Article

User-Friendly HPLC Method Development and Validation for Determination of Enalapril Maleate and Its Impurities in Enalapril Tablets

Srinivasarao Koppala*, V. Ranga Reddy, and Jaya Shree Anireddy

Centre for Chemical Sciences and Technology, Institute of Science and Technology, Jawaharlal Nehru Technological University Hyderabad, Kukatpally, Hyderabad 500085, Telangana, India

*Author to whom correspondence should be addressed. Email: srinivasaraokoppala@gmail.com

Received 7 June 2016; Revised 3 May 2017; Editorial Decision 12 June 2017

Abstract

The official method for the determination of Enalapril Maleate and its related substances in European Pharmacopoeia (EP) is a gradient liquid chromatographic method. The method used styrene–divinylbenzene copolymer column, mobile phase buffer pH 6.8 and column oven temperature 70°C.

In this method, the separation between main component Enalapril and Ph. Eur. Imp-A was not completed hence the achieving system suitability requirement is a tough task and it requires quite often adjustment in chromatographic parameters. Moreover, column oven temperature 70°C is not user friendly to HPLC instruments and users. In this study, several changes were introduced to the method in order to improve the separation, peak shapes and to overcome the column oven temperature. A new user-friendly stability-indicating RP-HPLC method was developed for Enalapril related substances analysis. The developed method uses a ZORBAX Eclipse XDB-C18 column with column oven temperature at 55°C and mobile phase containing acetonitrile and a phosphate buffer at pH 3.0. The method is capable of separating all the known impurities with resolution more than 3.5, which is much better than that obtained with the existing monograph methods. The optimized method was validated and demonstrated to have acceptable specificity, sensitivity, linearity, accuracy, precision, robustness, solution stability and equivalency to the EP method. The developed method proved to be applicable to a wide number of C18 reversed-phase columns. In addition, the Enalapril assay method also presented with 20 min run time.

Introduction

Enalapril Maleate (ENP) is chemically described as (2S)-1-[(2S)-2-[[[(1S)-1-(Ethoxycarbonyl)-3-phenylpropyl] amino]propanoyl]pyrrolidine-2-carboxylic acid (Z)-butenedioate (Figure 1A). Its empirical formula is C_{20}H_{28}N_{2}O_{5}·C_{4}H_{4}O_{4} and molecular weight is 492.53 gm/mol. The drug was approved by United States Food and Drug Administration with brand name of VASOTEC and also approved by European Medicines Agency with brand name RENITEC. Enalapril is a pro-drug that belongs to the angiotensin-converting enzyme (ACE) inhibitor class of medications. It is rapidly metabolized in the liver to enalaprilat following oral administration. Enalapril is used for the treatment of essential or renovascular hypertension and symptomatic congestive heart failure (1–2). The drug is available in the market with 2.5, 5, 10 and 20 mg dosage forms.

Extensive literature searches revealed only a few analytical techniques were found for determining ENP, including the following methods: UV spectrophotometry (3–5), HPLC estimation (6–15), High Performance Thin Layer Chromatography (16) and LC–MS estimation (17–19). All of the listed techniques are applied to the assay of ENP.

ENP related substances method is official in European Pharmacopoeia (EP) (20) and six impurities (Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-H) have been specified in EP. In these methods, a styrene–divinylbenzene copolymer column with mixture of
sodium dihydrogen phosphate (0.02 M, pH 6.8), acetonitrile (ACN), were used as the mobile phase. The mobile phase flow was 1.0 mL/min in gradient elution mode at a column temperature of 70°C.

The reported EP monograph method has the following drawbacks (i) Achieving EP system suitability requirement (peak-to-valley ratio: minimum 10 for Imp-A and Enalapril) is a tough task, even with a state-of-the-art instrument and column. It requires quite often adjustment in chromatographic parameters to meet the system suitability (ii) The used column oven temperature 70°C is not user friendly to instruments and users since the maximum limit of column oven temperature is 65°C only for Waters and Shimadzu HPLC instruments hence separate column oven box is required to maintain 70°C (iii) Noticed the placebo interference at Imp-C and Imp-A peaks (iv) After few injections broad peak shapes were observed for all known and unknown impurities hence the above method cannot be operated at the optimum conditions. In contrast, the present communication comprising of the new user-friendly HPLC method which is capable of separating all impurities with resolution more than 3.5 using ZORBAX Eclipse XDB-C18 column with column oven temperature at 55°C and mobile phase pH at 3.0. The developed was more superior to the existing monograph methods. The literature survey reveals that no reference exists for the quantitative determination of impurities by a stability-indicating HPLC method with the exemption of EP and USP monographs (20–21). Hence, it was felt necessary to develop the user friendly, selective, specific, sensitive and robust stability-indicating LC method for the

