Stability indicating RP-UPLC method for simultaneous quantification of bempedoic acid and ezetimibe in bulk and pharmaceutical formulations

Uma Sai Teja Yarra and Sowjanya Gummadi*

Abstract

Background: Bempedoic acid and Ezetimibe acid are used in combination for treatment of hypercholesterolemia. The current work was undertaken to develop a simple and rapid stability-indicating RP-UPLC method for the simultaneous estimation of Bempedoic acid and Ezetimibe in tablets as no such method was available. The chromatographic separation was performed with Waters Acquity C18 [50 × 2.1 mm, 1.7 μ] column using methanol: acetonitrile: water [50: 30: 20, by volume] as mobile phase pumped at a flow rate 0.5 mL/min. The separated analytes were detected at 260 nm using UV detector.

Results: The separation of Bempedoic acid (BA) and Ezetimibe (EZ) was done at a retention time of 1.827 min. and 3.577 min. respectively. The validation and stability studies of the present method were carried out according to the ICH guidelines. The linearity of the proposed method was in the range of 30–130 μg/mL and 5–50 μg/mL for Bempedoic acid and Ezetimibe respectively. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.1216 μg/mL and 0.3685 μg/mL for Bempedoic acid and 0.1189 μg/mL and 0.3602 μg/mL for Ezetimibe respectively. The recovery of the method was found to be in the range of 99.89—100.31% for Bempedoic acid and 98.14—99.94% for Ezetimibe while the % RSD for both drugs in the precision and robustness study was less than 2.0. The drugs did not show any major degradants in the exposed conditions.

Conclusion: The developed method was found to be simple, sensitive, accurate, precise, robust, rapid and yet stability indicating. The method can be adopted for simultaneous estimation of Bempedoic acid and Ezetimibe in the pharmaceutical formulation.

Keywords: UPLC, Bempedoic acid, Ezetimibe, ICH guidelines, Validation, Stability

Background

High LDL cholesterol (LDL-C) levels represent an important risk factor for cardiovascular diseases, which can be due to genetic mutations or lifestyle factors, hypercholesterolemia can substantially reduce life quality and increase the risk of cardiovascular disease mortality. Bempedoic acid (Fig. 1a) and Ezetimibe (Fig. 1b) acid are used in combination for treatment of hypercholesterolemia and ASCVD by reducing lipid parameters and attenuating hsCRP levels [1]. Ezetimibe is an azetidinone derivative, it prevents absorption of cholesterol by blocking the Niemann-Pick C1-like 1 (NPC1L1) protein on epithelial cells of gastrointestinal tract, and in hepatocytes. It also blocks aminopeptidase N and interfere with caveolin 1-annexin A2 complex which is in cholesterol trafficking. This decreases hepatic cholesterol and cuts the total cholesterol level in the blood [2]. Bempedoic
acid is a prodrug, the activated form inhibits adenosine triphosphate citrate lyase (ACL) inhibitor which is responsible for biosynthesis of cholesterol in liver [3].

A literature survey conveyed that, limited methods are available for simultaneous estimation of Bempedoic acid and Ezetimibe. A few articles reported spectrophotometric techniques for estimation of Ezetimibe alone and with other drugs [4–15]. Few HPLC methods were reported for the determination of Ezetimibe alone and in combination with other drugs [16–20]. One RP-HPLC method was reported for simultaneous estimation of Bempedoic acid Ezetimibe [21]. Few LC–MS methods were reported for determination of Ezetimibe alone and in combination with other drugs [22–25]. One LC–MS method was reported for estimation of Bempedoic acid in human plasma and urine [26]. To our knowledge, this is the first report of a stability indicating RP-UPLC method for simultaneous estimation of Bempedoic acid and Ezetimibbe in bulk and pharmaceutical formulations. The present work aims to develop stability indicating reverse-phase ultra-performance liquid chromatography (RP-UPLC) method for simultaneous estimation of Bempedoic acid and Ezetimibe in bulk and pharmaceutical formulations as per ICH guidelines.

Methods

Instrument
Acquity UPLC system equipped with quaternary pumps, Acquity TUV detector, and autosampler integrated.

Pure samples
Standard samples of Bempedoic acid (99.5% w/w) and Ezetimibe (98% w/w) were procured from Madras Pharmaceuticals, Chennai.

Formulation
Marketed formulation (tablets) of Bempedoic acid and Ezetimibe, NEXLIZET (Bempedoic acid 180 mg and Ezetimibe 10 mg) was purchased online.

Chemicals and reagents
Acetonitrile, methanol, and water (HPLC grade) were procured from Merck. Sodium hydroxide, hydrochloric acid, hydrogen peroxide (AR grade) obtained from Rankem were utilised in the study.
Preparation of mobile phase/Diluent
The mobile phase consists of methanol, acetonitrile and water in the ratio 50: 30: 20, by volume. The solvent is filtered through a 0.45 μm membrane filter and sonicated before use.

