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Development and validation of an HPLC method using an experimental design for analysis of amlodipine besylate and enalapril maleate in a fixed dose combination

Amlodipin besilat ve enalapril maleatın sabit dozlu kombinasyondan analizi için deney tasarımı yoluyla bir YBSK yöntemi geliştirilmesi ve validasyonu

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ABSTRACT
Objectives: The aim of this study was development and optimization a simple, cost-effective, and robust HPLC method with the experimental design approach in the assay and dissolution analysis of amlodipine besylate and enalapril maleate from a fixed-dose combination tablet.
Materials and Methods: The chromatographic analysis was performed on a C18 column (4.6 x 250 mm id., particle size of 5 µm). The injection volume was 5 µL and detection wavelength was 215 nm. The Box-Benkhen design was used to test robustness of the method.

Flow rate (1, 1.2, and 1.4 mL/min), column temperature (25ºC, 30ºC, and 35ºC), methanol ratio of the mobile phase (5, 10, and 15%), and pH of the mobile phase (2.8, 3, and 3.2) were selected as independent variables. The validation of the method was carried out according to ICH guidelines. Dissolution of the tablets was performed by using USP apparatus 2 and analysed using the optimized HPLC method. Multivariate linear regression analysis and ANOVA was used in the statistical evaluation.
Results: Linear models were fitted for all variables. The flow rate was the most significant factor affecting the API's concentrations. The optimized method included following parameters: 25ºC of column temperature, 10% of methanol ratio in the mobile phase, 2.95 of pH and 1.205 mL/min of flow rate. Retention times were 3.8 min and 7.9 min for enalapril and amlodipine, respectively. The method found to be linear in a range of 0.8-24 µg/mL (R^2>0.999) and 1.6-48 µg/mL (R^2>0.999) for amlodipine and enalapril, respectively. Both active substances were dissolved more than 85% within 10 minutes.
Conclusion: Experimental design was found a useful tool for determination and separation of enalapril maleate and amlodipine besylate in dosage forms. It was shown that the optimized method can be used for in vitro performance and quality control tests of fixed dose tablet combinations containing enalapril maleate and amlodipine besylate.
Key words: Amlodipine, Enalapril, design of experiment (DOE), HPLC, fixed dose combination (FDC)

ÖZ

Amaçlar: Bu çalışmanın amacı, amlodipin besilat ve enalapril maleat içeren sabit dozlu kombinasyon tabletinden disolüsyon ve miktar tayini analizi için deney tasarımı yaklaşımı ile basit, ekonomik ve sağlam bir YBSK yönteminin geliştirilmesi ve optimizasyonudur.
Gereç ve Yöntemler: Kromatografik analiz C18 kolonda (4.6 x 250 mm id., 5 µm partikül çapı) gerçekleştirilmiştir. Enjeksiyon hacmi 5 µL ve dalga boyu 215 nm'dir. Yöntemin sağlamlığının test edilmesinde Box-Behnken tasarımı kullanılmıştır. Akış hızı (1, 1.2, ve 1.4 mL/dk) , kolon sıcaklığı (25, 30 ve 35ºC), hareketli fazdaki metanol oranı (%5, 10 ve 15) ve hareketli fazın pH'sı (2.8, 3 ve 3.2) bağımsız değişkenler olarak seçilmiştir. Yöntemin validasyonu ICH kılavuzlarına göre gerçekleştirilmiştir. Tabletlerin çözünme hızı deneyleri USP cihaz 2 kullanılarak 75 devir/dakika hızda gerçekleştirilmiştir. Çözünme hızı çalışması 0.1 N HCl'de 37 ± 0.5˚C'de yapılmış ve optimize edilen HPLC yöntemi ile analiz edilmiştir. İstatistiksel değerlendirmede çok değişkenli doğrusal regresyon analizi ve ANOVA kullanılmıştır.
Bulgular: Tüm değişkenler için doğrusal modeller kullanılmıştır. Etkin madde konsantrasyonlarını etkileyen en anlamlı faktör akış hızıdır. Optimize edilen yöntem şu parametreleri içermektedir: 25˚C kolon sıcaklığı, hareketli fazda %10.6 metanol oranı, 2.95 hareketli faz pH'sı ve 1.205 ml/dk akış hızı. Alıkonma zamanları enalapril ve amlodipine için sırasıyla 3.8 ve 7.9 olarak bulunmuştur. Yöntem amlodipin ve enalapril için sırasıyla 0.8-24 µg/mL (R^2>0.999) ve 1.6-48 (R^2>0.999) µg/mL aralıkta doğrusal bulunmuştur. Her iki etkin madde de 10 dakika içinde %85'ten fazla çözünmüştür.
Sonuç: Enalapril maleate ve amlodipine besilatın dozaj formlarından analizinde deney tasarımı faydalı bir yaklaşım olarak görülmüştür. Optimize edilen yöntemin enalapril ve amlodipine içeren bir sabit dozlu kombinasyonun in vitro performansı ve kalite kontrol testlerinde kullanılabileceği gösterilmiştir.
Anahtar kelimeler: Amlodipin, Enalapril, deney tasarımı, YBSK, sabit dozlu kombinasyon (SDK)

INTRODUCTION

At the early stages of the treatment of hypertension, it can be useful to choose monotherapy to observe the effect and the side effects of the drug. However, monotherapy can be insufficient to reach the target blood pressure in majority of patients.[1-3] More efficient treatment can be performed with two or even more antihypertensive drugs.[4] Therefore, Fixed Dose Combinations (FDCs) are frequently used in the cardiovascular diseases such as hypertension. In order to develop an FDC product including two drugs, some certain conditions must be provided. For instance, a synergistic effect can be observed using two drugs together or a side effect related to a drug may be eliminated using the other drug concurrently.[5] In the treatment of hypertension there is a synergistic effect between the calcium channel blockers (CCBs) and angiotensin converting enzyme inhibitors (ACEIs). In addition, ACEIs such as enalapril prevents peripheral edema caused by CCBs such as amlodipine.[6]

