Efficient validated method of HPLC to determine amlodipine in combined dosage form containing amlodipine, enalapril and bisoprolol and in vitro dissolution studies with in vitro/in vivo correlation

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Abstract

Aim. A rapid and reproducible HPLC method has been developed for the determination of amlodipine in experimental combined dosage forms containing amlodipine, enalapril and bisoprolol and for drug dissolution studies.

Materials and methods. The separation was done using a column Phenomenex Polar Synergi, 5 μm, 4.6×50 mm and a mobile phase of methanol:phosphate buffer solution (65:35, v/v), flow-rate of 1.0 mL/min. The injection volume was 100 μL and the ultraviolet detector was set at 240 nm.

Results. The method was validated as per ICH guidelines. Under these conditions, amlodipine was eluted at 1.89 min. Total run time was shorter than 2.5 min. The linearity of the method had a good correlation with concentration and peak area. The correlation coefficient of amlodipine was found to be not less than 0.9991, which indicates good linear relationship over concentration range 0.625 mg/mL–5.000 mg/mL (1.250 mg/mL–5.000 mg/mL at pH 4.5). The % RSD values in intra-day and inter-day precision study were found to be less than 0.267 for amlodipine, which indicate method was precise. Hence, the present developed method was said to be suitable for the analysis of drugs in their pharmaceutical dosage form. Also, in vitro dissolution of amlodipine containing tablets were performed to validate the suitability of the proposed method. The dissolution pattern complies with the FDA standards, indicating suitability of the proposed method for the dissolution study of amlodipine. It will allow conducting comparative studies in vitro to confirm the equivalence of tablets containing amlodipine.

Conclusion. A simple and sensitive HPLC method was developed for the estimation of amlodipine in tablets containing amlodipine, enalapril and bisoprolol. The proposed method was applied successfully for quality control assay of amlodipine in experimental tablets and in vitro dissolution studies. In vitro/in vivo correlation of amlodipine has been conducted.

Keywords

Amlodipine, High performance liquid chromatography, Dissolution study, Validation, In vitro/in vivo correlation
Introduction

Dissolution testing is a requirement for all solid oral dosage forms and is used in all phases of development for product release and stability testing. It is a key analytical test used for detecting physical changes in an active pharmaceutical ingredient (API) and in the formulated product. At early stages of development, in vitro dissolution testing guides the optimization of drug release from formulations. Over the past 50 years, dissolution testing has also been employed as a quality control (QC) procedure, in R&D to detect the influence of critical manufacturing variables and in comparative studies for in vitro-in vivo correlation (IVIVC). The FDA guidance on dissolution testing for immediate release solid oral dosage forms includes the use of the Biopharmaceutics Classification System (BCS) guidelines for biorelevant dissolution tests, which is based upon API solubility and permeability. According to the BCS guidelines, in vitro dissolution testing may be a useful tool to forecast the in vivo performance of drug products and potentially reduce the number of bioavailability/bioequivalence studies required. The FDA guidance on scale-up and post-approval changes (SUPAC) for immediate release oral dosage forms recommends the use of in vitro dissolution to justify post-approval changes (ICH Harmonized Tripartite Guideline Q1A (R2) (2003), Q2A (1994), Q2B (1996)).

Amlodipine is a synthetic dihydropyridine and a calcium channel blocker with antihypertensive and antiangiinal properties. It is a dihydropyridine, a member of monochlorobenzenes, an ethyl ester, a methyl ester and a primary amino compound. Chemical name of amlodipine is 3-O-ethyl 5-O-methyl 2-(2-aminoethoxy methyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (Fig. 1).