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Name</th>
<th>Chemical Structure</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ENP</td>
<td><img src="ENP.png" alt="ENP" /></td>
<td>((2S)-1-([2S]-2-[([1S]-1-(Ethoxycarbonyl)-3-phenylpropyl]amino)propanoyl]pyrrolidine-2-carboxylic acid (Z)-butenedioate)</td>
</tr>
<tr>
<td>B</td>
<td>Imp-A</td>
<td><img src="Imp-A.png" alt="Imp-A" /></td>
<td>((2S)-1-([2S]-2-[([1R]-1-(Ethoxycarbonyl)-3-phenylpropyl]amino)propanoyl]pyrrolidine-2-carboxylic acid)</td>
</tr>
<tr>
<td>C</td>
<td>Imp-B</td>
<td><img src="Imp-B.png" alt="Imp-B" /></td>
<td>((2S)-2-[([1S]-1-(Ethoxycarbonyl)-3-phenylpropyl]amino)propanoic acid)</td>
</tr>
<tr>
<td>D</td>
<td>Imp-C</td>
<td><img src="Imp-C.png" alt="Imp-C" /></td>
<td>((2S)-1-([2S]-2-[([1S]-1-carboxy-3-phenylpropyl]amino)propanoyl]pyrrolidine-2-carboxylic acid)</td>
</tr>
<tr>
<td>E</td>
<td>Imp-D</td>
<td><img src="Imp-D.png" alt="Imp-D" /></td>
<td>Ethyl ((2S)-2-([3S,8aS]-3-methyl-1,4-dioxo octahydropyrrolo[1,2-a]pyrazin-2-yl]-4-phenylbutanoate)</td>
</tr>
<tr>
<td>F</td>
<td>Imp-E</td>
<td><img src="Imp-E.png" alt="Imp-E" /></td>
<td>((2S)-1-([2S]-2-[([1S]-3-phenyl-1-[(2phenylethoxy)carbonyl]propyl]amino)propanoyl]pyrrolidine-2-carboxylic acid)</td>
</tr>
<tr>
<td>G</td>
<td>Imp-H</td>
<td><img src="Imp-H.png" alt="Imp-H" /></td>
<td>((2S)-1-([2S]-2-[([1S]-3-cyclohexyl-1-(ethoxycarbonyl)propyl]amino)propanoyl]pyrrolidine-2-carboxylic acid)</td>
</tr>
</tbody>
</table>

**Figure 1.** Chemical structure and name of ENP and impurities. (A): ENP; (B): Imp-A; (C): Imp-B; (D): Imp-C; (E): Imp-D; (F): Imp-E; (G): Imp-F.
determination of ENP and its related compounds. This method was successfully validated according to the International Conference on Harmonization (ICH) guidelines (22) and also demonstrated equivalency to the EP method. Moreover, stability-indicating ENP assay method also developed within this publication with 20 min runtime.

Experimental

Materials and reagents
ENP working standard, ENP tablets (2.5, 5, 10 and 20 mg), placebo and standards of impurities were supplied by APL Research Centre Limited, Hyderabad, India. HPLC grade methanol (MeOH), and acetonitrile (ACN), analytical grade sodium dihydrogen phosphate (NaH₂PO₄) and ortho phosphoric acid (H₃PO₄), hydrochloric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide (H₂O₂) were purchased from Merck life science private limited, Vikhroli (East), Mumbai. Purified water was prepared by using of a Milli-Q plus water purification system from Millipore (Bedford, MA, USA).

Instrumentation and chromatographic method for HPLC
Waters HPLC system equipped with 2998 photo diode array (PDA) detector (Waters corporation, Milford, MA, USA) was used for specificity and development studies. The output signal was monitored and processed using Empower three software (Waters Corporation, Milford, MA, USA).

The related substances separation was accomplished on ZORBAX Eclipse XDB-C18 (250 mm × 4.6 mm, 5 μm). The mobile phase-A contains a mixture of 0.02 M NaH₂PO₄ buffer (pH-3.0 adjusted with H₃PO₄) and ACN in the ratio 95:5 (v/v). The mobile phase-B contains a mixture of 0.02 M NaH₂PO₄ buffer (pH-3.0 adjusted with H₃PO₄) and ACN in the ratio 34:66 (v/v). The mobile phases were filtered through nylon 0.2 μm membrane filter. The mobile phases were pumped with the flow rate of 1.2 mL/min using the gradient program (time in min)%B: 0.0/10, 35/55, 40/55, 41/10 and 50/10. The oven column temperature was maintained at 55°C and injection volume was set as 50.0 μL. The detection was monitored at wavelength 215 nm. About 950 mL of 0.02 M NaH₂PO₄ buffer (pH-2.5 adjusted with H₃PO₄) and 50 mL of ACN was used as a diluent.

