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ORIGINAL ARTICLE

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 to develop and optimize a simple, cost-effective, and robust high-performance liquid chromatography (HPLC) method by taking an experimental design approach to 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 (4.6x250 mm id., particle size of 5 µm). The injection volume was 5 µL, and the detection wavelength was 215 nm. A Box-Behnken design was used to test the robustness of the method. The flow rate (1, 1.2, and 1.4 mL/min), column temperature (25˚C, 30˚C, and 35˚C), 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 method was validated according to International Conference on Harmonization guidelines. Dissolution of the tablets was performed by using USP apparatus 2 and analyzed using the optimized HPLC method. Multivariate linear regression analysis and ANOVA were used in the statistical evaluation.

Results: Linear models were fitted for all variables. The flow rate was the most significant factor affecting the APIs' concentrations. The optimized method included the following parameters: Column temperature of 25˚C, 10% methanol as the mobile phase, pH of 2.95, and flow rate of 1.205 mL/min. Retention times were 3.8 min and 7.9 min for enalapril and amlodipine, respectively. The method was found to be linear in the 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 APIs were dissolved more than 85% within 10 min.

Conclusion: The experimental design was proved as a useful tool for the determination and separation of enalapril maleate and amlodipine besylate in dosage forms. 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, HPLC, fixed-dose combination

ÖZ

Amaç: 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 yüksek basınçlı sıvı kromatografisi (YBSK) yönteminin geliştirilmesi ve optimizasyonudur.

Gereç ve Yöntemler: Kromatografik analiz C18 kolonda (4,6x250 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°C,

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30°C ve 35°C), 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/dk hızda gerçekleştirilmiştir. Çözünme hızı çalışması 0,1 N HCl'da 37±0,5°C'de yapılmış ve optimize edilen YBSK yöntemi ile analiz edilmiştir. İstatistiksel değerlendirmede çok değişkenli doğrusal regresyon analizi ve ANOVA testi 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 metanol oranı, 2,95 hareketli faz pH'sı ve 1,205 mL/dk akış hızı. Alıkonma zamanları enalapril ve amlodipine için sırasıyla 3,8 dk ve 7,9 dk 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 maleat ve amlodipin besilatın dozaj formlarından analizinde deney tasarımı faydalı bir yaklaşım olarak görülmüştür. Optimize edilen yöntemin enalapril ve amlodipin içeren bir sabit dozlu kombinasyonun *in vitro* performansı ve kalite kontrol testlerinde kullanılabileceği gösterilmiştir.

Anahtar kelimeler: Amlodipin, enalapril, deney tasarımı, YBSK, sabit dozlu kombinasyon

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 a majority of patients.[1-3] A greater therapeutic benefit can be achieved with two or even more antihypertensive drugs.[4] Therefore, fixed-dose combinations (FDCs) are frequently used in cardiovascular diseases such as hypertension. In order to develop an FDC product including two drugs, certain conditions must be met. 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 calcium channel blockers (CCBs) and angiotensin-converting enzyme inhibitors (ACEIs). In addition, ACEIs such as enalapril prevent 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 when used 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. A dose of 6.94 mg of amlodipine besylate is equivalent to 5 mg of amlodipine base. The 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 the bioavailability of amlodipine is approximately 60%-65%, it is defined as a highly permeable drug because of the 90%-95% excretion rate as an inactive metabolite in the urine Shohin et al.[9] Amlodipine is a class 1 drug according to the Biopharmaceutics 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 high-performance liquid chromatography (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 the literature for analyses of amlodipine,[15] and enalapril,[16,17] individually or in combination with other 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 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.[27] recommended reducing the acetonitrile amount in the methods and using multiparameter methods such as design of experiment (DOE) instead of the one factor at a time (OFAT) approach.[28] In the method developed by Chaudhari[24], the mobile phase contains 50% acetonitrile and 40% methanol and a higher injection volume (20 µL), which increases the 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). In another method, the mobile phase includes 60% acetonitrile, 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.[26], 50% 1N HCl and 50% methanol were included in the mobile phase, and the injection volume was 10 µL. 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 the combined dosage form in the literature.