The State Pharmacopoeia of Ukraine (SPhU) does not have a monograph on the substance of amlodipine besylate on the prepared medical form. However, the United States Pharmacopoeia regulates the determination of amlodipine besylate in substances and tablets. For identification, UV-spectrophotometry and HPLC/UV are proposed. For quantitative determination of amlodipine besylate in tablets – HPLC/UV, respectively. Chromatographic conditions for the determination of amlodipine besylate, tablets are given in the monograph of the United States Pharmacopoeia, which is used the chromatographic column of category L1 (fixed phase C18) and mobile phase consisting of three components: buffer solution of pH 3.0 with triethylamine, acetonitrile and methanol. The solvent – mobile phase, mobile phase rate – 1 ml/min, detection wavelength – 237 nm. The proposed method of the United States Pharmacopoeia requires a long sampling. The European Pharmacopoeia has a monograph on the substance of amlodipine besylate. Identification of amlodipine besylate of the European Pharmacopoeia regulates the absorption spectrophotometry in the infrared region and quantitative determination – HPLC/UV. As a solvent, methanol is used, mobile phase is 2.3 g/l of ammonium acetate solution P: methanol P (30:70, V/V), mobile phase rate is 1.5 ml/min, detection wavelength is 237 nm. Methods of quantitative determination of amlodipine besylate by the method of spectrophotometry are described in the scientific literature. However, those methods are not developed for determination of amlodipine in dissolution studies while the dissolution profile of enalapril, bisoprolol and another API from the combination drug product has not hitherto been reported in the literature (Kondratova et al. 2016, 2017; Krynytska and Maruschak 2018; Liliya et al. 2016; Logoyda 2018a, b, c, 2019; Logoyda et al. 2017a, b, c, Mykhalkiv et al 2018a, b, c). In order to elucidate the dissolution profiles amlodipine, bisoprolol and enalapril, a validated HPLC method is required for determination of amlodipine from tablets in dissolution matrix.

Therefore, the aim of this study was to develop and validate an efficient HPLC method for determination of amlodipine and to introduce the dissolution profiles of tablets which contain amlodipine, bisoprolol and enalapril. Moreover, this new method could also be used for the routine analysis of amlodipine in dosage forms, provided it is completely validated and rapid. The method was validated according to guidelines and applied for assay of amlodipine from their combination tablet dosage form. Also, in vitro dissolution of amlodipine containing tablets were performed to validate the suitability of the proposed method. The study of profiles of tablets dissolution and in vitro/in vivo correlation was conducted.

Materials and methods

Instrumentation and chromatographic conditions

The HPLC system consisted of Agilent 1260. A C18 column (Phenomenex Polar Synergi, 5 μm, 4.6×50 mm) was
used for separation and quantification. The mobile phase consisted of methanol:phosphate buffer solution (65:35, v/v) and was filtered through a 0.45 μm filter and degassed before use. The injection volume was 100 μL and the ultraviolet detector was set at 240 nm. Analyses were run at a flow-rate of 1.0 ml/min at an ambient temperature (25 °C). The peak area was integrated automatically by using Empower software. Under these conditions, amlodipine was eluted at 1.89 min. Total run time was shorter than 2.5 min.

**Chemicals and reagents**

Standard amlodipine was supplied by Refik Saydam National Public Health Agency. Methanol and phosphate buffer solution were of HPLC grade from Merck (Darmstadt, Germany) and all other reagents were analytical grade. Water obtained from the Milli-Q water system (Barnstead, USA) was used for the preparation of buffer and other aqueous solutions. Experimental tablets with modified release containing 5 mg of enalapril, 2.5 mg amlodipine and 2.5 mg of bisoprolol were developed by Prof. Kachrimanis Kyriakos (Department of Pharmaceutical Technology, Aristotle University of Thessaloniki, School of Pharmacy, Thessaloniki, Greece).

**Preparation of Strock Solution**

Standard stock solutions of amlodipine were prepared separately by dissolving 10 μg of amlodipine besylate in 50 mL appropriate solvent. These solutions were prepared freshly every week, during method development and application period.

