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Determination of Amlodipine and Furosemide with Newly Developed and Validated RP-HPLC Method in Commercially Available Tablet Dosage Forms

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Introduction

Hypertension is an important problem that requires chronic treatment. Angiotensin-converting enzyme (ACE) inhibitors were primarily considered as antihypertensive drugs which are able to reduce significantly high blood pressures in hypertensive patients. Calcium channel blockers were initially considered for treatment of angina pectoris and for the treatment of vasospastic angina. These drugs are able to markedly reduce vascular resistance and for that reason they were extensively studied in the field of hypertension. ACE inhibitors and calcium channel blocker drugs are widely used for the treatment of many cardiovascular conditions including mild to moderate hypertension and heart failure, either alone or in combination with other drugs[1-3].

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Amlodipine (AML) (as besylate, mesylate or maleate) [(*RS*)-3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl) - 6-methyl - 1,4 – dihydropyridine -3 ,5- dicarboxylate], is a long-acting calcium channel blocker (dihydropyridine) used as an anti-hypertensive and in the treatment of angina. AML is one of the calcium channel blockers that works primarily on arterial muscle and it acts by relaxing smooth muscle in the arterial wall, decreasing peripheral resistance and hence reducing blood pressure; in angina it increases blood flow to the heart muscle[4].

Furosemide (FSM) [4-chloro-N-furfuryl-5-sulphamoylanthranilic acid], is a loop diuretic commonly used in adults, infants and children for the treatment of edematous states associated with congestive heart failure, cirrhosis of the liver and renal disease. Oral FSM may be used in adults for the treatment of hypertension alone or in combination with other antihypertensive agents[1].

FSM has been individually determined in pharmaceutical formulations by extractive spectrophotometry[5]: Also HPLC[6-9] and HPLC-mass spectrometric analysis[10] have been used for measurement of FSM in biological fluids and urine. On the other hand, several analytical methods including capillary electrophoresis[11], spectrophotometry[12,13] have been reported in the literature, for analysis of AML[11-20] in pharmaceutical dosage forms. A number of high performance liquid chromatographic (HPLC) methods[13-20], have also been reported for this drug using ultraviolet (UV) as well as fluorometric and mass (MS) detectors[14,19,20]. Some of the reported methods require solid-phase extraction, evaporation and time consuming preparation steps or expensive equipments, which are not economically feasible for routine use in pharmacokinetic and pharmaceutical studies, where numerous samples should be analyzed.

Owing to the widespread use of HPLC in routine analysis, it is important that specific HPLC methods are developed and thoroughly validated[21-23]. HPLC–UV detection offers important advantages, such as rapid set-up of instrumentation, versatility and low cost, and has proved to be a valuable method in the quality control of drug compounds.

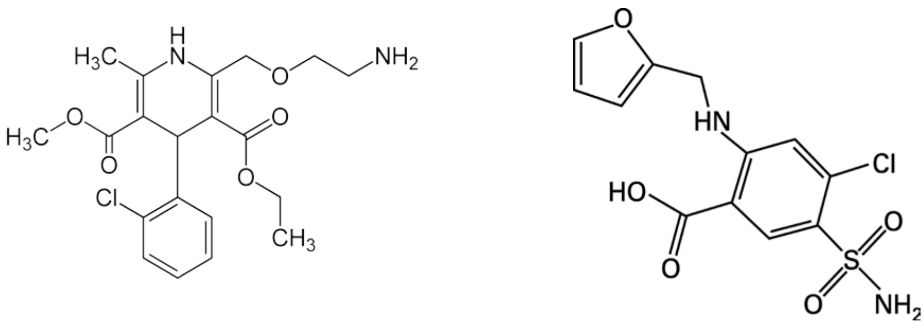
Although at present there is not any commercial pharmaceutical formulation containing both drugs for human use, some pharmacies in the USA are preparing fixed dose combination capsules containing 0.625 mg AML (as besylate) and FSM 6.25 mg for animals especially cats[24]. The

analytical simultaneous determination has not been reported yet in the actual USP[25]. A comprehensive literature search revealed the lack of a suitable procedure for the sensitive simultaneous determination of AML and FSM in pharmaceutical dosage forms. The present work was undertaken with an objective to develop a simple, sensitive, accurate and precise direct estimation RP-HPLC method for the simultaneous determination of AML and FSM in pharmaceutical dosage forms, for their pharmacokinetic studies and therapeutic drug monitoring.

Material and Methods

Chemicals and reagents

The chemical structures of the two studied protease inhibitors are shown in Figure 1. AML and FSM reference substances were kindly supplied by Pfizer (Turkey) and Sanofi-Aventis (Turkey) respectively. All reagents used were at least of analytical grade except acetonitrile. HPLC grade acetonitrile were obtained from Merck (Darmstadt, Germany). HPLC grade water was obtained following distillation in glass and passage through a Milli-Q® system (Millipore, Milford, MA, USA) and was used to prepare all necessary solutions. Ortho-phosphoric acid (min. 85%) was obtained from Riedel (Riedel-de Haen, Germany), and sodium hydroxide from Merck. Test and reference AML and FSM drug products Norvasc,



Amlodipine Furosemide

Figure 1
Chemical structures of AML and FSM.