The following chromatographic conditions were used for ENP assay method. Column: ZORBAX Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm), Injection volume: 10 μL, flow rate: 1.0 mL/min, gradient program (Time in min)%B: 0.0/10, 10/70, 14/70, 15/10, 20/10) and other chromatographic conditions were followed as per the above-related substances method.

Preparation of stock and standard solutions
A stock solution of ENP (500 μg/mL) was prepared by dissolving the drug in diluent. Working standard solutions of 2.5 μg/mL and 100 μg/mL were prepared from the stock solution for the determinations of the related compounds and assay, respectively. The individual stock solutions (100 μg/mL) of all impurities were prepared in the diluent. These solutions were prepared freshly and diluted further quantitatively to study the validation attributes. The specification limits considered for validation studies were 0.3% for Imp-A, Imp-B, Imp-E and Imp-H; 1.5% for Imp-C and 0.5% for Imp-D. Impurities mixture solution was prepared by dissolving ENP (500 μg/mL) and all impurities in diluent. These impurities mixture solution was used for method development. System suitability solution was prepared by spiking the Imp-A at specification level to ENP test solution (500 μg/mL) and used for method developed and validation studies.

Preparation of sample solution
ENP tablets (n = 20) were weighed and averaged before being crushed into fine powder. Transferred an accurately weighed amount of tablet powder equivalent to 25 mg of ENP into a 50 mL volumetric flask, added 30 mL of diluent and sonicated for 30 min with intermediate shaking, diluted to volume with diluent and mixed well. Filtered the solution through 0.45 μm Millipore PVDF/Nylon or Whatman GFC filters. The solution was further diluted to 100 μg/mL (5–25 μg with diluent) and used for assay analysis. Similarly, placebo (mixture of all excipients without ENP drug) solution was prepared as per above procedure. API test preparation was prepared by dissolving 25 mg of ENP to 50 mL with diluent.

Solution for forced degradation studies
Stress degradation studies was performed according to ICH guidelines Q1A (R2) (22) to demonstrate the stability-indicating nature and specificity of the proposed method.

Equivalent to 25 mg of each ENP tablet powder was transferred into four separate 50 mL volumetric flasks and subjected to forced degradation study under acid (1 M HCl at 60°C for 5 h), base (0.1 M NaOH at room temperature for 1 h), neutral (water at 60°C for 4 h) and oxidation (10.0% v/v H₂O₂ at room temperature for 72 h). The stressed samples of acid and base degradation were neutralized with 1 M NaOH and 0.1 M HCl, respectively, and made up to volume with diluent. The drug was placed in a thermally controlled oven at 105°C up to 24 h for thermal stress study. Photolytic degradation was performed by exposing the drug to visible light and UV with minimum exposure of 1.2 million lux-hours and 200w-hr/m², respectively.

All ENP degradation samples were prepared at 500 μg/mL concentration in the respective stressing medium. The same degraded solutions were diluted to the concentration of 100 μg/mL with diluent and used for assay of ENP.

Results

Evaluation of EP monograph method
As per EP monograph method, the following chromatographic conditions were used. Column: styrene–divinylbenzene copolymer (150 mm × 4.1 mm, 5 μm); Mobile phase A: mixture of 50 mL of ACN and 950 mL of buffer (0.02 M NaH₂PO₄, pH: 6.8); Mobile phase B: mixture of 660 mL of ACN and 340 mL of buffer (0.02 M NaH₂PO₄, pH: 6.8); Diluent: mixture of 50 mL of ACN and 950 mL of buffer (0.02 M NaH₂PO₄, pH: 2.5); Injection volume: 50 μL; flow rate: 1.0 mL/min; column oven temperature: 70°C; wavelength: 215 nm and gradient program (time in min)%B: 0.01/5, 20/60, 25/60, 26/5, 30/5.

During evaluation of EP method, the following drawbacks were observed: (i) The system meets suitability criteria for resolution is just nearer to the specified limit (peak-to-valley ratio: minimum 10 for Imp-A and Enalapril) and it indicates that resolution between Imp-A and Enalapril is tough tasks to achieve. It requires quite often adjustment in chromatographic parameters to meet the system suitability (Figure 2A). (ii) Separate column oven box is required to maintain column oven temperature at 70°C. (iii) Imp-C eluted early at 2.5 min and placebo interference was also noticed for Imp-C and
Imp-A peaks (Figure 2B) (iv) After few injections, broad peak shapes were noticed for all peaks (Figure 2C).