**Preparation of Calibration Standards**

Calibration standards for amlodipine besylate (1,250, 1.875, 2.500, 3.125, 3750 and 5.000 μg mL) were daily prepared from standard stock solutions by appropriate dilution processes using mobile phase.

**In vitro dissolution studies**

In vitro dissolution of twelve modified release tablets containing amlodipine, bisoprolol and enalapril were performed using buffer solutions (pH 1.2; 4.5; 6.8) as the dissolution media at 50 rpm using an USP Apparatus II. The dissolution study was carried out in a 900 mL volume of buffer solution at 37 °C (± 0.5) using the paddle method. One mL of sample was withdrawn and replaced with fresh dissolution medium at the time intervals of 5, 15, 30, 45, 60, 90, 120, 180 minutes. The concentrations of enalapril in samples were determined by the proposed HPLC method.

**Analytical method validation**

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters such linearity range, sensitivity, intermediate precision, accuracy, and specificity, as per ICH Q2 (R1) guidelines (Q1A (R2) (2003), Q2A (1994), Q2B (1996)).

**System suitability parameters**

The chromatographic systems used for analysis must pass system suitability before going to start the experiment. HPLC system was stabilized for forty minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters such as tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and retention time. The % RSD for the peak area of six replicate injections of amlodipine standard is NMT 2.0. The parameters such as tailing factor, % RSD and theoretical plates were studied.

**Linearity**

A standard stock solution of the amlodipine was prepared with the same solvent. To study the linearity range of drugs, serial dilutions were made from standard stock solution in the range of 25–200% of the nominal concentrations of amlodipine in the test solution.

**Specificity**

Specificity of an analytical method is its ability to measure accurately and specifically the analyte of interest without interference from placebo and degradation products. The specificity of the method was established by injecting blank, placebo and standard solution in triplicate and recording the chromatograms. Blank solution shows no interference with any HPLC system artifact peak. Placebo demonstrate the lack of interference from excipients.

**Precision**

The precision of the method was determined by repeatability (intraday) and intermediate precision (interday). Repeatability was determined by performing repeated analysis of the same working solution of amlodipine on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on different days and also by another analyst performing the analysis in the same laboratory (between-analysts).

**Accuracy**

The accuracy of the method was defined as the closeness of a measured value to the true value. The recovery studies were carried out at 25–200% of the target level in the tablet in triplicate each in the presence of placebo.
Results and discussion

Optimization of chromatographic conditions

Chromatographic condition was optimized in several trials with the variation of the composition of mobile phase and flow rate. Based on the theoretical plates, peak shape and back pressure, the column was selected. The optimized mobile phase composition produced stable and acceptable peak shapes for amlodipine, enalapril and bisoprolol with mixtures of methanol:phosphate buffer solution (65:35, v/v) pumped at 1.0 ml/min flow rate and 220 nm UV detection wavelength. Therefore, all the experiments were carried out on a C18 column (Phenomenex Polar Synergi, 5 μm, 4.6×50 mm). The present RP-HPLC method is a simple, express, precise, specific, accurate and linear method for analyzing of amlodipine in combined tablets containing amlodipine, enalapril and bisoprolol. The previous study had reported that UV spectrophotometry methods and HPLC methods for amlodipine, enalapril and bisoprolol individually and in combination with other drugs but not together. In the present RP-HPLC method, we used UV detector which prove selectivity of the method for APIs. The method was developed by using different buffer ratios at different flow rates. Finally methanol:phosphate buffer solution (65:35, v/v) as mobile phase, and Phenomenex Polar Synergi, 5 μm, 4.6×50 mm column as stationary phase was selected and separation were done for amlodipine at 1.89 min.

Representative chromatogram of amlodipine in tablets containing amlodipine, bisoprolol and enalapril is presented in Fig. 2.

Figure 2. Representative chromatogram of amlodipine in combined tablets (1- peak of bisoprolol, 2- peak of enalapril, 3 – peak of amlodipine).

