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Identification and characterization of a principal oxidation impurity in clopidogrel drug substance and drug product

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Abstract

The focus of this study is identification, isolation and characterization of a principal oxidation impurity of clopidogrel which ranged from 0.05 to 0.12% using high performance liquid chromatography. This impurity is considered as principal oxidation impurity as it is observed in oxidative degradation (stress) study. Preparative HPLC with Xterra MS C18 ODB column was used to isolate the impurity. The isolated impurity was co-injected with the sample containing impurities and found the retention time match of the spiked impurities. A thorough study was undertaken to characterize this impurity and based on their spectral data (UV, MS, MS n, 1H/13C, DEPT and 2D NMR) the structure was characterized as 5-[(1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-6,7-dihydrothieno[3,2-c]pyridin-5-ium with a molecular weight 320 amu.

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Keywords: Clopidogrel related compound; Degradation product characterization; LC–MS/MS

1. Introduction

Clopidogrel bisulfate, methyl (+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate sulfate (1:1), is a potent oral antiplatelet agent often used in the treatment of coronary artery disease, peripheral vascular disease and cerebrovascular disease. It is marketed by Bristol-Myers Squibb and Sanofi-Aventis under the trade name Plavix which is the world’s second highest selling pharmaceutical with sales of US$5.9 billion. The mechanism of action of clopidogrel is irreversible blockade of the adenosine diphosphate (ADP) receptor P2Y12 and is important in platelet aggregation, the cross-linking of platelets by fibrin. The blockade of this receptor inhibits platelet aggregation by blocking activation of the glycoprotein IIb/IIIa pathway. Platelet inhibition can be demonstrated 2 h after a single dose of oral clopidogrel, but the onset of action is slow, so that a loading-dose of 300–600 mg is usually administered [1].

Four impurities of clopidogrel have been already identified and documented in the literature [2–6] and named as clopidogrel related compound A, positional stereo isomers of clopidogrel named as clopidogrel related compounds B1 and B2 and a chiral isomer of clopidogrel named as clopidogrel related compound C. It has also been established that these positional stereoisomers (B1 and B2) are process impurities and other impurities are formed during the process and also self-degradation. Marketed samples of Plavix and few batches of drug substances were analyzed using reported method [3]. An Ultron ES-OVM L 57, chiral specific column (Shiwa chemical industries, Japan) with dimensions of 150 mm × 4.6 mm i.d. packed with 5.0 μ particle size was employed for separation. Acetonitrile–potassium phosphate buffer (10 mM) (75:25, v/v) was used as mobile phase and flow rate was kept at 0.8 ml/min with the detection at 220 nm for 30 min. Relative retention time (RRT) of the related compounds A, B1, clopidogrel B2 and C were found, respectively, at 0.46, 0.93, 1.0, 1.1 and 2.10. Related compound D was found at about 2.0 min (RRT of 0.30). As the related compound D elutes in the void volume of the system, various buffer composition, pH, gradients were attempted and found unsuccessful. The main problems that occurred were peak shape, peak purity

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and blank interference due to peroxide. Hence, conventional, cost effective, new method was developed wherein good peak shape and peak purity were achieved. The new method involves Hypersil BDS C8 column (Thermo Electron Corporation) with gradient conditions for the separations.

During the analysis, it has been observed that the new impurity content in clopidogrel tablets, were in the range of 0.05–0.07% (by area percentage) and in drug substance it ranges from 0.08 to 0.12% (by area percentage). Typical chromatograms of clopidogrel drug substance, drug product and drug product spiked with the related compound D were shown in Fig. 1. It is mandatory requirement from regulatory authorities, to identify and characterize any unknown impurity present in it at a level as low as 0.05% [7,8]. The presence of this impurity

Fig. 1. Typical HPLC chromatograms of (A) clopidogrel drug substance, (B) drug product and (C) drug products spiked with related compound D.
in tablets can have a significant impact on quality and safety of the important drug. The isolation of any impurity is required to find the response in an analytical method and also to validate the analytical procedure for its quantitative estimation. Even though the same impurity is formed during oxidation condition, there is no report on the isolation and characterization. Hence comprehensive study was undertaken to identify and characterize the oxidative impurity. The new oxidation impurity in this paper referred as related compound D. The chemical structures of clopidogrel, and related compound D were shown in Fig. 2.

2. Experimental

2.1. Materials and methods

Clopidogrel bisulphate was purchased from Dr. Reddys Laboratories Ltd., Hyderabad, India and clopidogrel tablets of brand name Plavix manufactured by Sanofi Pharma Bristol Myers Squibb Inc. were used. Potassium phosphate and ammonium acetate, GR grade was obtained from E. Merck, India. Methanol, acetonitrile of HPLC grade were obtained from E. Merck, India. Purified water was collected through Milli-Q water purification system (Millipore, USA). Dimethylsulphoxide-d$_6$ (DMSO-d$_6$) was purchased from Aldrich Chemical Co., USA and all other chemical used were of analytical grade.

2.2. Analytical methods

Analytical method was developed using Waters HPLC system consisting Alliance integrated hardware of quaternary solvent delivery module, auto sampler and PDA detector. Data was processed through Waters Empower software Version 1.63. Hypersil BDS C8 column (Thermo Electron Corporation) with dimensions of 250 mm x 4.6 mm i.d. packed with 5.0 µ particle size was employed along with gradient conditions for the separations. The gradient involves two mobile phases consisting of acetonitrile–potassium phosphate buffer (pH 2.3; 10 mM) (20:80, v/v) as solvent A and acetonitrile–potassium phosphate buffer (pH 2.3; 10 mM) (80:20, v/v) as solvent B. The gradient program employed with a timed gradient program of T (min)/%B (v/v): 0.01/0, 5/0, 15/15, 40/30, 45/0, 60/0 for the separations. Flow rate was kept at 1.0 ml/min and the column eluent was monitored at 220 nm for 60 min.