Amlodipine is a long-acting CCB that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. It is indicated for the treatment of hypertension and coronary artery disease by using alone or in combination with another antihypertensive agent.[7] Amlodipine is given orally as besylate in general, but doses are calculated in terms of amlodipine base. 6.94 mg of amlodipine besylate is equivalent to 5 mg of amlodipine base. Recommended dose of amlodipine is 5-10 mg once daily.[8] Since amlodipine is a weak base, it exhibits high solubility in physiological pH values. Although bioavailability of amlodipine is about 60-65%, it is defined as a highly permeable drug because of the 90-95% excretions as inactive metabolite by urine (Shohin, 2010). Amlodipine is a Class 1 drug according to the Biopharmaceutical Classification System (BCS).[9-11]

Enalapril is the ethyl ester of enalaprilat, an ACEI indicated for the treatment of hypertension and heart failure. Enalapril is available as maleate salt in the drug market. Enalapril maleate is a white crystalline powder sparingly soluble in water. Although the solubility is 25 mg/mL at pH 3.5, it increases to 200 mg/mL at pH 7.0. It is defined as BCS Class 3 with high solubility but low permeability properties.[12]

There are HPLC methods recommended in United States Pharmacopeia (USP42) for analysis of amlodipine besylate[13] and enalapril maleate[14], separately and a few liquid chromatography methods are available in literature for analyses of amlodipine[15], and enalapril[16,17], individually or in combination with another drugs.[18-23] However, these methods are not suitable for the separation of amlodipine and enalapril in the same dosage unit. Nevertheless, there are three published articles for HPLC analysis of amlodipine besylate and enalapril maleate together in dosage forms.[24-26] However these methods contain in a high ratio of organic solvents in the mobile phase which is environmentally inappropriate according to the green chemistry approach. An important principle of green chemistry is to reduce toxic organic solvents and to consume safer chemicals.[27,28] Relating to the green analytical chemistry approach, Korany et al recommended to reduce acetonitrile amount in the methods and to use multiparameter methods such as design of experiment (DOE) instead of one factor at a time (OFAT) approach.[27,28] In the method developed by Chaudhari et al, the mobile phase contains 50 % acetonitrile and 40 % methanol and a higher injection volume (20 µL) which causes more consumption of mobile phase and the linearity range was comparatively narrow (0.5 – 6 µg/mL and 0.5 – 8 µg/mL for enalapril and amlodipine, respectively).[24] In another method, mobile phase includes 60% of acetonitrile and the injection volume was 20 µL and the linearity range was not suitable for lower concentrations (20-100 µg/mL) which might be

essential for the initial points of the dissolution tests.[25] In the method developed by Masih et al, 50% 1N HCl and 50% methanol was included in the mobile phase and the injection volume was 10 µL.[26] Additionally, none of the studies include the application of DOE in robustness testing in validation for amlodipine besylate and enalapril maleate. Furthermore, there is no dissolution analysis of enalapril and amlodipine in combined dosage form in the literature.

DOE is a well-defined mathematical methodology to demonstrate how to get maximum reliable and valuable scientific information by performing minimum experiments.[29] In this technique the effects of the multiple variations on one or more response can be investigated at the same time, instead of changing OFAT. Although conventional developmental approaches are mainly empirical and are often conducted using the changing OFAT method, DOE provides the facility of performing systematic and multivariate experiments in order to entirely understand the process and to assess the statistical significance of the variables.[30,31] By creating experimental matrix, DOE allows faster visualization and determination of more factors at a time.[32] Besides, in OFAT approach factors are evaluated independently, so it is assumed that the factors do not influence each other. However, the potential interactions between the factors can be identified using appropriate DOE model.[33,34] In pharmaceutical area, DOE helps to understand the effects of the critical formulation and process variables on the final product.[35,36] DOE can be used for factor screening and characterization of a new system or for optimization of a characterized system. Factors are independent variables that might affect the results of critical responses. For instance, in an analytical method development process, flow rate can be an independent factor which has potential effect on the peak area of the analyte. In a screening design it is aimed to investigate numerous factors that might affect the response and to discover the factor which has the most significant influence on the responses.[37] On the other hand, in an optimization process, the main objective is to define the optimal conditions and settings for the factors.[38] In case more than one factor required to be examined, the multivariate optimization designs can be reasonable in order to evaluate different factors at the same time and to find out if there are interactions between the factors.[37,38]

In analytical chemistry, DOE can be used for chromatographic analytical method development to optimize the sampling preparational factors, column factors, detector factors, instrumental factors or environmental factors.[31,39] Similarly, analytical method validation parameters such as accuracy, linearity, precision or robustness can be performed by experimental design approaches.[29,40-46] Using DOE in validation studies is recommended in International Conference on Harmonization (ICH) guidelines.[27,47] There are so many researches in which DOE was applied to the robustness.[31,32,43,48,49] Experimental design in robustness is a good approach to fully understand the factors having effects on the responses and provide maximum information about the method on a short time. Robustness should be built into methods in pre-validation stages; otherwise lately evaluated robustness test has a risk of obtaining inappropriate results which can cause redevelopment and revalidation.[50] Therefore, a robustness test in the earlier stage of the method development process leads to save effort, time and money. Experimental data which was obtained from early stages can help to evaluate the performance of the method and can be used to guide for further method development.[51]

Optimization can be performed by using Response Surface Methodology (RSM) designs such as Box Behnken design (BBD) and Central composite design (CCD).[49,52] BBD is a second order design that allows investigating numerous factors with three levels. It is preferable to CCD by preventing unrealistic extreme scenario by creating the experimental matrix without

containing the extreme points at the same experiment.[33,52] BBD is used in analytical method optimization in many studies.[6,48,53-65]

In this study, a simple, rapid and robust HPLC method with photodiode array (PDA) detection at 215 nm was developed for the determination and separation of amlodipine besylate and enalapril maleate in FDC tablets. This method, which is available for assay and dissolution studies, was fast, environmentally-friendly and more cost-effective than the earlier published methods.[24-26] In this study, DOE was adapted to the robustness parameter of the analytical method for determining amlodipine and enalapril together. DOE principles were used in method development of amlodipine and enalapril for the first time. The validation of the method was performed according to ICH Q2(R1) guideline.[47] The BBD was used for optimization of the method. The optimized HPLC method was applied to dissolution and assay analysis of an in-house FDC tablet including amlodipine and enalapril.