The resultant chromatograms of blank, standard and sample were compared, the correlation was good between standard and sample and no interference of excipients in blank with drug was observed. Blank solution shows no interference with any HPLC system artifact peak. Placebo to demonstrate the lack of interference from excipients. The representative chromatogram (Fig. 2) shows no other peaks in retention time of amlodipine, bisoprolol and enalapril and retention times did not change. In addition, when the solution prepared from the blank tablet was injected into the HPLC system, no co-eluting peaks were obtained at the retention time of amloidipine, bisoprolol and enalapril.

Method validation

The proposed method was validated as to linearity range, sensitivity, repeatability, precision, accuracy, and specificity according to the ICH guidelines.

System suitability

A suitability test was applied to the chromatograms taken under optimum conditions to check various parameters such as column efficiency (plates), peak tailing, retention factor, and resolution. Suitable resolution (>2) and column efficiency (>1500 for both compounds) were achieved for the analysis method. The peak symmetries for both compounds were <1.2, whereas the capacity factors were >1.5. The analysis time was shorter than 2.5 minutes. LOD and LOQ were estimated a single to noise ratio of 3.3 σ/S and 10 σ/S where σ is the standard deviation of intercept, S is the slope derived from calibration curve. Limit of detection (LOD) – 0.021%, limit of quantification (LOQ) – 0.062%.

Specificity

Specificity was carried out by evaluation of blank, standard and sample were compared, the correlation was good between standard and sample and no interference of excipients in blank with drug was observed. Commonly used tablet excipients did not interfere with this method. It shows that the method is specific. The specificity results are tabulated in Table 1.

Table 1. Specificity study.

| Name of the solution          | Retention time (RT) min |
|------------------------------|-------------------------|
| mobile phase                  | No peaks                |
| placebo                      | No peaks                |
| Amlodipine besylate           | 1.89                    |
| bisoprolol fumarate (another API) | 0.85                 |
| Enalapril maleate (another API) | 1.09                   |

Linearity range

Calibration curve representing the relation between the concentrations of drugs versus the peak area were constructed. In triplicate run from which the linear regression equation was calculated. The results obtained were processed by the least squares method. The correlation coefficient of enalapril maleate was noted more than 0.9992 which states that the method was good linear to the concentration versus peak area responses. Results indicate high sensitivity of the proposed HPLC method. The calibration graph of amlodipine is presented in Figs 3–5 and characteristics of the linear dependence of amlodipine are listed in Table 2.

A linear relationship between the concentration and the area of chromatographic peaks of amlodipine in the range of 0.625 mg/mL –5.000 mg/mL (1.250 mg/mL –5.000 mg/mL at pH 4.5) was established (Table 2). Requirements for linear dependency parameters are performed in this case throughout the range of application of the technique. Li-
Table 3. Results of repeatability (n = 5).

| Sample            | Peak area | Injection no. | RSD, % |
|-------------------|-----------|---------------|--------|
| Amlodipine besylate | 1026.397 | 1             | 0.04   |
|                   | 1028.198 | 2             |        |
|                   | 1023.396 | 3             |        |
|                   | 1024.487 | 4             |        |
|                   | 1027.744 | 5             |        |

Note: n is number of determinations, RSD is relative standard deviation.

Table 4. Intra-day and Inter-day precision data of amlodipine (n = 5).

| Day | Intra-day precision | Inter-day precision |
|-----|---------------------|---------------------|
|     | Mean | RSD % | Mean | RSD % |
| 1   | 100.02 | 0.389 | 100.15 | 0.298 |
| 2   | 100.87 | 0.291 | 99.99  | 0.267 |
| 3   | 100.92 | 0.329 | 100.15 | 0.317 |

Note: n is number of determinations, RSD is relative standard deviation.