2.3. Preparative HPLC method

Preparative HPLC system used was a Waters system equipped with W 600 quaternary solvent delivery module Delta prep 2487 dual wavelength UV detector. Data was processed through Waters empower software. An Xierra MS C18 ODB HPLC column (Waters, Ireland) with dimensions 100 mm x 30 mm packed with 5.0 µ particle size was used for preparative work. The gradient conditions employed for the separations with a timed gradient program of T (min)/%B (v/v): 01/10, 06/10, 16/95, 17/95, 18/100, 23/100, 28/10, 32/10. Flow rate was kept at 20 ml/min and the column eluent was monitored at 220 and 300 nm for about 32 min.

2.4. Infra-red spectroscopy

The IR spectra were recorded in the solid state as KBr as dispersion using Shimadzu FT-IR 8700 with DRS technique.

2.5. Mass spectrometry

The isolated compound was dissolved (about 0.05 mg/ml) in methanol containing of 0.1% formic acid (v/v) and infused into the ion source by the syringe pump at the rate of 10 µl/min. The mass spectrum of the isolated degradation product was acquired on a Finnegan LCQ instrument from Thermoquest (San Jose CA) in positive spray ionization (ESI+) mode. The spray potential was set at 5.6 kV and the capillary temperature at 220 °C. Mass range was scanned between 100 and 500 amu. The mass spectrum was also recorded in negative spray ionization (ESI−) mode. The spray potential was set at 5.6 kV and the capillary temperature at 220 °C.

2.6. Nuclear magnetic resonance

$^1$H (400.13 MHz) and $^{13}$C (100.62 MHz) NMR spectra of isolated related compound D was recorded on an Avance DPX-400 MHz spectrometer Bruker (Germany). The probe was a $^1$H/$^{13}$C 5 mm, 3 axis gradients (x, y, z), optimized for inverse detection. Spectra were recorded in DMSO-d$_6$ (5-mm tubes) at 300 K. Sample concentration was 0.6 mg in 0.6 ml. The residual protonated resonance of the solvent (DMSO-d$_6$) was used as an internal chemical shift standard, which was related to tetramethylsilane with chemical shifts of 2.5 and 39.2 ppm, respectively, for $^1$H and $^{13}$C. Processing of the raw data were performed using Bruker XWinNmr software. The pulse conditions were 90° pulse, 9.4 µs (attenuation 0db) for $^1$H and 30° pulse, 11.75 µs (attenuation 0db) for $^{13}$C. Gradient pulses
used in this study were all shaped to a sine envelope with 1 ms duration (DQF-COSY, and $^1$H/$^{13}$C HSQC). Spectral width was 5431.88 Hz for proton and 18111.66 Hz for carbon.

2.7. Preparation of degradation samples of clopidogrel

2.7.1. Acid and base degradation
A solution of clopidogrel bisulphate (500 mg) in 50 mL of 0.1 N hydrochloric acid was kept at 80°C for 60 min. Another sample was prepared in a similar manner by treating clopidogrel bisulphate (500 mg) in 50 mL of 0.1 N sodium hydroxide.

2.7.2. Peroxide degradation
A solution of clopidogrel bisulphate (500 mg) in methanol (0.5 mL) and hydrogen peroxide (5% in water, 5 mL) was kept at 60°C for 3 h. Similarly 10 tablets of clopidogrel bisulphate were powdered (equivalent to 500 mg of clopidogrel bisulphate) was dissolved in 0.5 ml of methanol and 5 ml of hydrogen peroxide (5%) and the solution was kept at 60°C for 3 h.

2.7.3. Thermal degradation
Clopidogrel bisulphate (1.0 g) was moistened with water and was kept in an oven maintained at 120°C for 24 h.

2.8. Analysis of degradation samples by analytical LC

The degradation samples were diluted to the required concentration analyzed with analytical LC. 2 and 7% of related compound D was found in the drug substance and drug product (Plavix), respectively, at oxidative condition. In other con-
2.9. Isolation of degradation product(s) by preparative LC

Clopidogrel bisulphate (5 g) was treated with hydrogen peroxide (5%, 15 mL) and kept at 80 °C for 3 h. The aqueous layer was washed with dichloromethane to remove clopidogrel. The aqueous layer was subjected to preparative LC as described in Section 2.4 and as many as 10 fractions were collected separately. Purity of all these fractions were analyzed by analytical LC and found to be in the range of 99%. The fractions were pooled together, 100 mg of sulfuric acid was added and the solvent was evaporated. The resulted solid was reanalyzed on analytical LC and the purity of the same was found to be 99% which was good enough for carrying out the spectroscopic experiments.

2.10. Characterization of the degradation product

The isolated related compound D was injected in both the analytical HPLC methods. The retention time and UV spectrum obtained in the PDA detector matches with that of targeted impurity. Characterization of the related compound D was performed using analytical data obtained from IR, UV, Mass, MS\textsuperscript{n} experiments, \textsuperscript{1}H/\textsuperscript{13}C NMR spectrum, DEPT and 2D NMR experiments. The MS–MS spectrum is presented in Fig. 4.
3. Results and discussion

In forced oxidative degradation study 2 and 7% of related compound D was found in the drug substance and drug product (Plavix), respectively. Hence the experiment was used to enrich the impurity. It was also confirmed that related compounds A and C are the degradation products and related compounds B1 and B2 are process impurities based on their trend. The higher level of related compound D in the drug product reveals that the susceptibility is more in the drug product than that in drug substance.

The isolated related compound D was found as off white powder and shows UV absorbance maxima at 299.9 nm which is higher than that of clopidogrel (220 nm). The +ve ES–MS spectrum of the related compound D showed peaks at m/z 320 and 322 corresponding to the 35Cl and 37Cl isotope, respectively. The compound does not form Lithium adduct ion and −ve ES–MS spectrum showed no peaks states that the molecular ion obtained is positively charged, i.e., m/z 320/322 is due to M+.

In comparison with clopidogrel, the related compound D corresponds to 1 atomic mass unit (amu) less which can be presumed to have similar structure as that of clopidogrel but with short of one hydrogen atom.