Hence, the above-described monograph method cannot be operated at optimum conditions. To overcome the above drawback, a new user-friendly HPLC method was developed by optimizing the stationary phase, mobile phase pH, column temperature, flow rate and gradient program. The developed method was validated and found to be precise, accurate, linear, robust and stability indicating. The equivalence to EP method was demonstrated by analyzing the three batches of ENP tablets and impurities spiked to test sample at specification level. The complete method development, validation and equivalence to the EP method were briefly discussed in the Discussion section.

Discussion

Method development

The need for a rugged and selective method for ENP was understood during the evaluation of EP method. It was decided to develop a selective, sensitive and user-friendly method for quantitative determination of ENP and its impurities. The major challenges involved in the development are: (i) Achieve efficient separation between Imp-A and ENP by using C18 stationary phase (ii) Column oven temperature should be reduced within instrument specified limit (iii) Column performance should be good for long period (iv) All known and unknown peaks should be symmetrical.

Initial chromatographic separation has been verified on four different C18 stationary phases with slightly different selectivities and hydrophobicities while keeping the other chromatographic parameter as described in EP method. The following columns were verified to achieve baseline separation between ENP and Imp-A (i) X-Terra RP-18 (150 x 4.6 mm, 5 μm), (ii) Inertsil ODS 3 V (150 x 4.6 mm, 5 μm), (iii) Hypersil BDS (150 x 4.6 mm, 5 μm), (iv) YMC Pro C18 (150 x 4.6 mm, 5 μm). In these columns, satisfactory separation was not achieved between Imp-A and ENP. However, ZORBAX Eclipse XDB-C18 (250 mm x 4.6 mm, 5 μm) column provided little separation between Imp-A and ENP with narrow peaks for every impurity. Hence, this column was used for further optimization.

The hydrophilic ionizable functional groups (-COOH and -NH) of ENP and Imp-A are being expected to show separation with mobile phase buffer pH. Hence, the effect of the buffer pH on the retention times of ENP and its impurities was studied from pH 2.0 to 7.0 by using Zorbax Eclipse XDB-C18 (250 mm x 4.6 mm, 5 μm) column, gradient program (T/%B: 0/5, 40/50, 50/50, 51/5, 60/5), column temperature 55 °C and other chromatographic parameters followed as per EP method. At pH 2.0, Imp-A co eluted with ENP; Imp-C strongly retained and close elution for Imp-D & Imp-E was noticed. In pH 3.0 and 4.0, Imp-A showed good separation from ENP peak (Rs~2.0) and Imp-D & Imp-E closely eluted. In pH 6.5 and 7.0, Imp-A slightly separated from ENP (Rs~1.3), Imp-D & Imp-E well separated and Imp-C early elution was observed. However, the best retention of early eluting Imp-C with good

Figure 2. Evaluated EP monograph chromatogram. (A) EP specified system suitability chromatogram. (B) Overlaid chromatogram of placebo sample and impurities spiked to ENP test sample. (C) Distorted peak shapes chromatogram after few injections in EP monograph method.
resolution between Imp-A and ENP was achieved at pH 3.0. Therefore, the mobile phase pH was set at 3.0 for further optimization of the method, which also gives good column performance as operated in acidic pH.

To improve more resolution between ENP & Imp-A and Imp-D & Imp-E with good peak shapes, column temperature and mobile phase flow was studied. The column oven temperature studied from 30 to 60°C. During the study, it was observed that the resolution between Imp-A and ENP was increasing while increasing the column oven temperature. Moreover, impurities peak shapes were also improved with increasing the column temperature. However, the column oven temperature optimized to 55°C. At this temperature, the resolution between Imp-A and ENP was increased to 3.8; the resolution between Imp-D and Imp-E was observed as 3.0 and narrow peaks were found for all impurities.

The mobile phase flow rate was studied from 0.8 to 1.5 mL/min while keeping the column oven temperature 55°C. At mobile phase flow rate 1.2 mL/min, the separation between Imp-D and Imp-E was slightly enhanced (Rs~5.0). Hence, the chromatographic column oven temperature and mobile phase flow rate were set as 55°C and 1.2 mL/min, respectively. The selected column oven temperature (55°C) was user friendly and it can be operated for all kind of HPLC instruments without using separate column oven box.