Preparation of stock and standard solutions
Stock solutions of Bempedoic acid and Ezetimibe were prepared by dissolving 180 mg of Bempedoic acid and 10 mg of Ezetimibe in about 70 mL diluent, sonicated and further made up to volume in a 200 mL volumetric flask with the diluent. Pipetted 1 mL of the above solution and transferred into a 10 mL volumetric flask and made up to volume with diluent (standard solution). The stock solution was diluted as per requirement.

Preparation of sample solution
Twenty tablets (NEXLIZET) were weighed and crushed. Quantity of powder equivalent to 180 mg of Bempedoic acid and 10 mg of Ezetimibe was taken in a 200 mL volumetric flask, 70 mL of diluent was added, sonicated it for 30 min with intermittent shaking and the volume was made up with diluent. This solution was filtered through a 0.45 μm membrane filter and further diluted.

Determination of working wavelength
10 μg/mL solutions of both the components were prepared separately and scanned in the UV wavelength region of 200 nm to 400 nm. An overlaying spectrum obtained was used to determine the working wavelength (isoabsorptive point).

Method development
Various system suitability parameters were analysed to optimize chromatographic conditions to achieve symmetric peak shape and better resolution of drugs. Optimization of mobile phase was done by combining various suitable solvents in different ratios and finalized that methanol: acetonitrile: water (50:30:20%, v/v) as mobile phase at a flow rate of 0.5 mL/min. gave the most symmetric and resolved peaks detected at 260 nm. Optimized chromatogram is shown in Fig. 2 and chromatographic conditions are given in Table 1.

Method validation
The validation of the proposed method was carried out as per ICH Q2 (R1) guidelines [27] and the validation parameters include system suitability, linearity, precision, accuracy, robustness, and specificity.

Linearity
Linearity was determined by preparing aliquots at six different levels of calibration curve over the concentration range of 30–130 μg/mL for Bempedoic acid and 5–50 μg/mL for Ezetimibe. Solutions for calibration curve were prepared by serial dilutions of stock solution to obtain concentrations of 30, 50, 70, 90, 110, 130 μg/mL of Bempedoic acid and 5, 10, 20, 30, 40, 50 μg/mL of Ezetimibe. The solutions were analysed in triplicate. The correlation coefficient with the regression equation was determined from the calibration curve.

Table 1 Optimized chromatographic conditions

| Parameter       | Optimized condition                                      |
|-----------------|----------------------------------------------------------|
| Mobile phase    | Methanol: acetonitrile: water (50: 30: 20, by volume)    |
| Column          | Waters Acquity C18 (50 mm × 2.1 mm ID, 1.8 μm)           |
| Flow rate       | 0.5 mL/min                                               |
| Column temperature | 25 ± 2° C                                      |
| Wavelength      | 260.00 nm (UV Detector)                                 |
| Injection volume| 10 μL                                                    |
| Elution         | Isocratic mode                                          |
| Run time        | 5 min                                                   |
| Retention time  |                                                          |
| Bempedoic acid  | 1.827 min                                                |
| Ezetimibe       | 3.577 min                                                |

Table 2 Linearity data

| S. No | Bempedoic acid Concentration (μg/mL) | *Area ± SD | Ezetimibe Concentration (μg/mL) | *Area ± SD |
|-------|-------------------------------------|------------|---------------------------------|------------|
| 1     | 30                                  | 16,674,907 ± 12,562 | 5                               | 13,022,190 ± 7369 |
| 2     | 50                                  | 25,055,621 ± 18,075 | 10                              | 16,194,209 ± 10,207 |
| 3     | 70                                  | 36,009,142 ± 20,674 | 20                              | 25,035,716 ± 18,128 |
| 4     | 90                                  | 45,211,483 ± 44,895 | 30                              | 33,633,340 ± 24,323 |
| 5     | 110                                 | 53,519,670 ± 34,322 | 40                              | 41,910,697 ± 37,208 |
| 6     | 130                                 | 63,933,476 ± 49,587 | 50                              | 49,953,204 ± 48,521 |

*Mean of three determinations; SD = Standard Deviation
Precision

The precision of the method was studied in terms of intra-day precision and inter-day precision. Each phase of precision was investigated by six replicates of injections of sample solutions of Bempedoic acid (90 µg/mL) and Ezetimibe (5 µg/mL). Precision was expressed in terms of relative standard deviation.

Accuracy

Accuracy was expressed in terms of recovery. It was determined by spiking a known amount of standard Bempedoic acid and Ezetimibe to pre-analysed samples at three different levels such as 50%, 100%, and 150%, and the percentage recovery was determined.

Robustness

The robustness of the method was determined by bringing deliberate variations in the method parameters such as flow rate, temperature and detection wavelength.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were based on the standard deviation of the response and slope of the constructed calibration curve.
Sensitivity of the method was established with respect to LOD and LOQ for Ezetimibe and Bempedoic acid by slope method. Limit of detection and limit of quantitation was calculated using $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$. Where, $\sigma$ is the standard deviation of the response and $S$ is the slope of the standard curve.