MATERIALS AND METHODS

Materials and reagents

HPLC grade methanol, o-phosphoric acid and hydrochloric acid 37% were obtained from Merck, Germany. Amlodipine besylate (Hetero Drugs, India) and enalapril maleate (Zheijiang Huahai, China) were kindly gifted by Nobel Pharma, Turkey.

The FDC tablet contains 6.94 mg of amlodipine besylate and 10 mg of enalapril maleate as APIs.

Apparatus

The HPLC system was a Shimadzu chromatographic system (Japan) with LC-20AD pump, SPD-M20A PDA detector at a wavelength of 215 nm, a reversed phase C18 column (4.6 x 250 mm id., particle size of 5 µm) from Waters® (USA). The HPLC system was controlled by LC Solution Software. Design-Expert® Version 9 (Stat-Ease Inc, USA) was used for the experimental design and statistical analysis of data. A pHmeter (PASS1 P11-BNC-Bante, England) was used to control the aqueous buffer. Dissolution test was performed with Pharmatest® Dissolution System (Germany).

Chromatographic conditions

The mobile phase was a mixture of methanol and water (pH adjusted to 3.0 with o-phosphoric acid) in the proportion of 10:90 (v:v). The injection volume of the samples was 5 µL. The flow rate was 1.2 mL/min. The detector wavelength was 215 nm and the column temperature was 30˚C.

Preparation of standard solutions

The standard solution was prepared according to the following process: 6.94 mg of amlodipine besylate (equivalent to 5 mg amlodipine base) and 10 mg of enalapril maleate were weighed and transferred to a 50 mL volumetric flask and diluted to volume with 0.1 N HCl. This solution includes 0.1 mg/mL of amlodipine base and 0.2 mg/mL of enalapril maleate. The calculations were performed considering amlodipine base and enalapril as maleate salt because of the dose proportionality in market products.

Calibration procedure

Calibration series were prepared in volumetric flasks by the appropriate dilution of standard solution with 0.1 N HCl. The calibration curve was plotted with eight concentrations in the range of 0.8-24 µg/mL for amlodipine and 1.6-48 µg/mL for enalapril (as maleate). The experiments were performed in three replicates for each level. Linearity of the calibration curve was evaluated by the linear regression statistics of concentrations against peak area.

Experimental design and statistical analysis

Experimental plan, data analysis and optimization process were executed in Design Expert® Version 9 by using the BBD. The BBD is a three-level and multi-factor design which is a

combination of 2k factorial and balanced incomplete block designs. In this study, four factors with three levels for each were determined as given in Table 1.
The significant factors in the model were found by Multivariate linear regression analysis and analysis of variance (ANOVA) F-test and its lack of fit with a confidence interval of 95% for each response. Significant factors were determined by the probability level that the p value is less than 0.05 and one-factor graphs.

Assay in FDC tablets

The FDC tablet containing amlodipine besylate and enalapril maleate was prepared by using direct compression method. For assay of the tablets 10 tablets for each product were randomly taken and weighed. Then these tablets were powdered and a quantity of the powder (equivalent to 5 mg of amlodipine and 10 mg of enalapril maleate) were accurately weighed and transferred to a 50 mL volumetric flask. 30 mL of diluting solution (0.1 N HCl) was added and mixed for 15 minutes in magnetic stirrer. Then, it was diluted with the same solution to the volume and mixed in ultrasonic bath for 10 minutes. 4 mL of this solution was transferred to a 25 mL volumetric flask and diluted to the volume using the same solvent and was held in an ultrasonic bath for 5 minutes. The samples were filtered through a syringe tip filter of 0.45 µm pore size and then analyzed using the HPLC method.

Dissolution studies

Dissolution studies were performed using USP apparatus II (paddle method) in 0.1 N HCl (pH 1.2). Dissolution volume was 900 mL and temperature was 37˚C ± 0.5˚C. Paddle rotational speed was 75 rpm. Samples (2 mL) were withdrawn at 10, 20, 30, 45, and 60 min and same amount of fresh media was replaced. The samples were filtered through 0.45 µm membrane filters to vials and analyzed by the optimized HPLC method. The dissolution profiles were evaluated by cumulative drug dissolved (%) to time. All experiments were performed in n=3 and the cumulative amounts were evaluated as the mean ± SD.

RESULTS AND DISCUSSION

The chromatograms of diluting media (blank) and the chromatograms obtained from the standard solutions of amlodipine and enalapril were given in Figure 1 and Figure 2, respectively. The initial method provided a good separation in a short time of 3.8 min for enalapril and 8.0 min for amlodipine. It can be acceptable in a conventional method development process. The robustness study with DOE was also performed.

Robustness with DOE principles

According to the ICH Q2 (R1) in a robust method, making small variations of certain method parameters do not affect the reliability and results of the method.[47] These small variations are important for the pharmaceutical industry in terms of the transfer of the analytical method from Research and Development to Quality Control laboratory or from a company to another. In other words, it is the indication of the strength of the method.[51] In order to assess the concurrent change of the factors on the defined responses, a multivariate analysis by DOE is recommended in robustness studies.[43] DOE is used in analytical method development for two main purposes: to determine the most significant factor influencing the response of the study and to discover the optimized value of the factors for best results for the response.[37]
DOE plan in robustness test includes following stages:[31]

Selection of factors and their levels

Robustness studies are excellent opportunity to apply statistical experimental design to provide data-based control of the method.[51] Since there are a lot of factors that might affect the method, it is vital to choose the right factors. In robustness studies of liquid chromatography most frequently preferred factors are pH of the mobile phase, analysis time, flow rate, column type, temperature, composition of the mobile phase, detection wavelength, chosen filters, or the variations in sample preparation such as dilution, shaking time or heating

temperature.[39,51] It should be noted that there are no absolute truths in selecting factors in a DOE process; the chosen factors should comply with the purpose. According to ICH Q2 (R1) following variations were recommended for the robustness test of HPLC methods: 1) pH of the mobile phase, 2) composition of the mobile phase, 3) column type, 4) temperature and 5) flow rate. Except from the column type all recommended factors (mobile phase ratio, pH, flow rate and column temperature) were investigated in this study. The chosen factors with pre-defined levels have a potential to affect the method depending on the analyst, a different laboratory or equipment and different environmental conditions.[47]