Two daughter ions were obtained at m/z 183 and 155 when the molecular ion M+ 320 fragmented in MS/MS experiments. Both the daughter ions were contains chlorine atom as it was confirmed by MS/MS experiments of the chorine isotope molecular ion M+322 and the m/z values of the daughter ions were 185 and 157. As the peak intensity ratios are nearly identical, it was confirmed that the eliminated neutral fragments contains no chlorine atom. Further MS2 experiments of daughter ion (m/z 183) showed the formation of a fragmentation ion at m/z 155 and MS3 shows the formation of a ion at m/z 125. Similar experiments were performed with the 37Cl isotope of daughter ion. Of m/z 185 and fragmentation ions of m/z 157 and 127 were found. Based on the fragmentation data, the structure for related compound D was assigned as shown in Fig. 1 and its probable fragmentation pathway is given in Scheme 1.

The fragmentation pathway of related compound D was compared with the clopidogrel fragmentation pathway given in Scheme 2 [9]. It was found that m/z value of the clopidogrel daughter ion is 212/214 but the same was not observed in the case related compound D. The fragmentation ion obtained from MS2 experiments of clopidogrel were 183/185 and 155/157 which is similar to that of the daughter ion of related compound D. Similar fragmentation was not observed in related compound D, due to the double bond associated with nitrogen atom. However, the fragmentation ion obtained from MS2 experiments of clopidogrel were similar to that of the daughter ion obtained from related compound D. This was confirmed by MS3 and MS4 experiments.

1H NMR spectrum of related compound D is slightly different from that of clopidogrel 1H NMR spectrum and exhibits one hydrogen less than that of clopidogrel. Comparison of 1H, 13C and 135 DEPT NMR data with that of clopidogrel (Table 1) shows that the related compound D has one methylene proton less and one methine proton more than that of clopidogrel. The methine protons at 2nd and 3rd positions are deshielded to the extend of 0.3, 0.7 ppm, respectively. The methylene protons at 6th and 7th positions are deshielded to the extend of 0.5, 0.4 ppm, respectively. The presence of methylene protons at 6th and 7th positions was confirmed by irradiation (proton decoupling) experiment. Irradiation of protons at chemical shift δ 4.30 ppm (m) affects the multiplicity of protons at δ 3.94 and 3.80 ppm which further reveals that the double bond position. The appearance of one singlet at δ 9.19 ppm at 4th position and the deshielding of methine proton at 10th position from δ 5.60 to δ 6.66 ppm were also noticed. All the above deshielding con-

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Position</th>
<th>Clopidogrel bisulphate</th>
<th>Related compound D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H 7.43/m, J 4.8, 125.72 CH</td>
<td>H 7.69–7.71/d, 5.2, 128.66 CH</td>
</tr>
<tr>
<td>2</td>
<td>H 6.88/d, J 4.8, 126.03 CH</td>
<td>H 7.55–7.57/d, 5.2, 128.94 CH</td>
</tr>
<tr>
<td>3</td>
<td>2H 4.24/bs, J 50.89 CH2</td>
<td>H 9.21/s, 162.51 CH</td>
</tr>
<tr>
<td>4</td>
<td>– – – – – – – – – – –</td>
<td>– – – – – – – – –</td>
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<tr>
<td>5</td>
<td>– – – – – – – – – –</td>
<td>– – – – – – – – –</td>
</tr>
<tr>
<td>6</td>
<td>2H 3.43/bs, J 49.62 CH2</td>
<td>2H 3.88–3.91, 4.23–4.30/m, 7.6 and 14.8, 48.49 CH2</td>
</tr>
<tr>
<td>7</td>
<td>2H 3.06/bs, J 22.63 CH2</td>
<td>2H 3.45/t, 7.6 and 14.8, 23.31 CH2</td>
</tr>
<tr>
<td>8</td>
<td>– – – – – – – – –</td>
<td>– – – – – – – – –</td>
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<td>9</td>
<td>– – – – – – – – –</td>
<td>– – – – – – – – –</td>
</tr>
<tr>
<td>10</td>
<td>1H 5.60/s, J 65.87 CH</td>
<td>1H 6.65/s, 70.94 CH</td>
</tr>
<tr>
<td>11</td>
<td>– – – – – – – – –</td>
<td>– – – – – – – – –</td>
</tr>
<tr>
<td>12</td>
<td>1H 7.51/m, J 131.22 CH</td>
<td>1H 7.58–7.61/m, 132.79 CH</td>
</tr>
<tr>
<td>13</td>
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<td>1H 7.65/d, 7.2, 128.88 CH</td>
</tr>
<tr>
<td>14</td>
<td>1H 7.57/m, J 132.08 CH</td>
<td>1H 7.50–7.54/m, 133.14 CH</td>
</tr>
<tr>
<td>15</td>
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<td>1H 7.66/d, 7.2, 131.07 CH</td>
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<tr>
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<td>– – – – – – – – –</td>
<td>– – – – – – – – –</td>
</tr>
<tr>
<td>18</td>
<td>3H 3.75/s, J 54.33 CH3</td>
<td>3H 3.85/s, 54.46 CH3</td>
</tr>
</tbody>
</table>

a Refer structural formula (Fig. 1) for numbering: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad.
b Unresolved.
firmed that there is a structural change in the piperidine ring. It was also confirmed by $^1$H--$^1$H COSY spectrum. There is no appropriate change in the aromatic ring for the presence of the aromatic ring in the related compound D. $^{13}$C and DEPT 135 NMR spectra results indicate the presence of one methyl carbon, two methylene carbons, eight methine carbons and five quaternary carbons. Related compound D shows five quaternary carbons like that of clopidogrel but one methylene carbon less and one methine carbon more.

The methylene signal at $\delta$ 49.62 ppm disappeared and a new methine signal at $\delta$ 162.51 ppm is observed. The high deshielding chemical shift value of the methine signal indicates that carbon may be attached to an electronegative atom or attached to aromatic ring or under the anisotropic influence of an aromatic ring. This led to the hypothesis of existence of C=N in the piperidine ring of related compound D structure. The position of the double bond was assigned to 4th carbon due the possibility of extended conjugation explained for the shifting of UV absorbance maxima to the higher wavelength. This was also confirmed by the deshielding of aliphatic methine carbon at 10th position to about 5 ppm and the deshielding of one of the quaternary carbon at 8th position to about 20 ppm. The presence of C=N was further confirmed by the IR characteristic absorption peak at 1469 cm$^{-1}$.