DOE is a well-defined mathematical methodology to demonstrate how to obtain maximum reliable and valuable scientific information by performing minimal experiments.[29] In this technique, the effects of multiple variations on one or more responses 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 the appropriate DOE model.[33,34] In the pharmaceutical field, 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 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, the flow rate can be an independent factor that has potential effects 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 of which is to define the optimal conditions and settings for the factors.[38] In case more than one factor must be examined, the multivariate optimization designs can be reasonable in order to evaluate different factors at the same time and to determine if interactions exist between factors.[37,38]

In analytical chemistry, DOE can be used for chromatographic analytical method development to optimize the sampling preparational, column, detector, instrumental, 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 the International Conference on Harmonization (ICH) guidelines.[27,47] There have been many studies in which DOE was applied to robustness.[31,32,43,48,49] Experimental design targeting robustness is a good approach to fully understand the factors with effects on the responses and provide maximum information about the method in a short time. Robustness should be built into methods in the pre-validation stages; otherwise, a robustness test performed too late 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 a saving of effort, time, and money. Experimental data obtained from early stages can aid in performance method evaluation and can be used to guide further method development.[51]

Optimization can be performed by using response surface methodology (RSM) designs such as the Box-Behnken design (BBD) and the central composite design (CCD).[49,52] The BBD is a second-order design that allows investigation of numerous factors with three levels. It is preferable to the CCD because it prevents an unrealistic extreme scenario by creating the experimental matrix without containing extreme points in 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 the method development of amlodipine and enalapril for the first time. The validation of the method was performed according to the ICH Q2 (R1) guideline.[47] The BBD was used for the 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 (4.6x250 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 pH meter (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 the appropriate volume with 0.1N HCl. This solution included 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 salts 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 0.1N 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. The linearity of the calibration curve was evaluated by the linear regression statistics of concentrations against peak area.

Statistical analysis

Experimental design

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 determined by multivariate linear regression analysis and 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 selected at random 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) was accurately weighed and transferred to a 50 mL volumetric flask. A 30 mL volume of diluent solution (0.1N HCl) was added and mixed for 15 min in magnetic stirrer. Then, it was diluted with the same solution to the volume and mixed in an ultrasonic bath for 10 min. A 4 mL volume 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 min. The samples were filtered through a syringe tip filter of 0.45-µm pore size and then analyzed using HPLC.

Dissolution studies

Dissolution studies were performed using USP apparatus II (paddle method) in 0.1N HCl (pH 1.2). The dissolution volume was 900 mL, and the temperature was 37°C±0.5°C. The paddle rotational speed was 75 rpm. Samples (2 mL) were withdrawn at 10, 20, 30, 45, and 60 min, and the 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 as the cumulative drug dissolved (%) over time. All experiments were performed in n=3 and the cumulative amounts were evaluated as the mean ± standard deviation (SD).

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

RESULTS AND DISCUSSION

The chromatograms of diluent (blank) and those obtained from the standard solutions of amlodipine and enalapril are given in Figure 1, 2 respectively. The initial method provided good separation in a short time of 3.8 min for enalapril and 7.9 min for amlodipine. This level of separation is acceptable in a conventional method development process. A robustness study with DOE was also performed.