After an extensive study, the method has been finalized on ZORBAX Eclipse XDB-C18 (250 mm × 4.6 mm, 5 μm) column using buffer (0.02 M NaH2PO4, pH:3.0), ACN in the ratio of 95:5 (v/v) as mobile phase-A; buffer (0.02 M NaH2PO4, pH:3.0), ACN in the ratio of 340:660 (v/v) was mobile phase-B. The mobile phase pumped through the column at a flow rate of 1.2 mL/min and column temperature kept at 55°C. The gradient elution was set as (T(min)/%B) 0.0/10, 35/55, 40/55, 41/10 and 50/10. The typical HPLC chromatogram represents the enabled separation for all pair of components with good peak shapes (Figure 3A).

Blank and placebo interference was also verified and found that no interference was observed at the retention time of ENP and its impurities (Figure 3A). The developed method was considered specific for the determination of the ENP and its impurities and no difficulty in peak integration.

The developed method has been verified on different stationary phases and proved to be applicable for X-Terra C18, X-bridge C18, Inertsil ODS-3V, YMC pro C18, ACE C18 reversed-phase columns (with dimension of 250 × 4.6 mm, 5 μm).

The chromatographic performance data were evaluated for ENP and its impurities. The symmetry factor (Sₜ) for all impurities and ENP were found to be <1.2. The resolution (Rs) between all components was greater than 3.5 (Table I).

In addition, a shorter run time assay method was developed for the determination of ENP in ENP tablets. To reduce the run time of assay method, a gradient program was optimized using a shorter dimensions ZORBAX Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column and the other chromatographic condition was followed as per the above-related substances method. After several attempts, a shorter run time (20 min) assay method was developed. The final assay chromatographic conditions were presented in the “chromatographic method for HPLC” section. All known impurities were spiked to assay test sample (100 μg/mL) and analyzed in the assay method. It was observed that all impurities were separated from ENP peak. Placebo interference verified and no peaks observed at retention time of ENP peak. The ENP peak was found to be spectrally pure in analyzed ENP tablets. Hence, developed assay method found to be specific for the determination of ENP in ENP tablets. The optimized chromatogram presented in Figure 3B and it indicated that ENP peak was observed as symmetrical peak shape (Figure 3B).

**Forced degradation study**

The results of the degradation studies of ENP samples confirmed that there is no co-elution of ENP and its impurities. Significant degradation was observed when the drug was subjected to base hydrolysis (0.1 M NaOH for 1 h) leading to the formation of Imp-C (10%). Partial degradation of Imp-C (1.8%) and Imp-D (0.6%) was observed under acid hydrolysis (1 M HCl, 60°C for 5 h). Low-level Imp-C (0.15%) and Imp-D (0.14%) were generated under peroxide degradation (10% H2O2, 72 h). The thermal degradation condition (105°C for 36 h) caused the formation of Imp-D as major degradation product (15%). Photolytic and hygroscopic degradation did not lead to any impurities generation. The chemical purity of ENP was the same before and after the experiment. Some low-level unknown impurities were generated at thermal degradation conditions but those are found below 0.15% level. The above results confirmed that ENP drug product was very sensitive toward base hydrolysis and thermal degradation (Figure 3C–F). All the forced degradation samples were analyzed using a PDA detector to ensure the homogeneity and purity of the ENP peak. The results from the peak purity assessment revealed that the purity angle was less than the purity threshold in all of the stressed samples. The mass balance (% assay + % sum of all impurities) results were calculated and found to be more than 98.0% (Table II). The purity of ENP was unaffected by the presence of its impurities, degradation products and other excipients (placebo) and thus confirms the stability-indicating power of the method.

All diluted degraded samples (100 μg/mL) were analyzed in the assay method and the calculated assay results were found to be similar with above-related substance method. Peak purity of ENP was determined for all degraded samples and the ENP peak was found to be spectrally pure. The assay of ENP was not affected in the presence of impurities and placebo. Hence, the developed assay method considered as stability-indicating nature.

**Method validation**

After achieving the optimal chromatographic separations, the method was subjected to the validation process in order to prove its reliability and suitability for its intended purpose. The validation procedure was performed as described in ICH Q2 guidelines (23) for determination of ENP and its impurities in ENP tablets. The described method was extensively validated in terms of system suitability, specificity, precision, accuracy, linearity, limits of detection and quantification (LOD & LOQ), solution stability and robustness.