All the above observations can be well explained on the basis of the proposed structure (Fig. 1) with quaternary nitrogen and a double bond. All proton signals were assigned on the basis of $^1$H NMR and $^1$H--$^1$H COSY spectral results. Carbon signals were assigned on the basis of DEPT135 and $^1$H--$^{13}$C HSQC spectral results.

Based on the above spectral data, the structure was characterized as 5-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-6,7-dihydrothieno [3,2-c] pyridin-5-ium with a molecular weight of 320 amu.

4. Conclusions

The major oxidative degradation product related compound D in clopidogrel drug substance as well as drug product was isolated by preparative LC and was characterized by using spectroscopic techniques namely NMR, MS and MS$^n$.

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The authors wish to thank the management of Torrent Pharmaceuticals Ltd. and Torrent Research Center for allowing us to carry out the present work. The authors are also grateful to the Director Dr. C. Dutt of Torrent Research Center for his constant encouragement. The authors also wish to thank friends and the other colleagues of Torrent Research Center for their cooperation.

References

Identification, Isolation, and Characterization of Five Potential Degradation Impurities in Candesartan Cilexetil Tablets

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Abstract

Five impurities were observed when candesartan cilexetil tablets were subjected to stability and forced degradation studies. These impurities were successfully isolated and characterized as desethyl candesartan cilexetil, 1N-ethyl candesartan cilexetil, 2N-ethyl candesartan cilexetil, 1N-ethyl oxo candesartan cilexetil, and 2N-ethyl oxo candesartan cilexetil. A gradient reverse phase liquid chromatography (LC) and an isocratic preparative LC method were used to detect and isolate all five degradation products impurities simultaneously. Mass spectrometry, 1H/13C, DEPT and 2D NMR experiments were extensively utilized to characterize these impurities. Even though desethyl candesartan cilexetil, 1N-ethyl candesartan cilexetil were 2N-ethyl candesartan cilexetil were documented in the literature as known impurities, the regioisomers 1N-ethyl oxo candesartan cilexetil and 2N-ethyl oxo candesartan cilexetil were never noticed. Single-crystal diffraction data has been used to confirm their structure unambiguously and synthetic preparations of all known and unknown impurities were also presented.

Keywords

Column liquid chromatography-mass spectrometry
NMR spectroscopy
Candesartan cilexetil degradation products

Introduction

Candesartan cilexetil, (±)-1-(cyclohexyl-oxycarbonyloxy)-ethyl-2-ethoxy-1-[2'- (1H-tetrazol-5-yl) biphenvl-4-yl] methyl] benzimidazole-7-carboxylate (Fig. 1), is a nonpeptide antihypertensive drug and used either single or in combination with other drugs, to treat high blood pressure. In the gastrointestinal tract candesartan cilexetil is converted to candesartan, an angiotensin II receptor antagonist (ARA II) which blocks the ability of angiotensin II to raise blood pressure by constricting or squeezing arteries and veins and ultimately leads to a reduction in blood pressure. It also reduced the work of heart by reducing the pressure against which the heart must pump blood, and is useful in patients with heart failure [1–3]. It is marketed under the brand name of Atacand and in combination with a thiazide diuretic drug hydrochlorothiazide (HCTZ) as Atacand HCT by Astra Zeneca.

Several impurities of candesartan cilexetil have been identified and documented in the literature [4–6]. Identification of the impurities and analytical method for quantitation in drug substances, drug products and plasma samples has been described. Stenhoff et al. have reported the identification of hydroxy candesartan or desethyl candesartan (metabolite) and candesartan in plasma samples [4]. Ferreirós et al. have reported the base hydrolytic impurities of candesartan cilexetil, i.e.; candesartan and its transesterification product of methyl ester [5]. Recently Rao et al. [6] established candesartan, candesartan methyl ester, candesartan ethyl ester, hydroxy candesartan cilexetil or desethyl
candesartan cilexetil, 1-N-ethyl candesartan cilexetil and 2-N-ethyl candesartan cilexetil as impurities using an isocratic RP-LC method. Candesartan is known to be an active drug and candesartan methyl and ethyl ester are process impurities, and the rest are degradates.

During accelerated stability studies (40 °C and 75% relative humidity) for 3 months and forced degradation study (thermal, 60 °C) for 2 weeks, three degradates were found to be the same as those reported by Rao et al. Surprisingly, two additional unknown degradates were also observed which are found to be the reporting threshold (0.05%) as per ICH guidelines [7, 8]. In terms of efficacy and patient safety it is important to isolate and identify impurities and/or degradates to ensure that their presence will not evoke any form of adverse response either pharmacologic or toxicologic in a patient taking medication. Moreover reference materials of all individual impurities were required to validate the method. Hence a comprehensive study was undertaken to isolate all impurities simultaneously and characterize them.

**Experimental**

**Materials**

Atacand tablets were purchased from the market and candesartan cilexetil were synthesized at Torrent Research Centre, Gandhi Nagar, India. LC grade acetonitrile, analytical grade ammonium acetate, acetic acid, spectroscopic grade deuteriated dimethylsulphoxide-d6 (DMSO-D6) and chloroform (CDCl3) were purchased from Merck, Darmstadt, Germany. High pure water was prepared by using Millipore Milli-Q plus water purification system.

**Chromatography**

Samples were analyzed using Waters Alliance 2695 LC system (Waters Corporation, Milford, MA, USA) consisting of an integrated hardware of quaternary solvent delivery module, auto sampler and a 2996 photo diode array detector. Data was processed through Waters Empower software. A gradient analytical method with Purosphere Star RP18e column of 150 x 4.6 mm i.d packed with 5 μm particle size was employed for separation. The gradient involved two mobile phases consisting of acetonitrile–ammonium acetate (pH 5.0; 20 mM) (10:90 v/v) as solvent A and acetonitrile–ammonium acetate (pH 5.0; 20 mM) (90:10 v/v) as solvent B. The gradient program employed a timed gradient program of T (min)/%B (v/v): 0.01/0, 5/5, 15/35, 35/65, 40/75, 45/100, 55/100, 60/0, 65/0, for the separations. Flow rate was kept at 1.2 mL min⁻¹ and the column eluent was monitored at 254 nm for 65 min. The analytical method was validated as per ICH guidelines for various parameters such as specificity, precision, linearity, accuracy, limit of detection, limit of quantitation, ruggedness and robustness [9].