After selecting the factors it is needed to define the levels of the factors. In a two-level model such as PBD or two-level factorial designs, a maximum and a minimum limit are required for the factor values. In three-level designs, additional middle values, which generally represent the target or the expected value, are added to the design. Defining the levels is a critical step in experimental design. Particularly in two-level designs in which inappropriate levels were used, it can be obtained inaccurate and low-quality results.[33] In order to avoid this problem, three-level BBD design was preferred. The levels of the factors are usually defined symmetrically around the nominal level which is the middle level in a three-level design. The interval chosen between the levels is generally decided according to the operator's personal experiences or anticipated changes from a laboratory to another. For example, if the developed method will be transferred to another laboratory, the pH meter can measure with a small deviation so pH should be considered as critical. A pH of a solution varies with a deviation of 0.02 with a confidence limit of 95 %.[50] Therefore this limit is acceptable for the pH in a robustness test. The interval of pH was ±0.02 in this study. The levels of column temperature were decided ±5˚C as recommended in the article by Vander-Heyden et al which was aimed to guide a robustness parameter in method development.[50] The levels of other factors were selected as 5% for mobile phase composition and 0.2 mL/min for flow rate comply with the previous similar studies.[32,43,65]

Defining responses to be investigated

In the HPLC studies where robustness were investigated by DOE various responses such as peak area, peak height, found concentration, retention time, tailing factor, theoretical plate number, resolution were used. The most important criteria to choose the response used in the evaluation of the factors is that the selected response should be easy to measure.[39] Additionally, using large number of responses can be confusing in interpreting the results. Therefore, API concentrations calculated from the peak areas are selected as responses in this study.

Choosing an experimental design

The suitable experimental design should be selected based on the aim of the study. In case there are a large number of factors might affect the method, it can be aimed to discard some factors which have not a significant effect on the response. For this purpose a screening design such as PBD can be used. On the other hand, if the main objective is to investigate the effects of the relatively lower number of factors deeply, or optimize the most effective factors, the optimization designs should be preferred.[31] Generally, optimization is carried out following the determination of most significant factors by screening design. In cases there is a known factor to be highly effective in the separation (such flow rate or temperature), optimization designs can be preferred directly.[37] In this study, factors that may affect the results, such as column temperature, flow rate, and composition of mobile phase were chosen with the purpose of performing an optimization. Another reason for choosing RSM design is to observe if any interaction between the factors.

The mostly used RSM designs are CCD and BBD. BBD is required the least amount of experiment number among the RSM designs because it does not contain the values which are

at their highest or lowest values in the experimental matrix.[33] Since BBD requires fewer experiments and experimental matrix does not contain the highest or lowest level in combination this experimental design leads to prevent the unrealistic extreme scenario. Therefore the experiment number, time and the cost were reduced. BBD can evaluate the linear and nonlinear effects of factors.[34,66] Thus, BBD was selected for the experimental plan, data analysis and optimization process using the Design Expert® Version 9 software.

Execution of experiments

Experimental executions were computed by Design Expert Software. Robustness was assessed by using BBD with 29 runs. Experimental design and calculated concentrations of enalapril (as maleate) and amlodipine with obtained responses were given in Table 2.

Statistical evaluation of the responses and interpretations

The best fitted model was linear for all factors on the responses. In the literature linear analysis is frequently indicated and recommended in the robustness tests.[29-30] Therefore, our results were as expected. Linear models are used to show the main effects of factors.

The equation model for Y_1 (enalapril concentration) and Y_2 (amlodipine concentration) were as follows:

$$Y_1 = 32.32 + 0.079X_1 - 5.32X_2 + 0.11X_3 + 0.51X_4 \quad \text{(Equation 1)}$$

$$Y_2 = 16.19 + 0.12X_1 - 2.72X_2 + 0.020X_3 + 0.021X_4 \quad \text{(Equation 2)}$$

Where, X_1 is column temperature, X_2 is flow rate, X_3 is methanol ratio in mobile phase, and X_4 is pH of the mobile phase.

The ANOVA results were given in Table 3. The significant effects showed p value less than 0.05, a low standard deviation (CV%) and a high adjusted R-square (adj R^2) value indicated a good relationship between the experimental data and those of the fitted model. The predicted R-square (pred R^2) value was acceptable with the adj R^2 for all responses.

The one factor graphs (Figure 3 and Figure 4.) demonstrated that the flow rate was the most significant factor on the responses; inverse proportionality was found ($p<0.05$). It was revealed that the most critical factor in the robustness is the flow rate. Methanol ratio in mobile phase, temperature, and pH had no significant effect on calculated concentrations of amlodipine and enalapril in defined levels. Kovacs et al have evaluated the same factors on their robustness test with different responses like peak asymmetry and retention time. They found the methanol proportion of mobile phase was significant on retention time of strontium ranelate.[30] Similarly, Dharmal et al found that methanol proportion in mobile phase and flow rate had a negative effect while pH had a positive effect on peak area and found tapentadol concentration.[32] In another study, in which the same factors and different responses (tailing factor, retention time and theoretical plate) were used, the most effective factors were found as methanol composition and pH.[45] However, significance of factors is dependable to APIs and the chromatographic conditions. If we had defined our levels more broadly for other factors (methanol ratio, temperature, and pH) or if we had assessed more responses such as tailing factor or resolution we might have observed a meaningful effect with other factors. However, this was not considered to be an error in the design because the DOE is specific to the purpose. In this study, we would like to see how possible rational changes would affect the analysis results, rather than creating a design space based on extreme values of factors. Two-way interactions between independent variables were found insignificant ($p>0.05$). Therefore, a simple screening design such as Plackett-Burman Design (PBD) which is the most popular design in robustness evaluation might be used in this study.[37] However, since PBD is a two-level design, it can cause inaccurate statistical evaluations when unsuitable factor levels are selected or when there might be an interaction between the factors. If an

experimental model needs to determine the tolerable variations an optimization design is recommended by Sahu et al.[31] For this reason, as discussed before, we preferred BBD with three-level which contained a third level (target middle level) and provided more information about the method. There are similar researches with other drugs in which calculated drug concentrations were the only response and flow rate was the only significant factor in the response.[43,46]

Optimization

Following linear model fitting, an optimization run was performed and factor settings were defined using the prediction spreadsheet of the software (Figure 5). Final optimized parameters were flow rate of 1.205 mL/min, 2.95 of pH and 25˚C of column temperature. The factors described in the optimization were very close to the nominal levels in BBD design. Nonetheless, these minor changes caused a better peak shape for amlodipine and a lower tailing factor (from 1.417 to 1.164, $p<0.05$) (Figure 6). Retention times were not changed in the method with 3.8 min and 7.9 min for enalapril and amlodipine, respectively.