**System suitability**

The results of the system suitability experiment provide the information about the separation efficiency, reproducibility and the state of HPLC instrument. The SST solution and diluted standard solution were used for the evolution of system suitability. The resolution between ENP and Imp-A was chosen as the parameter to indicate the efficiency of the separation—the resolution more than 3.0 indicated good separation. The system repeatability was also verified as the diluted standard (1.5 μL of ENP) was analyzed six times and calculated RSD was well within the acceptance criteria of 3.0%. The peak symmetry (Sₜ) and plate count (N) was also verified for ENP peak in diluted standard and it was fulfilled the acceptance criteria of
symmetry factor—<1.5 and plate count—should be more than 5,000. The system suitability results were presented in Table 1.

Specificity
The specificity of the analytical method was examined by analyzing the ENP test solution containing all impurities at the specification level. An equivalent placebo concentration was prepared and injected to evaluate the interference with the analyte peaks. No peak at retention time of any known impurity and ENP was seen in the diluent and placebo. Moreover, the resolution values between each of the adjacent impurity peaks were greater than 3.5. ENP peak in the analyzed tablets was spectrally pure and that there were no co-eluting peaks and no difficulty in peak integration. Therefore, the method is considered specific for the determination of the ENP and its impurities.

Sensitivity
The LOD & LOQ were calculated for ENP and its impurities. The results were estimated and afterwards empirically verified. The concentration is considered as the LOD if the detector response expressed as the signal-to-noise ratio is not <3.0. The concentration of the analyte could be quantitated with good sensitivity.

Figure 3. Chromatograms in optimized method condition. (A) ENP spiked with impurities mixture chromatogram. (B) ENP Assay chromatogram with 20 min run time. (C) ENP Acid hydrolysis. (D) ENP Base hydrolysis. (E) ENP Peroxide degradation. (F) ENP Thermal degradation.
when setting the LOQ should be higher than the signal-to-noise ratio of 10. The LOQ of ENP and its related compounds was estimated at the level of 0.15 μg/mL (0.03%). The analytical method was sensitive enough as the LOQ was well below the reporting threshold (0.05%). The LOD for ENP and its impurities was 0.0045 μg/mL (0.009%).

Precision study was performed at the LOQ concentration level by injecting six individual preparations of ENP and its impurities and calculated the %RSD for the areas of each peak. The RSDs were found to be below the acceptance criteria of 10% (Table III).

Accuracy at LOQ level was verified by injecting three individual preparations of ENP spiked with impurities at the speciation level. The %RSD was calculated for each impurity (Table III). The method was assumed to be precise since the calculated RSD was not more than the acceptance criteria of 10%.

The precision of ENP assay method was verified in the similar way. The sample of ENP tablets was prepared at 100 μg/mL (100%) concentration level and analyzed. The results of the precision and intermediate precision were found to be below 0.5% which is less than the acceptance criteria of 2%. These results confirmed the high precision.

Linearity
Linearity test solutions were prepared from impurity stock solution at seven different concentration levels ranging from LOQ to 200% of the specification level (i.e. LOQ, 0.30, 0.75, 1.05, 1.5, 2.25 and 3.0 μg/mL for Imp-A, Imp-B, Imp-E, Imp-H and ENP; LOQ, 1.5, 3.75, 5.25, 7.5, 11.25 and 15.0 μg/mL for Imp-C; LOQ, 0.5, 1.25, 1.75, 2.5, 3.75 and 5.0 μg/mL for Imp-D).

<table>
<thead>
<tr>
<th>S. no</th>
<th>Impurity name</th>
<th>RT (in min)</th>
<th>Resolution</th>
<th>Symmetry factor</th>
<th>Plate count</th>
<th>%RSD&lt;sup&gt;a&lt;/sup&gt; (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Imp-C</td>
<td>6.463</td>
<td></td>
<td>1.1</td>
<td></td>
<td>4,193</td>
</tr>
<tr>
<td>2</td>
<td>Imp-B</td>
<td>15.553</td>
<td>28.08</td>
<td>1.0</td>
<td></td>
<td>93,441</td>
</tr>
<tr>
<td>3</td>
<td>ENP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.092</td>
<td>10.09</td>
<td>1.0</td>
<td></td>
<td>9,847</td>
</tr>
<tr>
<td>4</td>
<td>Imp-A</td>
<td>23.842</td>
<td>3.76</td>
<td>1.1</td>
<td></td>
<td>27,875</td>
</tr>
<tr>
<td>5</td>
<td>Imp-H</td>
<td>30.190</td>
<td>9.26</td>
<td>1.1</td>
<td></td>
<td>22,560</td>
</tr>
<tr>
<td>6</td>
<td>Imp-D</td>
<td>34.773</td>
<td>8.41</td>
<td>1.0</td>
<td></td>
<td>231,973</td>
</tr>
<tr>
<td>7</td>
<td>Imp-E</td>
<td>37.037</td>
<td>5.06</td>
<td>1.1</td>
<td></td>
<td>59,224</td>
</tr>
<tr>
<td>8</td>
<td>ENP&lt;sup&gt;b&lt;/sup&gt; (Assay)</td>
<td>9.1</td>
<td>1.0</td>
<td></td>
<td></td>
<td>8,652</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of determinations; RSD, Relative standard deviation.