Preparative LC was performed in a Waters system equipped with W 600 quaternary solvent delivery module Delta prep 2487 dual wavelength UV detector using an YMC pack ODS A column of 250 x 20 mm i.d packed with 5 μm particle size. Data was
processed through Waters Empower software. Acetonitrile–trifluoroacetic acid buffer (0.1%) (60:40. v/v) was used as mobile phase and flow rate was kept at 30 mL min⁻¹ with the detection at 254 nm for 70 min.

**Infra-Red Spectroscopy**

The IR spectra were recorded in the solid state as KBr dispersion (DRS technique) using Bruker FT-IR Tensor 27 (Ettlingen, Germany) in the range of 400–4,000 cm⁻¹ with a resolution of 4.0 cm⁻¹.

**Mass Spectrometry**

Pneumatically assisted electrospray ionization (ESI) mass spectrometric analyses were performed using a single-quadrupole mass spectrometer (Micro-mass ZQ-2000, Beverly MA, USA). Operating in the positive ion mode, the spray needle was held at a potential of 5.6 kV. The skimmer cone potential, which affects the degree of collisional activation in the source, was set to 45 V. The source temperature was maintained at 150 °C. It was also operated in the negative ion mode with −4.5 kV spray needle potential. Spectra were collected at a rate of 12 points per peak while scanning from m/z 100 to 1,000.

**Nuclear Magnetic Resonance**

¹H (400.13 MHz) and ¹³C (100.62 MHz) NMR spectra of isolated impurities were recorded on an Avance DPX-400 MHz spectrometer Bruker (Silberstreifen, Germany). The probe was a ¹H/¹³C 5 mm, 3 axis gradients (x, y, z), optimized for inverse detection. Spectra were recorded in deuteriated solvents (5 mm tubes) at 300 K. Sample concentration was 0.6 mg in 0.6 mL. The residual protonated resonance of the solvent was used as an internal chemical shift standard, which was related to tetramethylsilane with chemical shifts of 2.5 and 39.2 ppm, respectively for ¹H and ¹³C. Processing of the raw data was performed using Bruker XWinNmr software. The pulse conditions were 90° pulse, 9.4 μs (attenuation 0db) for ¹H and 30° pulse, 11.75 μs (attenuation 0db) for ¹³C. Gradient pulses used in this study were all shaped to a sine envelope with 1 ms duration (DQF-COSY, and ¹H/¹³C HSQC). Spectral width was 5431.88 Hz for proton and 18111.66 Hz for carbon.

**X-Ray Crystallographic Analysis**

The single crystal was grown by slow evaporation technique using ethanol solvent system. X-ray diffraction analysis was carried out on a Bruker Smart Apex CCD diffractometer at room temperature. The crystal was monoclinic, (space group C2/c) with a unit cell a = 16.3770 (7) Å, b = 8.5928 (4) Å, c = 43.7735 (19) Å, β = 91.150(1)°, V = 6158.7 (5) Å³, Z = 8. Density 1.317 mg/m³. Absorption coefficient (μ) = 0.093 mm⁻¹, CuKα radiation (λ = 0.71073 Å). A total of 5,399 reflections with F > 2σ (F) gave R = 0.0452 with 412 parameters refined. The structure was elucidated by direct methods (Siemens SHELXTL PLUS). Final refinement of the non hydrogen atoms was done by full-matrix least-square refinement.

**Analysis of Degradation Samples by Analytical LC and LC-MS**

The drug product samples kept at an accelerated stability condition for 3 months, and exposed directly at 60 °C for 2 weeks were diluted to the required concentration and analyzed with analytical LC. All the five impurities were enriched and found in the range of 6–40%. The autoclaved material was dissolved in the mobile phase to obtain a concentration of 50 mg mL⁻¹ and the solution (2 mL) was subjected to preparative LC and as many as 25 fractions were collected separately. The typical chromatogram of preparative LC is given in the Fig. 4. Purity of all these fractions were analyzed by analytical LC and found to be in the range of 99%. The eluent was evaporated and the resultant solution of Impurities-II and III were filtered individually to separate solid and washed with chilled water to remove traces of trifluoro acetic acid. The solid was dried at 60 °C under vacuum for 3 h. The impurities from other resultant solutions were isolated using a freeze drier maintained at −48 °C for 8 h. Thus the isolated impurities were reanalyzed on analytical LC and the purity of the same was found to be 99% which was good enough for carrying out the spectroscopic experiments.

**Isolation of Degradation Product(s) by Preparative LC**

Candesartan cilexetil (5 g) was autoclaved for 3 h and the material was diluted to the required concentration and analyzed with analytical LC. All the five impurities were enriched and found in the range of 6–40%. The autoclaved material was dissolved in the mobile phase to obtain a concentration of 50 mg mL⁻¹ and the solution (2 mL) was subjected to preparative LC and as many as 25 fractions were collected separately. The typical chromatogram of preparative LC is given in the Fig. 4. Purity of all these fractions were analyzed by analytical LC and found to be in the range of 99%. The eluent was evaporated and the resultant solution of Impurities-II and III were filtered individually to separate solid and washed with chilled water to remove traces of trifluoro acetic acid. The solid was dried at 60 °C under vacuum for 3 h. The impurities from other resultant solutions were isolated using a freeze drier maintained at −48 °C for 8 h. Thus the isolated impurities were reanalyzed on analytical LC and the purity of the same was found to be 99% which was good enough for carrying out the spectroscopic experiments.
**Fig. 2.** Typical LC chromatograms of candesartan cilexetil tablets.
Synthesis of Impurities

Candesartan cilexetil (50 g) was dissolved in dichloromethane (250 mL) at 25–30 °C and hydrochloric acid (25%) was added. The mixture was stirred for 24 h and the solvent was evaporated. The solid was filtered and washed with chilled water. Impurity I thus obtained was dried at 60 °C under vacuum for 3 h to give 45 g with a chromatographic purity of 99% by area normalization.