The optimized method was validated based on international guidelines.

Linearity

The linearity of the peak area versus concentration was shown in the range of 0.8-24 µg/mL for amlodipine and 1.6-48 µg/mL for enalapril (as maleate). Linearity results were given in Table 4. The linearity range was kept wider than the previously published methods.[24-26].The lower concentrations are considered for the first minutes of the dissolution study and higher values are for assay.

Accuracy

Accuracy was demonstrated using six different solutions, containing 1.39, 2.78, 5.56, 12, 16 and 19.2 µg/mL of amlodipine and 2.78, 5.56, 11.12, 24, 32 and 38.4 µg/mL of enalapril maleate. Recovery values were obtained within the range of 98.6-101.6%. The low value of RSD less than 1% indicates that the proposed method is accurate. Results were presented in Table 5.

Repeatability

Repeatability is also termed intraday precision and provides information about the precision under the same operating conditions in a short interval of time.[47] Repeatability was assessed using 10 determinations of the solutions including 16 µg/mL of amlodipine and 32 µg/mL of enalapril maleate. The recovery values were 99.9 ± 0.31% and 100 ± 0.07% for amlodipine and enalapril maleate, respectively.

The RSD were 0.307% and 0.0711% for amlodipine and enalapril maleate, respectively.

Intermediate precision

Intermediate precision was assessed using the inter-day variations. Two different concentrations (4 and 16 µg/mL for amlodipine and 8 and 32 µg/mL for enalapril maleate) were analyzed at consecutive three days. The RSD values of interday precision were less than 1%, verifying the method precision. The results were given in Table 6.

The low RSD value for intermediate precision and repeatability of method as well as within a day and day to day variation suggested that method was found to be precise in the range of measurement.

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

LOD and LOQ were calculated based on the standard deviation of the response and the slope by using the equations below:

$$LOD = \frac{3.3\sigma}{S} \qquad \text{(Equation 3)}$$

$$LOQ = \frac{10\sigma}{S} \quad \text{(Equation 4)}$$

where σ is the standard deviation of the response and S is the slope of the calibration curve. According to the equations, LOD values were 0.0631 µg/mL and 0.0424 µg/mL and LOQ were 0.19 µg/mL and 0.129 µg/mL for amlodipine and enalapril maleate, respectively. The LOD and LOQ results suggested that the method was highly sensitive.

Stability

The drugs dissolved in 0.1 N HCl were stable when stored at 25˚C for 72 hours. After 72 hours, drug recovery values were 99.7% for amlodipine and 99.4% for enalapril maleate.

Assay in tablets

The optimized method was used for the assay of amlodipine and enalapril in FDC tablets. An additional peak from excipients was not observed. The results were in the range of labelled amount ± 5% for both drugs (Table 7).

Dissolution

Dissolution was performed with the in-house FDC tablet by using USP apparatus II in 0.1 N HCl. 0.1 N HCl was selected as the model dissolution medium. The proposed HPLC method was available for dissolution of FDC tablets. Both amlodipine and enalapril were dissolved more than 85% within 10 minutes. Dissolution profiles of amlodipine and enalapril were given in Figure 7. The dissolution media of 0.1 N HCl replaces the artificial stomach medium that is frequently used with the purpose of formulation development and quality control. For using this analytical method for other dissolution media such as pH 4.5 or pH 6.8 there might be small modifications in chromatographic conditions.

In conclusion, an accurate, precise, specific and environmentally appropriate HPLC method was developed and validated for amlodipine besylate and enalapril maleate in the dosage unit. BBD, an optimization design, was used to evaluate the operational factor in robustness test and validation was performed according to international guidelines. The developed method was more economic and suitable for green chemistry with less solvent consumption that allowed better column performance. The method was applied to assay and dissolution studies and was found suitable for quality control tests and in vitro performance of pharmaceutical dosage forms for a fixed dose tablet combination containing amlodipine besylate and enalapril maleate for treatment of hypertension.

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FIGURES

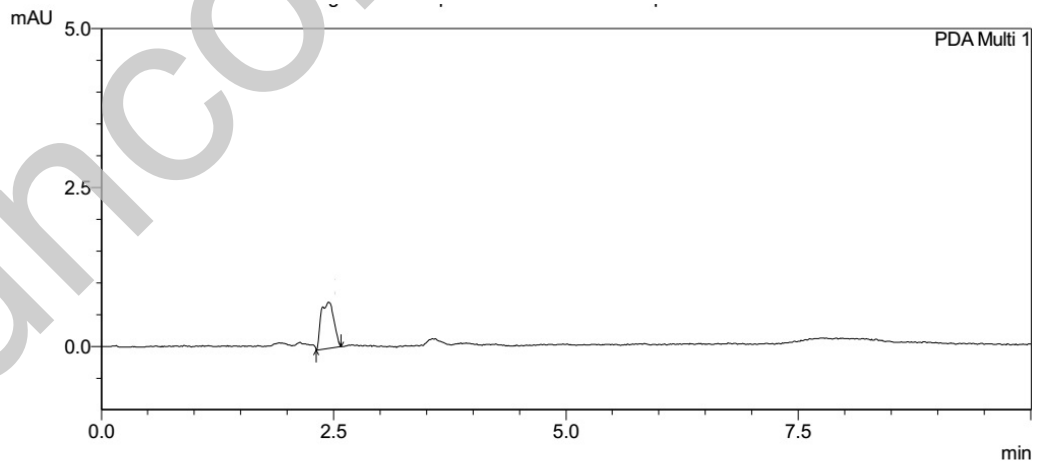

Figure 1. Chromatogram of the placebo (blank medium) for specificity.