<sup>b</sup>ENP diluted standard (1.5 μg/mL).

<sup>c</sup>ENP Assay standard (100 μg/mL).
The calibration curve was drawn by plotting impurity area versus the concentration. The calculated correlation coefficient was greater than 0.999 for all the impurities (Table III). Similarly, the linearity of ENP in the assay method was also established at five concentrations (from 25 to 150 μg/mL). The result of correlation coefficient (r) for ENP was 0.9995.

The acceptance criteria for linearity (assay and purity methods) were considered as the correlation coefficient should be more than 0.995. The obtained results indicated there was an excellent linearity relationship between the peak area and concentration of all the components.

During the linearity studies relative response factors (RRF) were established for all known impurities as the ratio of the slope of impurities and the slope of ENP. The slope value obtained with the linear calibration plot was used for the determination of RRF (Table III).

**Accuracy**

Accuracy of the proposed method was evaluated by spiking with known amounts of impurities to the ENP tablets test sample (500 μg/mL) at the level of 50%, 100% and 150% of specifications.

Each level of the preparation was prepared in triplicate. The percent recoveries were calculated for all related substances (Table III) and those are well within the limit of acceptance criteria (90–110%).

**Robustness**

To determine the robustness of the method, experimental conditions were deliberately altered. The factors chosen for this study, which were the critical sources of variability in the operating procedures such as flow rate (1.2 ± 0.1 mL/min), mobile phase pH (3.0 ± 0.2), mobile phase composition (mobile phase A; ± 5% ACN, mobile

**Table II. Summary of ENP Forced Degradation**

<table>
<thead>
<tr>
<th>Degradation condition</th>
<th>%Assay</th>
<th>RS by HPLC</th>
<th>Mass balance (%Assay + %deg. products)</th>
<th>Remarks/observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolysis (1 M HCl, 60°C, 5 h)</td>
<td>98.1</td>
<td>2.4</td>
<td>100.5</td>
<td>Imp-C and Imp-D degradation product were formed</td>
</tr>
<tr>
<td>Base hydrolysis (0.1 M NaOH, 25°C, 1 h)</td>
<td>89.1</td>
<td>11.1</td>
<td>100.2</td>
<td>Imp-C major degradation product was formed</td>
</tr>
<tr>
<td>Oxidation (10.0% H2O2, 25°C, 72 h)</td>
<td>99.5</td>
<td>0.29</td>
<td>99.8</td>
<td>Low-level Imp-C and Imp-D degradation products were formed</td>
</tr>
<tr>
<td>Photo degradation: (Exposed to 200 watt hour/m² and 1.2 million lux hour in Photo stability chamber for 16 h)</td>
<td>99.4</td>
<td>0.21</td>
<td>99.6</td>
<td>No significant degradation observed</td>
</tr>
<tr>
<td>Thermal degradation (105°C, 36 h)</td>
<td>83.4</td>
<td>16.1</td>
<td>99.5</td>
<td>Imp-D major degradation product and few low-level unknown degradation products were formed No significant degradation observed</td>
</tr>
<tr>
<td>Humidity at 90% RH (25°C, 5 Days)</td>
<td>99.6</td>
<td>0.2</td>
<td>99.8</td>
<td>No significant degradation observed</td>
</tr>
</tbody>
</table>