The % RSD values in intra-day and inter-day precision study were found to be less than 0.267 for amlodipine, which indicate method was precise. Hence, the present developed method was said to be suitable for the analysis of drugs in their pharmaceutical dosage form.

**In vitro dissolution studies**

In vitro dissolution of amlodipine containing tablets were performed to validate the suitability of the proposed method. The dissolution pattern complies with the FDA standards, indicating suitability of the proposed method for the dissolution study of amlodipine. It will allow conducting comparative studies in vitro to confirm the equivalence of tablets containing amlodipine. The average percentage drugs released as detected by the proposed HPLC method after in vitro dissolution of tablets containing combination drug product are depicted in Figs 6–8.

In order to prepare delayed release tablets, as can be seen from Figs 6–8, in the medium with pH 1.2 release of amlodipine tablets in 5 minutes is 38.71%, after 15 minutes – 59.50%, after 30 minutes – 68.48%, after 45 minutes – 69.87%, after 60 minutes – 70.50%, after 90 minutes – 69.82%, after 120 minutes – 67.92%, after 180 minutes – 80.42%; in medium with pH 4.5 the release of amlodipine from tablets in 5 minutes makes 36.47%, after 15 minutes – 76.24%, after 30 minutes – 85.77%, after 45 minutes – 88.47%, after 60 minutes – 87.91%, after 90 minutes – 98.84%, after 120 minutes – 93.40%, after 180 minutes – 84.48%; in a medium with pH 6.8, the release of amlodipine from tablets in 5 minutes is 36.51%, after 15 minutes – 76.24%, after 30 minutes – 85.77%, after 45 minutes – 88.47%, after 60 minutes – 87.91%, after 90 minutes – 98.84%, after 120 minutes – 93.40%, after 180 minutes – 84.48%.

**In vitro / in vivo correlation**

Nowadays, in vitro / in vivo correlation has become one of the key trends of modern pharmacy. Detection of a reliable in vitro / in vivo correlation allows one to predict the behavior of API in vivo by studying the kinetics of

![Calibration Curve in (pH 1.2) (Figure 3)](image1)

![Calibration Curve in Acetate Buffer (pH 4.5) (Figure 4)](image2)

![Calibration Curve in Buffer (pH 6.8) (Figure 5)](image3)

Table 2. Characteristics of the linear dependence of amlodipine.

| pH   | Concentration range (μg/mL) | Regression equation | R² | Compliance with eligibility criteria |
|------|-----------------------------|---------------------|----|-------------------------------------|
| 1.2  | 0.625 – 5.000               | y = 109.2999x - 35.3668 | 0.9991 | Corresponds |
| 4.5  | 1.250 – 5.000               | y = 97.5017x - 18.2518 | 0.9998 | Corresponds |
| 6.8  | 0.625 – 5.000               | y = 97.9425x - 22.4737 | 0.9992 | Corresponds |

Linearity studies were conducted in a wide range of concentrations (25–200% at pH 1.2 and 6.8, 50–150% at pH 4.5).

**Accuracy and Precision**

System precision is shown in Table 3. Intra-day and inter-day % RSD values lower than 2% clearly assuring that this method was found to be fairly precise (Table 4). Regarding accuracy, a known amount of the standard drug was added to the fixed amount of preanalyzed sample solution. % recovery was calculated by comparing the area before and after addition of the standard drug. The standard addition method was performed at 25–200% levels concentrations. The high value of recoveries obtained for bisoprolol indicates that the proposed method was found to be accurate.

![Figure 3. Linearity on profiles of dissolution test at pH 1.2.](image4)

![Figure 4. Linearity on profiles of dissolution test at pH 4.5.](image5)

![Figure 5. Linearity on profiles of dissolution test at pH 6.8.](image6)
The developed method can also be conveniently adopted for dissolution testing of tablets containing amlodipine. The proposed method can help research studies, quality control and routine analysis with lesser resources available.

3. In vitro / in vivo correlation of amlodipine has been conducted.

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