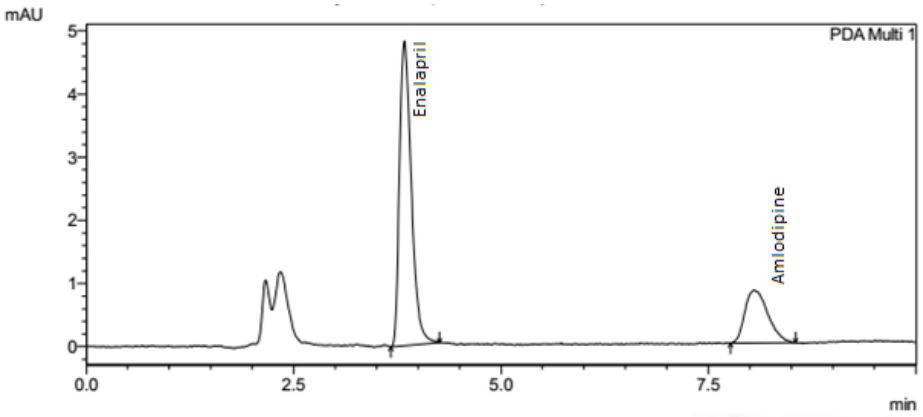

Figure 2. Chromatogram of enalapril (8 µg/mL, as maleate) and amlodipine (4 µg/mL) in initial method.

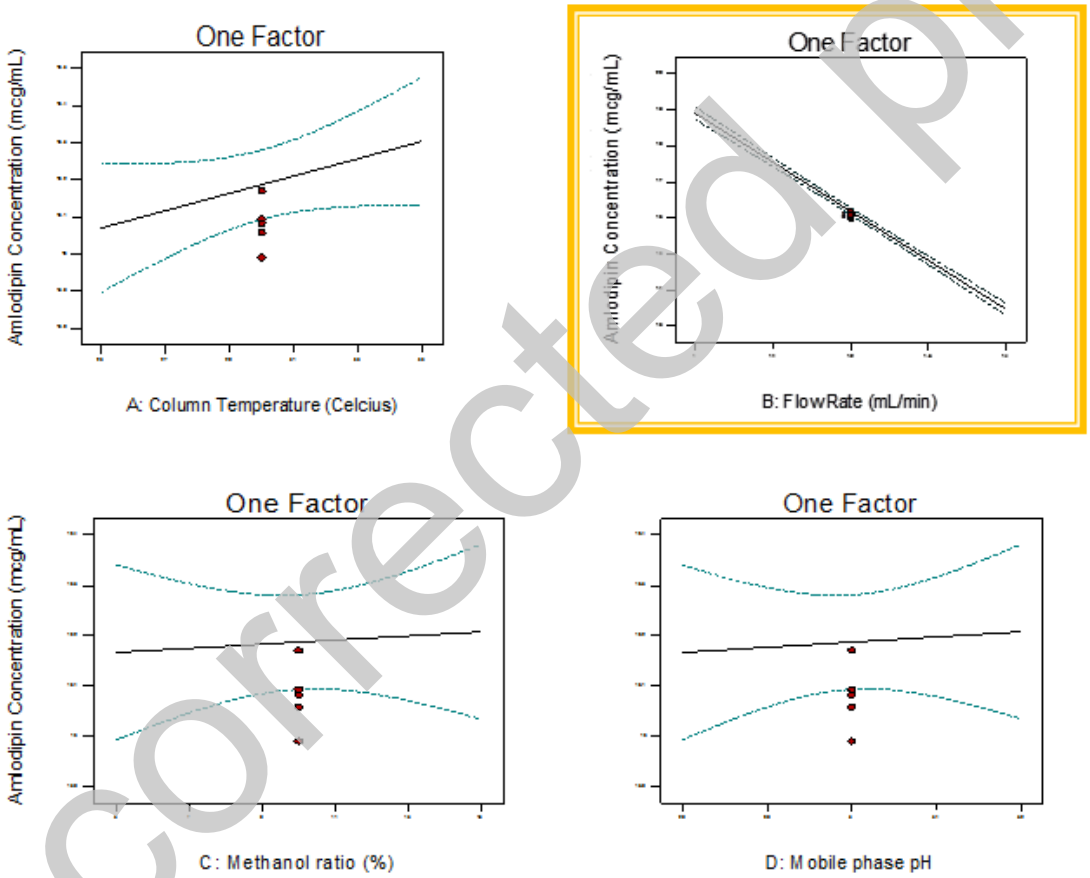

Figure 3. One factor graphs of the main effects of the factors on amlodipine concentration

