Fac. Pharm. 26 (2006) 1–12.
[6] M.K. Srinvasu, M.B. Rao, B.S.S. Reddy, P.R. Kumar, K.B. Chandrasekhar, P.K. Mohakhud, A validated chiral liquid chromatographic method for the enantiomeric separation of rivastigmine hydrogen tartarate, a cholinesterase inhibitor, J. Pharm. Biomed. Anal. 38 (2005) 320–325.
[7] M.S. Rao, S.V. Rao, D.V.N.S. Rao, Ch. Bharathi, P. Rajput, H.K. Sharma, Identification and characterization of new impurities in rivastigmine, Pharmazie 65 (2010) 336–338.
[8] A. Karthik, P. Musmade, G. Subramanian, M.S. Kiado, Stability-indicating HPTLC determination of rivastigmine in the bulk drug and in pharmaceutical dosage form, J. Plan. Chromatogr. Mod. TLC (2007) 933–4173.
[9] K. Arumugam, M.R. Chamallamudi, R.R. Gilibili, R. Mullangi, S. Ganesan, S.S. Kar, R. Averineni, G. Shavi, N. Udupa, Development and validation of a HPLC method for quantification of rivastigmine in rat urine and identification of a novel metabolite in urine by LC–MS/MS, Biomed. Chromatogr. 25 (2010) 353–361.
[10] J. Bhatt, G. Subbaiah, S. Kambli, B. Shah, S. Nigam, M. Patel, A. Saxena, A. Baliga, H. Parekh, G. Yadav, A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the estimation of rivastigmine in human plasma, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 852 (2007) 115–121.
[11] United States Pharmacopoeia-34, vol. 3, 2011, pp. 4176–4178.
[12] Pharmaeuropa, vol. 23, no. 2, 2011, pp. 379–381.
[13] ICH Guidelines, Q3A (R2), Impurities in New Drug Substances, October 25, 2006.
[14] E.M. Sheldon, J.B. Downar, Development and validation of a single robust HPLC method for the characterization of a pharmaceutical starting material and impurities from three suppliers using three separate synthetic routes, J. Pharm. Biomed. Anal. 23 (2000) 561–572.
[15] E.M. Sheldon, Development of a LC–LC–MS complete heart-cut approach for the characterization of a pharmaceutical compounds using standard instrumentation, J. Pharm. Biomed. Anal. 31 (2003) 1153–1166.
[16] D. Bartos, S. Gorog, Recent advances in the impurity profiling of drugs, Curr. Pharm. Anal. 4 (2008) 215–230.
[17] R.N. Rao, V. Nagaraju, An overview of the recent trends in development of HPLC methods for the determination of impurities in drugs, J. Pharm. Biomed. Anal. 33 (2003) 335–377.
[18] S. Gorog (Ed.), Determination of Impurities in Drugs, Elsevier Sciences, Amsterdam, 1999.
[19] S. Ahuja, Impurities Evaluation in Pharmaceuticals, Marcel Dekker, New York, 1998.
[20] S. Husain, R.N. Rao, Monitoring of process impurities in drugs, in: Z. Dey1, I. Miksik, F. Taglirao, E. Tesarova (Eds.), Advanced Chromatographic and Electromigration Methods in Biosciences, Elsevier Science, Amsterdam, 1998, pp. 834–888.
[21] L.A.B. Moraes, A.A. Sabino, E.C. Meurer, M.N. Eberlin, Absolute configuration assignment of ortho, meta or para isomers by mass spectrometry, J Am. Soc. Mass Spectrom. 16 (2005) 431–436.
[22] K. Jiang, G. Bian, Y. Pan, G. Lai, Recognising *ortho*-, *meta*- or *para*-positional isomers of S-methyl methoxyphenylmethylenehydrazine dithiocarboxylates by ESI-MS^2: the positional effect of methoxyl substituent, Int. J. Mass Spectrom. 299 (2011) 13–19.
[23] V.V. Reddy, M.V.N.B. Rao, V. Ganesh, A.V. Kumar, C. Praveen, K. Mukkanti, G.M. Reddy, G.M. Reddy, An improved process for the production of rivastigmine tartrate, a cholinesterase inhibitor, Lett. Org. Chem. 7 (2010) 149–154.
[24] ICH Guidelines, Q2 (R1), Validation of Analytical Procedures: Test and Methodology, November, 2005.