To a suspension of impurity I (40.0 g) in N,N-dimethyl formamide (250 mL) potassium carbonate (19 g) and ethyl iodide (16.0 g) were added. The mixture was heated and stirred for 2.5 h at 70–75 °C. The mixture was then cooled and quenched with water. The solid was filtered and washed with cold water. The material was analyzed and found to have impurity II (30%) and impurity III (40%) in LC analysis.

The above solid material (20 g) was subjected to conventional column chromatography using 2:1 v/v hexane/ethyl acetate as eluent. The appropriate fractions were collected separately for impurities-II and III. The combined fractions were evaporated and the
impurities were solidified from the ethyl acetate solution at $\sim 0^\circ C$. White powder was collected by filtration and dried at 60 $^\circ C$ under vacuum for 3 h to give 3.0 g of Impurity II with chromatographic purity of 99% (by area normalization) and 4.0 g of Impurity III with chromatographic purity of 99% (by area normalization).

Impurities IV and V were synthesized in the similar manner as impurities II and III, using candesartan cilexetil as a starting material instead of impurity I. The route of synthesis is represented in the Fig. 5.

**Results and discussion**

**Detection of Impurities**

The initial study of the tablet showed five impurities in the levels of 0.01–0.3%. The stability study of the same tablet showed that all five impurities were increased to levels of 0.05–0.4%. The same trend was also observed during the thermal degradation studies. This showed that the molecule was prone to degradation. These five targeted impurities were marked as impurity I (RRT: 0.83, MW: 582), impurity II (RRT: 1.14, MW: 610), impurity III (RRT: 1.26, MW: 610), impurity IV (RRT: 1.36, MW: 638), and impurity V (RRT: 1.43, MW: 638).

**Structural Elucidation of Impurity-I**

The isolated impurity-I was found as a white powder and showed similar UV absorbance spectra to parent candesartan cilexetil. In the FT-IR spectrum, a characteristic absorption band appeared at 1,726 cm$^{-1}$ for $-\text{C=O}$ stretching and at 1,460 cm$^{-1}$ for $-\text{N H}$ stretching vibration. The negative ES-MS spectrum of impurity-I showed peaks at $m/z$ 581 atomic mass unit (amu) and the positive ES-MS spectrum showed peaks at $m/z$ 605 amu corresponding to the sodium adduct of the impurity. Hence molecular weight of impurity-I is confirmed as 582. In comparison with parent, impurity-I had 28 amu less which can be presumed to have a similar structure skeleton as that of parent but with short four hydrogen atoms and two carbon atoms.

In $^1$H spectrum of the impurity-I in dimethyl sulfoxide (DMSO), 16 protons appeared in the up-field region ($\delta$ 1.17–5.34 ppm), 12 in the downfield region ($\delta$ 6.69–7.67 ppm) and two acidic protons appeared at ($\delta$ 11.56 and 16.23 ppm). Deuterium oxide (D$_2$O) exchange experiment showed the presence of two exchangeable protons in the impurity whereas only one exchangeable proton was found for the parent. Comparison of $^1$H, $^{13}$C and 135 DEPT NMR data (Table I) showed that the methylene signal at $\delta$ 67.77 ppm and methyl signal at $\delta$ 14.40 ppm disappeared in the impurity-I spectrum which corresponded to 32 and 33 position in the parent. This has been supported by the mass data which was 28 amu smaller than that of the parent. This led to the conclusion that an ethyl group attached to an oxygen atom in the benzimidazole group is hydrolyzed, subsequently undergoes keto-enol tautomerism and stabilized as stable keto form. This was confirmed by the IR spectrum which revealed the
presence of –C=O stretching, –N H stretching vibration and absence of –O H stretching vibration.

Based on the above spectral data the molecular formula of impurity-I was confirmed as C_{31}H_{30}N_{6}O_{6} and the corresponding structure was characterized as 1-\{[(cyclohexyloxy) carbonyl] oxy\} ethyl-2-oxo-1-\{2-(1H-tetrazol-5-yl) biphenyl-4-yl\}methyl]-1H-benzimidazole-7-carboxylate. This impurity is referred as CNS desethyl (desethyl candesartan cilexetil) in the reported literature [10].

Structural Elucidation of Impurity-II

The isolated impurity-II was found as a white powder and showed similar UV absorbance spectra of parent compound. In FTIR spectrum a characteristic absorption band appeared at 1,750 cm\(^{-1}\) for –C=O stretching and at 1193 cm\(^{-1}\) for –N–C stretching vibration. The negative ES-MS spectrum of impurity-II showed a peak at \(m/z\) 609 amu and the positive ES-MS spectrum showed peaks at \(m/z\) 633 amu corresponding to the sodium adduct of the impurity. Hence molecular weight of impurity-II was confirmed as 610. In comparison to the parent, impurity-II had the same molecular weight, which can be presumed to have a similar structure as that of the parent with some possible positional isomerism.

In the \(1H\) spectrum of impurity-II in CDCl\(_3\), 21 protons appeared in the upfield region (\(\delta\) 0.84–5.54 ppm), 12 in the downfield region (\(\delta\) 6.82–7.64 ppm). One exchangeable proton (\(\delta\) 10.75 ppm) was observed in the impurity in the D\(_2\)O exchange experiment as that of the parent but the chemical shift shielded from \(\delta\) 13.95 to 10.75 ppm. Comparison of \(1H\), \(13C\) and \(135\) DEPT NMR data (Table 1) showed that the methylene signal at \(\delta\) 67.77 ppm disappeared and a new methylene signal at \(\delta\) 45.68 ppm was observed. This led to the hypothesis of possible rearrangement of the ethyl group from benzimidazole to tetrazole. The long range correlation (LRC) experiment predicted that the ethyl group attached to the first “N” atom of the tetrazole group.