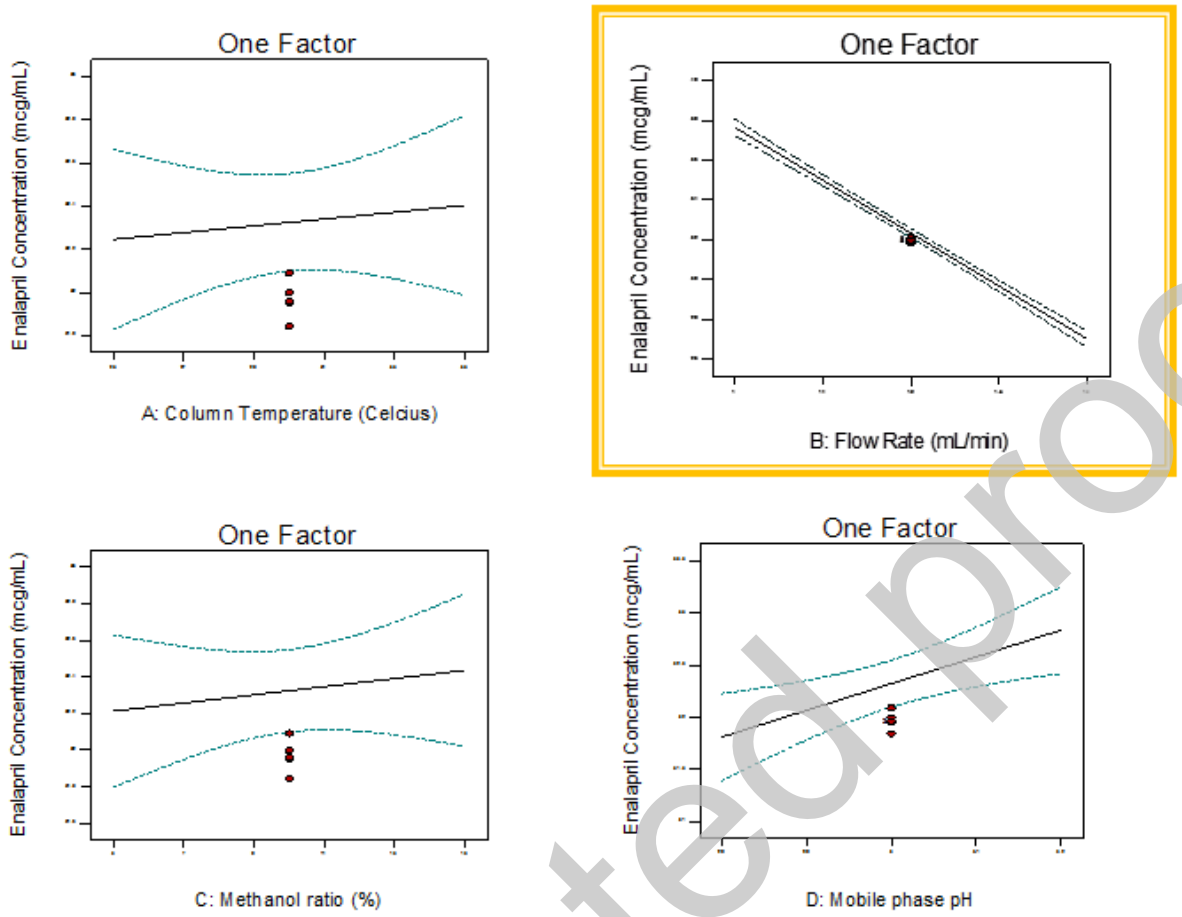

Figure 4. One factor graphs of the main effects of the factors on enalapril concentration

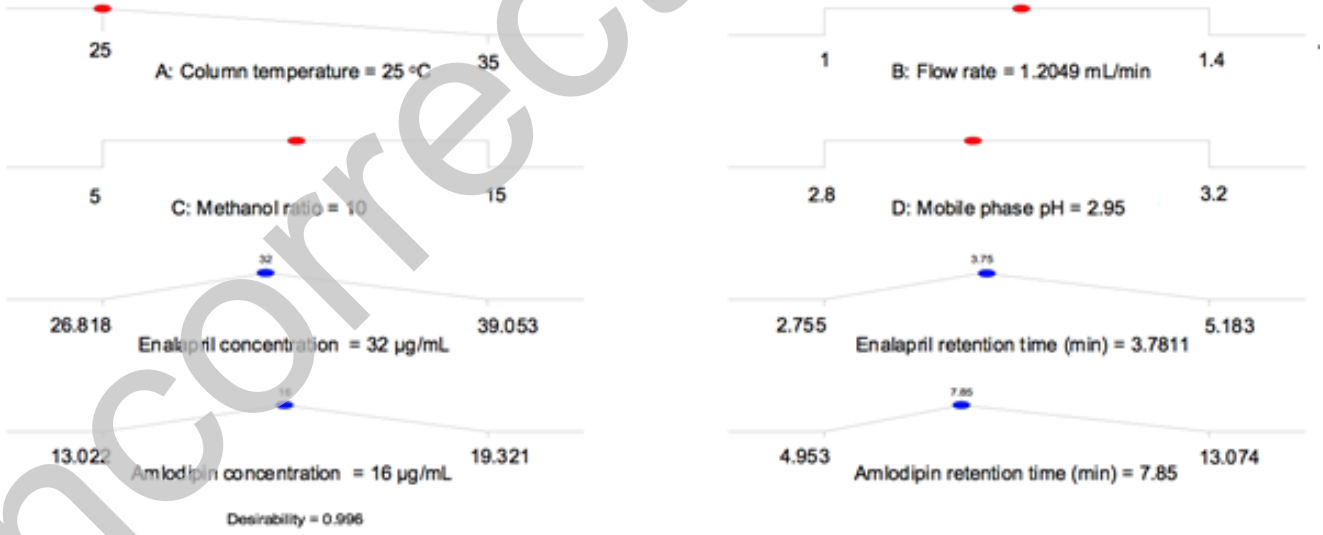

Figure 5. Optimization conditions of independent variables according to the Design Expert® Software.

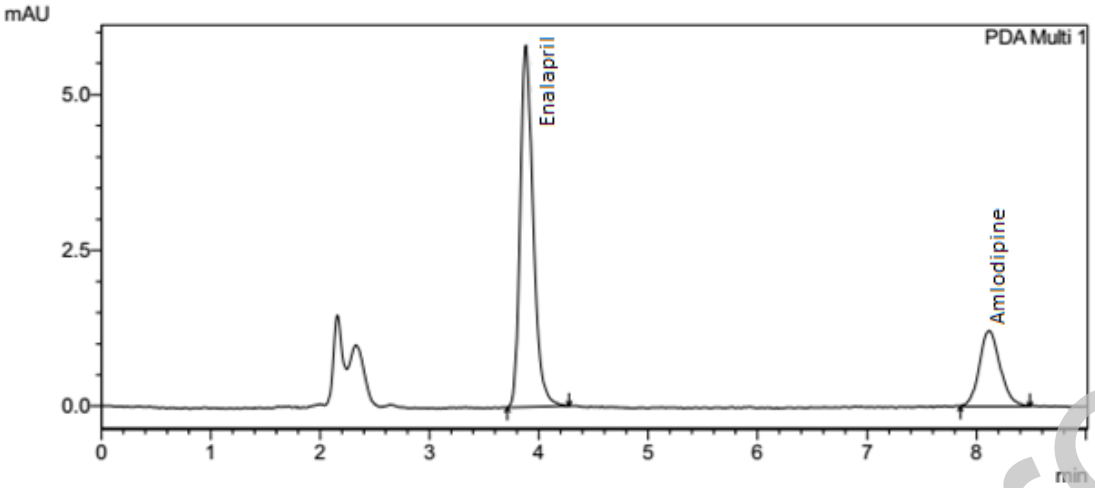

Figure 6. Chromatograms of enalapril (8 µg/mL, as maleate) and amlodipine (4 µg/mL) in optimized method.