Robustness with DOE principles

According to the ICH Q2 (R1), in a robust method, small variations in 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 the quality control laboratory or from one company to another. In other words, it is the indication of the strength of the method.[51] In order to assess the concurrent influences of the changes in 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]

The DOE plan in a robustness test includes the following stages:[31]

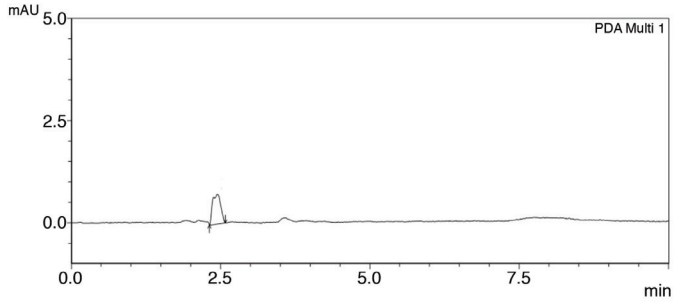

Figure 1. Chromatogram of the placebo (blank medium) for specificity testing

PDA: Photodiode array

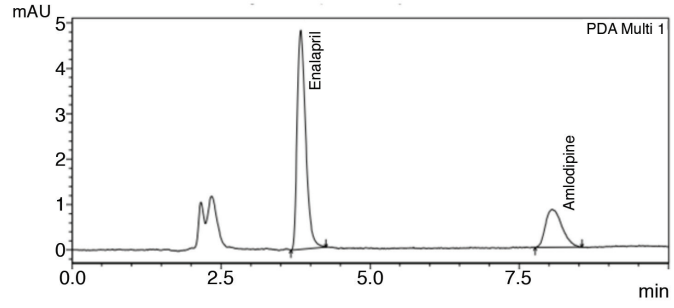

Figure 2. Chromatogram of enalapril (8 µg/mL, as maleate) and amlodipine (4 µg/mL) in the initial method

PDA: Photodiode array

Selection of factors and their levels

Robustness studies are an excellent opportunity to apply statistical experimental design to provide data-based control of the method.[51] Since there are many factors that might affect the method, it is vital to choose the right factors. In robustness studies of liquid chromatography, the most frequently preferred factors are the 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), the 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 for the column type, all recommended factors (mobile phase ratio, pH, flow rate, and column temperature) were investigated in this study. The chosen factors and their pre-defined levels have the potential to affect the method depending on the analyst, laboratory or equipment, and environmental conditions.[47]

After selecting the factors, it is necessary to define their levels. In a two-level model such as Plackett-Burman Design (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, inaccurate and low-quality results can be obtained.[33] In order to avoid this problem, a three-level BBD design is 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 one laboratory to another. For example, if the developed method will be transferred to another laboratory, the pH can be measured using a pH meter with a small deviation, so pH should be considered as critical. The 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.[50], which was aimed to guide a robustness parameter in method development. The levels of other factors, selected as 5% for mobile phase composition and 0.2 mL/min for flow rate, were in agreement with previous similar studies.[32,43,65]

Defining responses to be investigated

In the HPLC studies where robustness was investigated by DOE, various responses such as peak area, peak height, determined concentration, retention time, tailing factor, theoretical plate number, and resolution were used. The most important selection criterion for a response to use in factor evaluation is ease of measurement.[39] Additionally, using a large number of responses can lead to confusion when interpreting the results. Therefore, API concentrations calculated from the peak areas were selected as responses in this study.

Choosing an experimental design

A suitable experimental design should be selected based on the aim of the study. In case a large number of factors might affect the method, the aim can be to discard some factors that have no 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, optimization designs should be preferred.[31] Generally, optimization is carried out following determination of the most significant factors by screening design. In case there is a factor known to be highly effective in the separation (such a flow rate or temperature), optimization designs can be preferred directly.[37] In this study, factors that may affect the results, such as the column temperature, flow rate, and composition of the mobile phase, were chosen with the purpose of performing an optimization. Another reason for choosing an RSM design is to observe any interaction between the factors.

The most used RSM designs are CCD and BBD. BBD requires the fewest experiments among the RSM designs because it does not contain values that are maximum or minimum values in the experimental matrix.[33] Since BBD requires fewer experiments, and the experimental matrix does not contain the highest or lowest level in the combination, this experimental design prevents an unrealistic extreme scenario. Therefore, the experiment number, time, and cost are reduced. BBD can evaluate the linear and non-linear 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 and the corresponding responses are given in Table 2.