**Table III. Summary of Method Validation for ENP and its Impurities**

<table>
<thead>
<tr>
<th>Validation parameter</th>
<th>Imp-C</th>
<th>Imp-B</th>
<th>ENP</th>
<th>Imp-A</th>
<th>Imp-H</th>
<th>Imp-D</th>
<th>Imp-E</th>
<th>ENP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response factor (Rf)</td>
<td>0.78</td>
<td>1.14</td>
<td>1.03</td>
<td>1.98</td>
<td>0.92</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specifications (%)</td>
<td>1.5</td>
<td>0.3</td>
<td>1.0</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOQ accuracy</td>
<td>95.1</td>
<td>97.8</td>
<td>98.8</td>
<td>101.2</td>
<td>106.2</td>
<td>96.2</td>
<td>94.1</td>
<td></td>
</tr>
<tr>
<td>%RSD (n = 6)</td>
<td>2.8</td>
<td>1.1</td>
<td>0.8</td>
<td>2.1</td>
<td>4.2</td>
<td>1.2</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>%RSD (n = 6)</td>
<td>1.4</td>
<td>2.6</td>
<td>3.2</td>
<td>2.2</td>
<td>3.7</td>
<td>3.4</td>
<td>3.1</td>
<td>0.29</td>
</tr>
<tr>
<td>%RSD (n = 6)</td>
<td>1.5</td>
<td>2.4</td>
<td>3.5</td>
<td>2.0</td>
<td>3.6</td>
<td>3.1</td>
<td>3.3</td>
<td>0.39</td>
</tr>
<tr>
<td>Accuracy at 50%</td>
<td>97.2</td>
<td>98.2</td>
<td>99.1</td>
<td>99.1</td>
<td>105.1</td>
<td>101.2</td>
<td>102.1</td>
<td>100.7</td>
</tr>
<tr>
<td>Accuracy at 100%</td>
<td>97.5</td>
<td>98.8</td>
<td>99.4</td>
<td>99.4</td>
<td>105.5</td>
<td>102.1</td>
<td>102.5</td>
<td>100.2</td>
</tr>
<tr>
<td>Accuracy at 150%</td>
<td>97.3</td>
<td>99.0</td>
<td>99.2</td>
<td>99.4</td>
<td>106.1</td>
<td>101.6</td>
<td>101.9</td>
<td>99.3</td>
</tr>
<tr>
<td>Linearity correlation</td>
<td>0.9996</td>
<td>0.9992</td>
<td>0.9996</td>
<td>0.9993</td>
<td>0.9996</td>
<td>0.9998</td>
<td>0.9997</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Linearity range is LOQ–200% with respect to specification level for ENP and its Impurities.

*Assay of ENP.

**LOQ Precision.**

*Repeatability.

*Intermediate precision.
phase B: ± 5% ACN) and column oven temperature (55 ± 5°C) were identified.

The ENP test solution (500 μg/mL) spiked with all known impurities at specification level was analyzed and the separations among the impurities were verified. During the changed experimental conditions, resolution between ENP and its impurities was found to be more than 3.0 and there was no significant change in relative retention time for all the impurities in spiked test sample. Similarly, ENP assay method was evaluated and there was no significant change in retention time and symmetry factor. Therefore, the developed methods were demonstrated to be robust over the deliberately changed chromatographic conditions.

Evaluation of equivalency with EP method
The equivalency to the EP method was demonstrated in this study since the stationary phase (C18), mobile phase pH (3.0), flow rate (1.2 ml/min), sample concentration (500 μg/mL) and column temperature (55°C) in the optimized method differ from those in the EP method. Three batches ENP tablet test samples and three preparations of spiked test sample (500 μg/mL) containing each impurity at specification level were analyzed by both methods. Experimental results for each impurity in % weight/weight, using the optimized HPLC method, were compared against the results obtained using the EP method. The % difference was calculated as follows:

\[
\text{Difference, } \% = \frac{(\text{wt/wt, optimized method}) - (\text{wt/wt, EP})}{\text{wt/wt, optimized method}} \times 100
\]

where % wt/wt are the results obtained using the respective methods.

The results showed that the experimentally determined concentrations of all impurities were within ±0.03% wt/wt (less than the method LOQ) for the two methods confirming that the optimized method is equivalent to the EP method for determining the ENP and its impurities.

As expected, resolution for Imp-A and ENP in the optimized method was more efficient than that resolution in EP method. The optimized column temperature 55°C was user friendly to operate the HPLC instrument than that column temperature 70°C was used in EP method. The optimized method showed constant rejections and resolution for all impurities with good peak shapes.

Conclusion
Developed method is simple, rugged and selective for quantification of impurities and ENP. The method is capable of separating all the known impurities with resolution more than 3.5. Key critical parameters like stationary phase, mobile phase pH, column temperature and flow rate were effectively optimized. The method is equivalent to EP methods in terms of producing result during regular analysis and superior to EP methods in terms of selectivity. The developed method was validated as per the ICH guidelines and found to be selective, specific, precise, accurate, linear and robust. The developed method was user friendly and can be used for quality control and stability study analysis of ENP drug substance and drug product. Stability-indicating assay method was also developed within 20 min runtime for determination ENP in ENP tablets.

Acknowledgments
This paper was supported by Jawaharlal Nehru Technological University Hyderabad which is gratefully acknowledged.

Conflict of interest statement
None declared.

References