Specificity
Specificity of the method was studied in the presence of excipients and degradants. Blank and placebo solutions were injected into the UPLC system and observed for any interfering peaks.

Forced degradation studies
Forced degradation studies were conducted to assess the stability-indicating property of the proposed method [28, 29]. The standard was exposed to various degradation conditions like acidic (0.1 N HCl, 60 °C, 1 h), alkaline (0.1 N NaOH, 60 °C, 1 h), peroxide (20% $H_2O_2$), thermal (105 °C, 6 h) and photolytic (254 nm, 1 day) stress. The exposed solutions were injected in replicates and the system suitability parameters were studied along the percentage degradations.
Fig. 5 Chromatograms of Bempeidoic acid (90 µg/mL) and Ezetimibe (5 µg/mL) stressed samples under: a Acidic, b Basic, c Peroxide, d Thermal and e Photolytic degradations
**Table 8** Results for assay

| Marketed formulation (tablet) | Drug       | Label claim (mg) | Amount found (mg) | *Assay (% w/w) ± SD |
|------------------------------|------------|------------------|-------------------|---------------------|
| NEXLIZET™                    | Bempedoic acid | 180              | 180.92            | 100.51              |
|                              | Ezetimibe  | 10               | 9.91              | 99.11               |

**Assay**

Assay of the marketed formulation (NEXLIZET, Bempedoic acid 180 mg and Ezetimibe 10 mg) was performed by using the standard and sample solutions. 1 mL of the clear and filtered sample solution as discussed above was pipetted in to a 10 mL volumetric flask and made up to volume with diluent. The resulting solution was used to record the chromatogram.

**Results**

Several trials were performed using a combination of mobile phases some of which include phosphate buffer: acetonitrile (60:40), phosphate buffer, pH 4.0: methanol: triethylamine (45:35:15), methanol: water (70:30), acetonitrile: water (60:40) and methanol: acetonitrile: water (50:30:20). A variety of stationary phases such as Phenomenex, Zorbax and Waters Acquity columns of varying dimensions were used. All these combinations resulted in chromatographic peaks with different system suitability parameters from which the optimized condition has been chosen as methanol: acetonitrile: water (50:30:20) and a Waters Acquity C18 column (50 × 2.1 mm ID, 1.8 µm) at a flow rate of 0.5 mL/min.

**Linearity**

Bempedoic acid and Ezetimibe obeyed linearity over the concentration range of 30–130 μg/mL and 5–50 μg/mL respectively. The regression equation and correlation coefficient was found to be $y = 472,696.19x + 2,251,687.96$ (0.999) for Bempedoic acid and $y = 833,313.27x + 8,430,966.32$ (0.9994) for Ezetimibe. Linearity data of Bempedoic acid and Ezetimibe is listed in Table 2. Calibration curves for Bempedoic acid and Ezetimibe are provided in Fig. 3a and b.

**Precision**

For intraday precision % RSD was calculated and it was 0.12% for Bempedoic acid and 0.38% for Ezetimibe, while % RSD value for interday precision was obtained in the range of 0.13—0.17% for Bempedoic acid and 0.20%—0.35% for ezetimibe. All the values of % RSD were within the limits indicating a precise method. Results of intraday and interday precision are given in Tables 3 and 4, respectively.

**Accuracy**

The accuracy of the proposed method was assessed at three levels. The % recovery of Bempedoic acid was found to be in the range of 99.89%—100.31% and the % recovery of Ezetimibe was found to be in the range of 98.14%—99.94% indicating the method is accurate. The accuracy results are listed in Table 5 and the data for robustness is given in Table 6.

**Limit of detection (LOD) and limit of quantification (LOQ)**

Limit of detection for Bempedoic acid and Ezetimibe was found to be 0.1216 μg/mL and 0.1189 μg/mL respectively. Limit of quantification for Bempedoic acid and Ezetimibe was found to be 0.3685 μg/mL and 0.3602 μg/mL respectively.

**Specificity**

No peaks were found in the diluent or placebo chromatograms at the retention times of Bempedoic acid and Ezetimibe indicating the specificity of the method in the presence of excipients as given in Fig. 4a and 4b.

![Fig. 6 Chromatogram for assay (Sample)](image)
Conclusion
The current UPLC method was developed and validated for the simultaneous estimation of Bempedoic acid and Ezetimibe in tablets. The stability indicating nature of the UPLC method was established from the degradation studies as all the peaks were resolved within the limits of system suitability. The proposed method is simple, rapid, economical, accurate, precise and yet stability indicating. This UPLC method be successfully employed for routine simultaneous analysis of Bempedoic acid and Ezetimibe in pharmaceutical formulations.

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Authors’ contributions
UY was experimental in performing the study and generating the required data. SG was involved in the preparation of manuscript. The authors read and approved the final manuscript.

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Availability of data and material
All the required data and material are available on request.

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