Statistical evaluation of responses and their interpretations

The best fit model was linear for all factors and their responses. In the literature, linear analysis is frequently indicated and recommended in 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) was 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 the methanol ratio in the mobile phase, and X_4 is the pH of the mobile phase.

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

The one-factor graphs (Figure 3, 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

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 precision	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

SD: Standard deviation, CV: Cardiovascular, Adj R^2: Adjusted R-square

most critical factor in robustness is the flow rate. The methanol ratio in mobile phase, temperature, and pH had no significant effect on the calculated concentrations of amlodipine and enalapril in defined levels. Kovacs et al.[30] have evaluated the same factors in their robustness test with different responses such as peak asymmetry and retention time. They found that the proportion of methanol in the mobile phase had a significant effect on the retention time of strontium ranelate. Similarly, Dhumal et al.[32] found that the proportion of methanol in the mobile phase and the flow rate had a negative effect, while the pH had a positive effect on the peak area and the determined tapentadol concentration. 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 to be the methanol composition and pH.[45] However, the significance of factors depends on the APIs and 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 analytical results, rather than creating a design space based on the extreme values of factors.

Two-way interactions between independent variables were found to be insignificant ($p>0.05$). Therefore, a simple screening design, such as a 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 is needed to determine tolerable variations, an optimization design is recommended by Sahu et al.[31] For this reason, as discussed before, we preferred a BBD that contained a third level (target middle level) and provided more information about the method. There have been similar studies 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]