Based on the above spectral data the molecular formula of impurity-II was confirmed as C_{33}H_{34}N_{6}O_{6} and the

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| 34 | 13.95(bs) NH | 10.75(bs) NH |}

\(^a,\) Refer structural formula (Fig. 1) for numbering: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad; \(^b\), Unresolved: dt-doubtlet of triplet
corresponding structure was characterized as 1-[(cyclohexyloxy) carbonyl]oxyethyl-2-oxo-1-[(2-0-(1-ethyl-1H-tetrazol-5-yl) biphenyl-4-yl)]methyl]-1H-benzimidazole-7-carboxylate. This impurity is named as 1N-ethyl oxo candesartan cilexetil.

**Structural Elucidation of Impurity-III**

The isolated impurity-III was found as white powder and showed similar UV absorbance spectra of the parent as well as Impurity-II. All the spectral data of impurity-III were similar to that of impurity-II. Comparison of $^1$H, $^{13}$C and DEPT NMR data (Table 2) showed that the methine signal corresponds to the first position at δ 154.31 ppm and deshielded to δ 165.01 ppm. Similarly the methylene signal corresponds to δ 45.68 ppm to 48.08 ppm. This led to the hypothesis of possible regio isomerism of the ethyl group attached to another “N” atom of tetrazole group. The existence of tetrazole regioisomers was reported in the literature [11]. Further this regio isomerism has been confirmed by single-crystal diffraction analysis. The single-crystal diffraction study confirmed that impurities II and III were regio isomers.

**Structural Elucidation of Impurity-IV**

The isolated impurity-IV was found as white powder and showed similar UV absorbance spectra as that of Table 2. NMR assignments for candesartan cilexetil and its impurities

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<td>28</td>
<td>1.23–1.31(m)</td>
<td>31.38 CH₂</td>
<td>1.21–1.43(m)</td>
</tr>
<tr>
<td>29</td>
<td>1.50–1.54(m)</td>
<td>25.18 CH₂</td>
<td>1.56 &amp; 1.51(m)</td>
</tr>
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<td>30</td>
<td>1.23–1.31(m)</td>
<td>23.6 CH₂</td>
<td>1.21–1.43(m)</td>
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<tr>
<td>31</td>
<td>1.70–1.93(m)</td>
<td>31.38 CH₂</td>
<td>1.73 &amp; 1.95(m)</td>
</tr>
<tr>
<td>32</td>
<td>4.39–4.45(m)</td>
<td>48.08 CH₂</td>
<td>4.60–4.67(m)</td>
</tr>
<tr>
<td>33</td>
<td>1.35(t)</td>
<td>14.4 CH₃</td>
<td>1.47(m)</td>
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<tr>
<td>34</td>
<td>9.75(bs)</td>
<td>NH CH₃</td>
<td>3.43–3.49(q)</td>
</tr>
<tr>
<td>35</td>
<td>0.83(t)</td>
<td>14.39 CH₃</td>
<td>1.3(t)</td>
</tr>
</tbody>
</table>

a. Refer structural formula (Fig. 1) for numbering: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad; b, Unresolved: dt-doublet of triplet

Based on the above data the molecular formula of impurity-III was confirmed as C₃₃H₃₄N₆O₆ and the corresponding structure was characterized as 1-[(cyclohexyloxy) carbonyl]oxyethyl-2-oxo-1-[(2-0-(1-ethyl-1H-tetrazol-5-yl) biphenyl-4-yl)]methyl]-1H-benzimidazole-7-carboxylate. This impurity is named as 2N-ethyl oxo candesartan cilexetil.
parent. In the FTIR spectrum, a characteristic absorption band appeared at 1,742 cm\(^{-1}\) for –C=O stretching. The negative ES-MS spectrum of impurity-IV showed a peak at \(m/z\) 637 amu and the positive ES-MS spectrum showed peaks at \(m/z\) 661 amu corresponding to the sodium adduct of the impurity. Hence molecular weight was confirmed as 638. The mass of impurity-IV was 28 amu higher than the parent, which can be presumed to have a similar structure skeleton as that of the parent with additional four hydrogen atoms and two carbon atoms.

In the \(^1\)H spectrum of impurity-I in CDCl\(_3\), 26 protons appeared in the upfield region (\(\delta\) 0.88–5.66 ppm), 12 in the downfield region (\(\delta\) 6.88–7.74 ppm). D\(_2\)O exchange showed absence of any exchangeable proton in the impurity. Comparison of \(^1\)H, \(^13\)C and 135 DEPT NMR data (Table 2) showed that impurity-IV has three methyl protons, eight methylene protons, thirteen methine protons and eleven quaternary carbons. All the spectral data has been compared to the parent and the presence of an extra methyl group is confirmed. The absence of acidic proton and LRC experiment confirmed that the additional ethyl group attached to the first “N” atom of the tetrazole group.

Based on the above spectral data the molecular formula of impurity-IV was confirmed as \(C_{35}H_{38}N_6O_6\) and the corresponding structure was characterized as 1-[(cyclohexyloxy) carbonyl] oxy]-ethyl-2-ethoxy-1-[(2’-(2-ethyl-1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benimidazole-7-carboxylate. This impurity is referred as 1N-ethyl CNS (1N-ethyl candesartan cilexetil) in the reported literature [10].

### Structural Elucidation of Impurity-V

Comparison of \(^1\)H, \(^13\)C and 135 DEPT NMR data (Table 2) showed that impurity-V was similar to that of impurity-IV. The methine carbon at first position was deshielded to extend of 5.2 ppm and the methylene carbon at 34th position was deshielded to an extent of 4.5 ppm compared to impurity-IV. Similar deshielding were found between regio isomers of impurities-II and III revealing that impurity IV and V are also another set of regioisomers. Based on the above spectral data the molecular formula of impurity-V was confirmed as \(C_{35}H_{38}N_6O_6\) and the corresponding structure was characterized as 1-[(cyclohexyloxy) carbonyl] oxy]-ethyl-2-ethoxy-1-[(2’-(2-ethyl-1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-1H-benimidazole-7-carboxylate. This impurity is referred as 2N-ethyl CNS (2N-ethyl candesartan cilexetil) in the reported literature [10].

