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

Diren SARISALTIK YAŞIN¹, Alev ARSLANTÜRK BİNGÜL², Alptuğ KARAKÜÇÜK³,⁴, Zeynep Şafak TEKSİN³*

¹Dicle University Faculty of Pharmacy, Department of Pharmaceutical Technology, Diyarbakır, Turkey
²Dicle University Faculty of Science, Department of Chemistry, Diyarbakır, Turkey
³Gazi University Faculty of Pharmacy, Department of Pharmaceutical Technology, Ankara, Turkey
⁴Ankara Medipol University Faculty of Pharmacy, Department of Pharmaceutical Technology, Ankara, Turkey

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 (R² >0.999) and 1.6-48 µg/mL (R² >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 analizini için deney tasarımı yaklaşımlı ile basit, ekonomik ve sağlam bir yüksek basınçlı svi kromatografisi (YBSK) yönteminin geliştirilmesi ve optimizasyonudur.

Gereç ve Yöntemler: Kromatografik analiz C18 kolonla (4,6x250 mm id., 5 µm partikül çapi) 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,
30°C ve 35°C'ye, hareketli fazdaki metanol orani (%5, %10 ve %15) ve hareketli fazın pH'si (2,8, 3 ve 3,2) bağımız değişkenler olarak seçilmiştir. Yöntemin validasyonu ICH kılavuzlarına göre gerçekleştirilmiştir. Tabletlerin çözünme hızı 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 (R² > 0,999) ve 1,6-48 (R² > 0,999) µg/mL araştırma doygunal davranış. Her iki etkin madde de 10 dakika içinde %68'ten fazla çözünmüştür.

Sonuç: Enalapril maleat ve amlodipin besilati dozaj formulardan analizinde deney tasarımında faydaları bir yaklaşım olarak görülmüştür. Optimize edilen yöntem enalapril ve amlodipin içeren bir sabit dozlu kombinasyonun in vitro performansı ve kalite kontrol testlerinde kullanılabileceği gösterilmiştir.

Anahtar kelimeler: Amlodipin, enalapril, deney tasarım, 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.1-13 A greater therapeutic benefit can be achieved with two or even more antihypertensive drugs.9 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 besylate13 and enalapril maleate,14 separately and a few liquid chromatography methods are available in the literature for analyses of amlodipine,15-17 and enalapril,18,19 individually or in combination with other drugs.20-22 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.23-25 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.26,27 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 Chaudhari24, 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 Mash 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
In this study, a simple, rapid and robust HPLC method with optimization in many studies.\textsuperscript{24-26} 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.\textsuperscript{33,34} In the pharmaceutical field, DOE helps to understand the effects of the critical formulation and process variables on the final product.\textsuperscript{29,36} 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.\textsuperscript{27,37} On the other hand, in an optimization process, the main objective of which is to define the optimal conditions and settings for the factors.\textsuperscript{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.\textsuperscript{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.\textsuperscript{30,39} Similarly, analytical method validation parameters such as accuracy, linearity, precision, or robustness can be performed by experimental design approaches.\textsuperscript{29,40-46} Using DOE in validation studies is recommended in the International Conference on Harmonization (ICH) guidelines.\textsuperscript{27,47} There have been many studies in which DOE was applied to robustness.\textsuperscript{20,32,43,48,49} 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 the method in a short time. Robustness should be built into the system or optimization of a characterized system. Factors are independent variables that might affect the results of critical responses. 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.\textsuperscript{27,37} On the other hand, in an optimization process, the main objective of which is to define the optimal conditions and settings for the factors.\textsuperscript{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.\textsuperscript{37,38}

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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.\textsuperscript{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.\textsuperscript{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\textsuperscript{®} (USA). The HPLC system was controlled by LC Solution Software. Design Expert\textsuperscript{®} 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\textsuperscript{®} 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).

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. 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. 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. 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.

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

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         |
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. 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. 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.

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. 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%. 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. 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.

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. 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. 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 as a flow rate or temperature), optimization designs can be preferred directly. 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. 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. 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. 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 ($^\circ$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** | **R²** | **Adj R²** | **Pred R²** | **Adeq precision** | **p value** |
| Amlodipine | 0.24 | 16.19 | 1.51 | 2.21 | 0.984 | 0.982 | 0.976 | 0.972 | 55.91 | <0.0001 |
| Enalapril maleate | 0.59 | 32.32 | 1.82 | 12.69 | 0.976 | 0.964 | 19.084 | 19.149 | 39.053 |

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. 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. 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. 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. 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. 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.

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. 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. 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.

![Figure 4](image-url)

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

| API            | Equation       | R²   |
|----------------|---------------|------|
| Amlodipine     | y=4253.2x-796.1 | 0.9998 |
| Enalapril maleate | y=6272.4x-1177.1 | 0.9995 |

| R²: R-square |
Repeatability

Repeatability is also termed intraday precision and provides information about the precision under the same operating conditions in a short time interval. 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 \(\sigma\) 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
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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