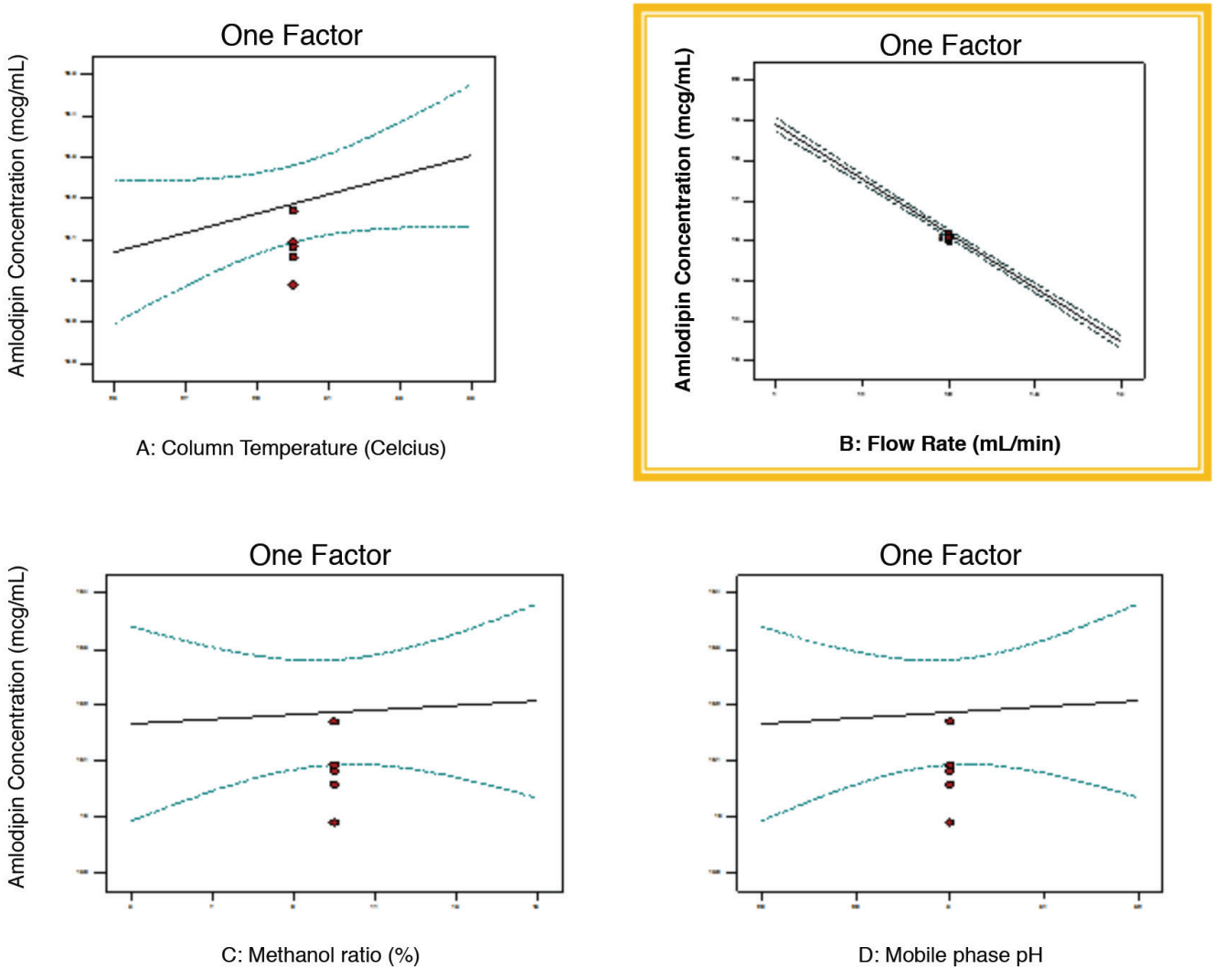

Figure 3. A-D) One-factor graphs of the main effects of the factors on amlodipine concentration

Optimization

Following linear model fitting, an optimization run was performed, and factor settings were defined using the prediction spreadsheet of the software (Figure 5). The final optimized parameters were a flow rate of 1.205 mL/min, pH of 2.95, and column temperature of 25°C. The factors described in the optimization were very close to the nominal levels in the BBD design. Non-etheless, 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 the 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 relative standard deviation (RSD) less than 1% indicates that the proposed method is accurate. Results are presented in Table 5.

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

APIs	Equation	R^2
Amlodipine	y=4253.2x-796.1	0.9998
Enalapril maleate	y=6272.4x-1177.1	0.9995