### Validation of Analytical Method

Analytical LC method has been extensively validated for the quantitation of all the five degradates. Specificity was verified by means of blank, placebo interferences and forced degradation studies. The results of various validation parameters such as precision, linearity, accuracy, limit of detection and limit of quantitation are summarized in the Table 3. The data suggests that the method met the requirement of validation. Ruggedness and robustness of the method has also been confirmed. The relative response factors were established and values given in the Table 3.

### Formation of Impurities

The parent drug molecule undergoes hydrolysis which results the formation of impurity I and ethyl cation. The ethyl cation further reacts with impurity I to form impurities II and III which are regioisomers. The content of impurity III was observed to be higher in the stability samples than that of impurity II. The rationale for this might be due to the steric hindrances present in impurity II. The ethyl cation reacted with the parent drug molecules leading to the formation of another set of regioisomers (impurities IV and V). In this case also the impurity V content is higher than that of impurity IV.

### Conclusions

The five major degradation products of candesartan cilexetil were isolated by preparative LC and characterized by using spectroscopic techniques namely IR, UV, NMR, MS and single-crystal X-ray diffraction data. The structures...
were supported by synthetic ethylating reaction of candesartan cilexetil and impurity I which allowed preparation of four impurities.

Acknowledgments

The authors wish to thank the management of the Torrent Research Center for allowing us to carry out the present work. The authors are also grateful to Dr. C. Dutt, Director, Dr. Sunil S. Nadkarani, Vice President and Mr. P. C. Gandhi, Vice President of Torrent Research Center for their constant encouragement. The authors also wish to thank friends and the other colleagues of the Torrent Research Center for their cooperation.

References

3. www.Rxlist.com
1-[(Cyclohexyloxy)carbonyl]oxy]ethyl 3-[[2’-(2-ethyl-2H-tetrazol-5-yl)biphenyl-4-yl]methyl]-2-oxo-2,3-dihydro-1H-benzimidazole-4-carboxylate

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Key indicators: single-crystal X-ray study; T = 293 K; mean σ(C–C) = 0.003 Å;
R factor = 0.054; wR factor = 0.143; data-to-parameter ratio = 17.6.

In the title compound, C35H34N4O6, the dihydrobenzimidazole-2-one ring system is essentially planar (r.m.s. deviation = 0.021 Å). The cyclohexane ring adopts a chair conformation. In the 5-(biphenyl-2-yl)-2H-tetrazole fragment, the tetrazole ring is twisted away from the attached benzene ring by 35.73 (11)° and the two benzene rings form a dihedral angle of 68.00 (9)°. An intramolecular C—H···O interaction is observed. In the crystal, the molecules are linked into a zigzag chain running along the b axis by intermolecular N—H···O hydrogen bonds.

Related literature
For applications of tetrazole derivatives in coordination chemistry, medicinal chemistry and materials science, see: Dunica et al. (1991); Wittenberger & Donner (1993); Xiong et al. (2002); Xue et al. (2002). For metal-organic coordination compounds with tetrazole ligands, see: Hu et al. (2007); Lü (2008). For puckering parameters, see: Cremer & Pople (1975).

Experimental
Crystal data
C35H34N4O6
M r = 610.66
Monoclinic, C2/c
α = 16.3770 (7) Å
b = 8.5928 (4) Å
c = 45.7335 (19) Å
β = 2915.0 (1)°
V = 6158.7 (5) Å 3
Z = 8
Mo Kα radiation
μ = 0.09 mm−1
T = 293 K
0.19 × 0.14 × 0.08 mm

Data collection
Bruker Kappa APEX2 area-detector diffractometer
Absorption correction: multi-scan (SADABS; Sheldrick, 2001)
T min = 0.984, T max = 0.993
34307 measured reflections
7255 independent reflections
5559 reflections with I > 2σ(I)
R int = 0.026

Refinement
R[F 2 > 2σ(F 2)] = 0.054
wR(F 2) = 0.143
S = 0.99
7255 reflections
412 parameters

Table 1
Hydrogen-bond geometry (Å, °).

<table>
<thead>
<tr>
<th>D—H···A</th>
<th>D—H</th>
<th>H···A</th>
<th>D···A</th>
<th>D—H···A</th>
</tr>
</thead>
<tbody>
<tr>
<td>N16—H16⋯O6</td>
<td>0.85 (2)</td>
<td>2.04 (2)</td>
<td>2.8588 (19)</td>
<td>161 (2)</td>
</tr>
<tr>
<td>C20—H20A⋯O5</td>
<td>0.97</td>
<td>2.22</td>
<td>3.004 (2)</td>
<td>137</td>
</tr>
</tbody>
</table>

Symmetry code: (i) −x+1/2, y+1/2, −z+1/2

Data collection: APEX2 (Bruker, 2004); cell refinement: SAINT (Bruker, 2004); data reduction: SAINT; program(s) used to solve structure: SHELXS97 (Sheldrick, 2008); program(s) used to refine structure: SHELXL97 (Sheldrick, 2008); molecular graphics: ORTEP-3 (Farrugia, 1997); software used to prepare material for publication: SHELXL97 and PLATON (Spek, 2009).

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Supplementary data and figures for this paper are available from the IUCr electronic archives (Reference: CI5048).

References
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30-Oct-2010

Dear Dr. Mohan:

It is a pleasure to accept your manuscript entitled "Development of a validated Reversed Phase UPLC Method for Related Substances and Assay of Lacidipine" in its current form for publication in the Journal of AOAC INTERNATIONAL.

Thank you for your fine contribution. On behalf of the Editors of the Journal of AOAC INTERNATIONAL, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Samir Wahab
Section Editor, Journal of AOAC INTERNATIONAL
szw@usp.org
Dear Dr. Mohan,

This is to inform you that your revised manuscript titled "Development and Validation of a Dissolution Method for novel fixed dose combination of Etodolac and Propranolol hydrochloride Tablets by RP-HPLC" underwent the consecutive evaluation procedure and finally it was found as valid and acceptable for publication in *Acta Chromatographica*. It was scheduled for issue no. 4 /2011 of our journal (i.e., for the December, 2011 issue).

Thank you for publishing with us.

Kind regards,

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