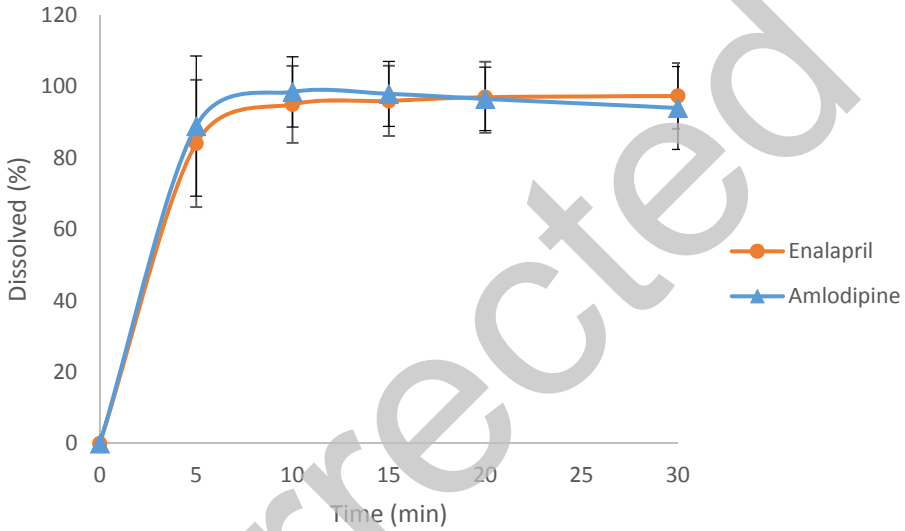

Figure 7. Dissolution results of amlodipine and enalapril in an in-house FDC product (n=3)

TABLES

Table 1. Experimental design

Factors	Low level	Nominal level	High level
Methanol ratio in the mobile phase (%)	5	10	15
Flow rate (mL/min)	1.0	1.2	1.4
pH of the mobile phase	2.8	3.0	3.2
Column temperature (˚C)	25	30	35

Table 2. Experimental plan for robustness and calculated responses

	Factors				Responses	
Run	**Column temperature (˚C)**	**Flow rate (mL/min)**	**Methanol ratio (%)**	**Mobile phase pH**	**Amlodipine concentration (µg/mL)**	**Enalapril maleate concentration (µg/mL)**
1	30	1.2	5	3.2	15.888	32.058
2	30	1.2	10	3.0	16.171	32.090
3	35	1.4	10	3.0	13.729	27.696
4	25	1.0	10	3.0	18.749	37.797
5	30	1.2	10	3.0	15.991	31.951
6	25	1.2	5	3.0	15.998	31.954
7	30	1.4	10	3.2	13.837	28.039
8	35	1.2	15	3.0	16.102	32.001
9	30	1.2	15	2.8	15.954	31.684
10	25	1.2	15	3.0	16.047	32.003
11	25	1.2	10	3.2	16.051	32.185
12	35	1.2	5	3.0	16.078	31.909
13	25	1.4	10	3.0	13.022	27.539
14	30	1.4	5	3.0	13.822	27.465
15	30	1.0	5	3.0	19.209	38.283
16	30	1.2	15	3.2	16.084	32.385
17	30	1.2	10	3.0	16.059	31.844
18	35	1.2	10	2.8	16.045	31.391
19	35	1.2	10	3.2	16.099	32.295
20	30	1.2	10	3.0	16.083	31.960
21	30	1.2	5	2.8	16.137	31.772
22	35	1.0	10	3.0	19.132	38.345
23	30	1.2	10	3.0	16.094	31.998
24	30	1.4	15	3.0	13.868	27.869
25	25	1.2	10	2.8	15.920	31.214
26	30	1.0	15	3.0	19.321	38.836
27	30	1.4	10	2.8	13.721	26.818
28	30	1.0	10	2.8	19.084	36.981
29	30	1.0	10	3.2	19.149	39.053

Table 3. ANOVA results

Responses	**±SD**	**Mean**	**CV%**	**Press**	**R^2**	**Adj.R^2**	**Pred R^2**	**Adeq.Prec.**	**p value**
Amlodipine	0.24	16.19	1.51	2.21	0.984	0.982	0.976	55.91	<0.0001
Enalapril maleate	0.59	32.32	1.82	12.69	0.976	0.972	0.964	47.76	<0.0001

Table 4. Calibration data for amlodipine and enalapril maleate (n=3 for each level) for optimized method

APIs	**Equation**	**R^2**
Amlodipine	y=4253.2x-796.1	0.9998

Enalapril maleate	y=6272.4x-1177.1	0.9995

Table 5. Accuracy results for amlodipine and enalapril maleate (n=3 for each level)

	Concentration (µg/mL)	Recovery (% ± SE)	RSD (%)
Amlodipine	1.39	99.0 ± 0.70	0.68
	2.78	98.6 ± 1.60	1.59
	5.56	100.0 ± 0.40	0.42
	12.0	100.1 ± 0.30	0.27
	16.0	99.7 ± 0.16	0.16
	19.2	101.1 ± 0.40	0.40
Enalapril maleate	2.78	100.4 ± 0.60	0.64
	5.56	99.6 ± 0.10	0.08
	11.12	100.6 ± 0.10	0.10
	24.0	100.0 ± 0.20	0.19
	32.0	99.7 ± 0.25	0.26
	38.4	101.6 ± 0.30	0.28

Table 6. Interday precision results of amlodipine and enalapril maleate (n=3)

	Concentration (µg/mL)	1st day (% ± SE)	2nd day (% ± SE)	3rd day (% ± SE)	RSD (%)
Amlodipine	4.0	99.0 ± 0.04	98.3 ± 0.02	99.0 ± 0.02	0.754
	16.0	99.9 ± 0.06	99.4 ± 0.04	99.7 ± 0.03	0.248
Enalapril maleate	8.0	99.3 ± 0.02	99.1 ± 0.02	99.0 ± 0.10	0.816
	32.0	99.8 ± 0.02	99.8 ± 0.02	100.0 ± 0.02	0.111

Table 7. Assay for FDC tablets (n=3)

	Labeled amount (mg/tablet)	Observed amount (mg/tablet)	RSD (%)
Amlodipine	5.00	4.95 ± 0.03	0.52
Enalapril maleate	10.00	10.17 ± 0.06	0.63