R^2: R-square

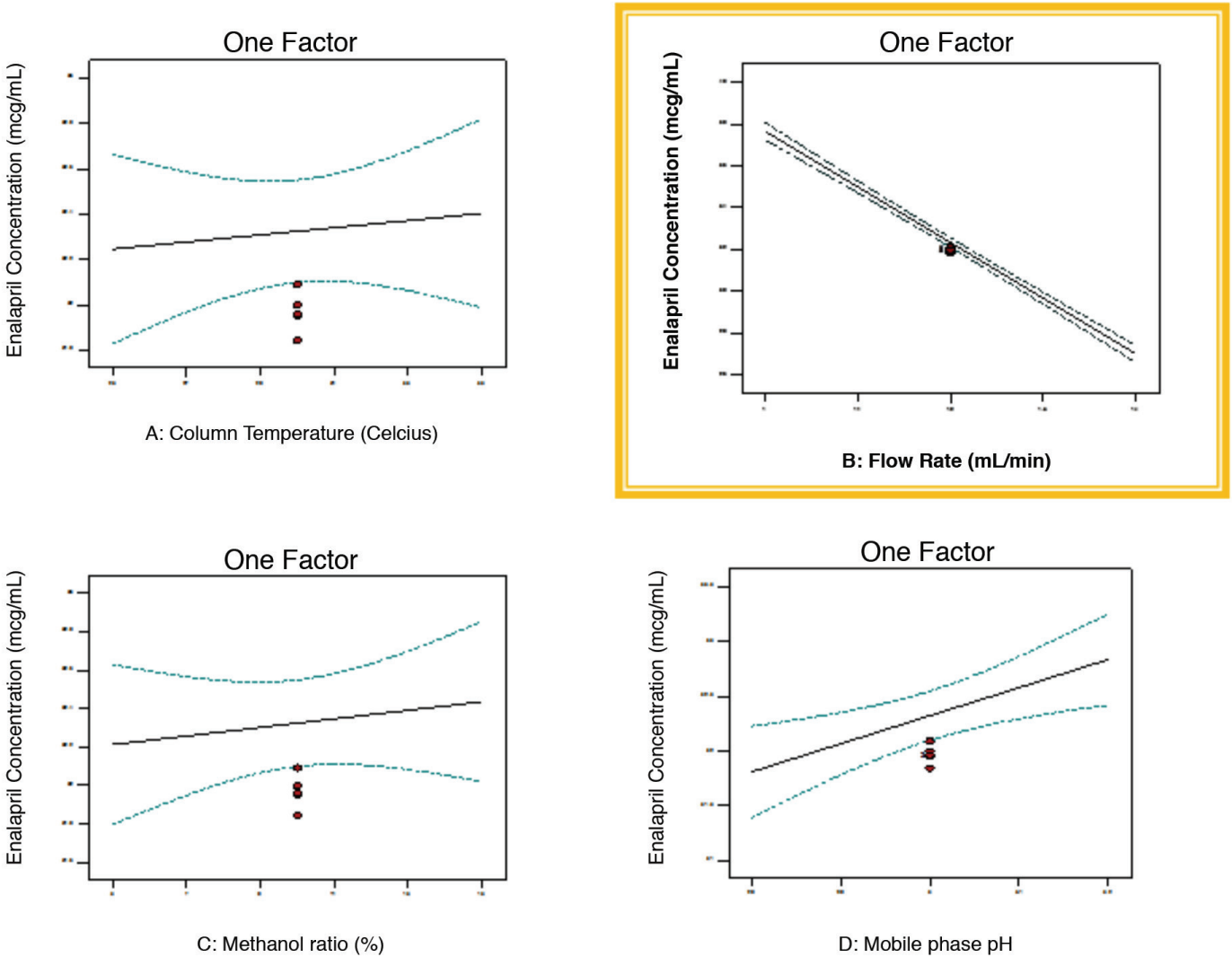

Figure 4. A-D) One-factor graphs of the main effects of the factors on enalapril concentration

Repeatability

Repeatability is also termed intraday precision and provides information about the precision under the same operating conditions in a short time interval.[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 RSDs were 0.307% and 0.0711% for amlodipine and enalapril maleate, respectively.

Intermediate precision

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

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

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

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

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

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

where σ is the SD 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 0.1N 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 the labeled amount ±5% for both drugs (Table 7).

Dissolution

Dissolution was performed with the in-house FDC tablet by using USP apparatus II in 0.1N HCl. 0.1N 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 min. Dissolution profiles of amlodipine and enalapril were given in Figure 7. The dissolution media of 0.1N HCl replaces the artificial stomach medium that is frequently used with the purpose of formulation development and quality control. For

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

SE: Standard error, RSD: Relative standard deviation

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

SE: Standard error, RSD: Relative standard deviation

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

FDC: Fixed-dose combination, RSD: Relative standard deviation

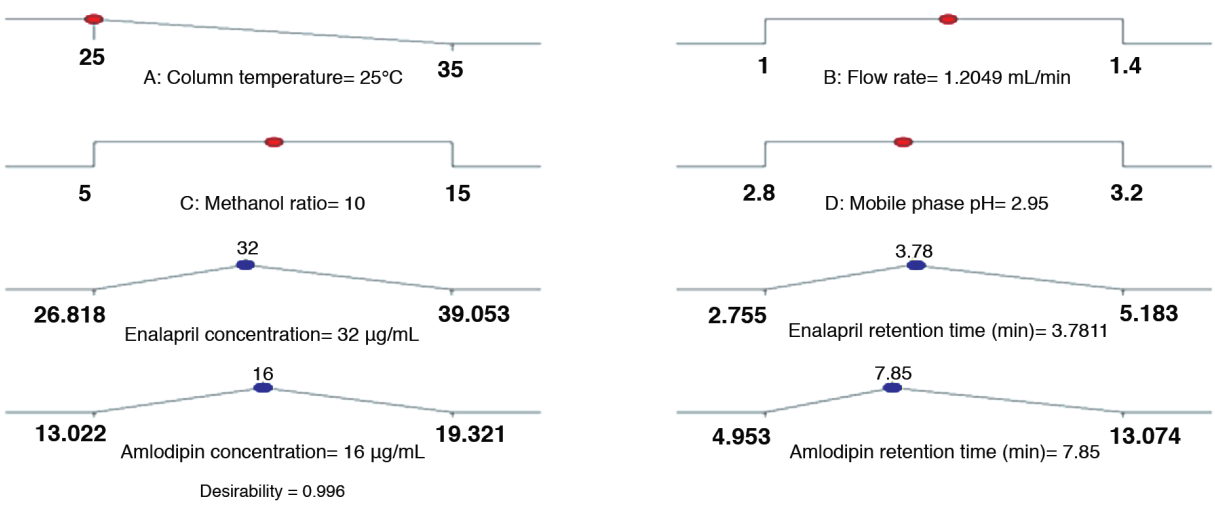

Figure 5. Optimization conditions of independent variables according to the Design Expert® Software

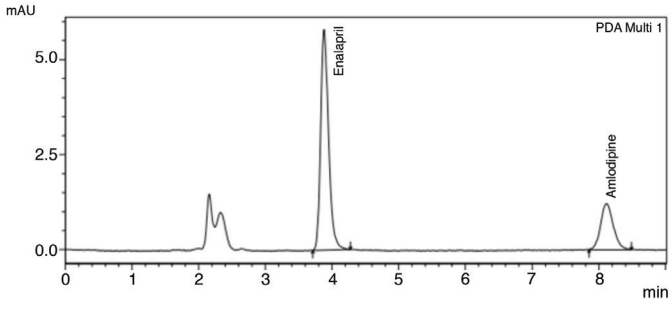

Figure 6. Chromatograms of enalapril (8 µg/mL, as maleate) and amlodipine (4 µg/mL) in the optimized method

PDA: Photodiode array

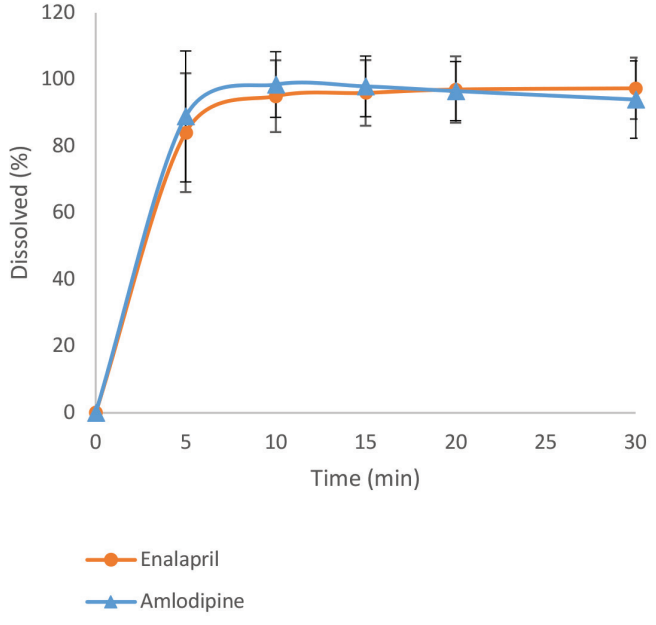

Figure 7. Dissolution results of amlodipine and enalapril in an in-house FDC product (n=3)

FDC: Fixed-dose combination

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.

CONCLUSION

In conclusion, an accurate, precise, specific, and environmentally appropriate HPLC method was developed and validated for amlodipine besylate and enalapril maleate in the typical dosage unit. The BBD, an optimization design, was used to evaluate the operational factors in a 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, which improved 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 the treatment of hypertension.

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