Penvasc, Dilapin, Monovasc, Lasix and Desal were obtained from Turkish market.

Chromatographic separation of AML and FSM was achieved on a reversed-phase C_{18} column (Fortis™ 250x4.60 mm 5 μm). The column was heated at 45°C during the analysis. The mobile phase consisted of water (15 mM o-phosphoric acid) and acetonitrile (50:50 v/v). The pH of the mobile phase was adjusted at 5.0 by addition of sodium hydroxide. Working solutions were diluted with the mobile phase for preparing the necessary concentrations. All stock and working solutions were protected from light and stored in fridge at about 4°C. The dead time (to) was measured by injecting potassium bromide solution [0.01% (v/w), in water].

A Hewlett Packard 1100 series HPLC system with ternary solvent pump online degasser, rhodyn injection valve, equipped with 20 μL loop, column heater and DAD detector was used. UV detection was performed at 238 nm. Analyses were run at a flow rate of 1 $mL.min^{-1}$. The injection volume was 20 μL and total run time for an assay was approximately 5 min. FSM was chosen as the internal standard for AML analysis because it showed a shorter retention time with better peak shapes and better resolution, compared to other potential internal standards. Also, AML was chosen as the internal standard for FSM analysis.

Validation of the developed method

Stock standard solutions of AML and FSM were prepared in methanol at concentrations of accurately 1000 $\mu g.mL^{-1}$ for AML and 100 $\mu g.mL^{-1}$ for FSM. All solutions were protected from light and were used within 24 h to avoid decomposition.

A similar method validation protocol was followed for both drugs. Linearity of the methods was established by triplicate injections of solutions containing AML and FSM in the range of 1.0–16 $\mu g.mL^{-1}$ and 0.1–12 $\mu g.mL^{-1}$, respectively. The linearity plots were constructed and the acceptable fit to the linear regression was demonstrated and reported by the necessary parameters. To determine intra-day precision (repeatability), six injections of selected concentrations were given on the same day and the values of relative standard deviation were calculated. These studies were repeated with different weightings on different days to determine inter-day precision (reproducibility).

The resolution factor of the drug peak from the nearest resolving peak was also determined. Also all necessary system suitability test parameters were reported.

Analysis of tablets

10 tablets containing individually 10.0 mg of AML and 40.0 mg of FSM were finely powdered separately. An accurate weight of the powder equivalent to one tablet content was weighed, transferred into a 100 mL calibrated flask, diluted with methanol, stirred for about 10 min and then completed to volume with the same solution. After filtration, appropriate solutions were prepared by taking aliquots from clear filtrate (for LC study, fixed amount of IS added) and diluting them with mobile phase and methanol. AML and FSM contents of the tablets were calculated from corresponding regression equations.

Results and Discussion

Optimization of Chromatographic Conditions

A Fortis™ reversed phase column (250 × 4.6 mm, 5 μm particle size) maintained at 45^0C was used for the separation and the method was validated for the simultaneous determination of AML and FSM in tablet dosage forms. The composition, pH and the flow rate of the mobile phase were changed to optimize the separation conditions. A mobile phase consisting of acetonitrile–water (50:50; v/v), containing 15 mM phosphoric acid was used. The pH of the mobile phase was adjusted at 5.0 by the addition of sodium hydroxide and a flow rate of 1 $mL.min^{-1}$ was selected for further studies after several preliminary investigatory chromatographic runs. This column and operating conditions gave us both sharp and symmetric peaks. Under the described experimental conditions, all the peaks were well defined and free from tailing. The effects of small deliberate changes in the mobile phase composition, pH and flow rate were evaluated as a part of testing for method robustness. The proposed method was successfully used for the simultaneous determination of AML and FSM in their dosage forms.

With the optimized operating conditions, the retention time corresponding to AML and FSM was 3.69 and 4.28 min. respectively, being ex-

tremely stable among injections. The total run time for an assay was approximately 5.0 min. However, the analysis time was set to 10.0 min. allowing elution of all possible excipients which could be retained, without the need of a further stabilization time between injections. FSM shows two absorption maxima nearly at 235 and 275 nm. The wavelength was set at 238 nm, the absorption maximum of FSM and AML in the elution solvent.

The proposed RP-HPLC method provided a simple procedure to simultaneously determine the concentrations of AML and FSM in drug formulations by DAD detection at 238 nm. After determining the optimum conditions, a satisfactory resolution was obtained in a short analysis time (about 5.0 min.). As can be deduced from Table 1 and Figure 2, sharp and symmetrical well-resolved peaks were obtained for all compounds.

Method validation

The optimized RP-HPLC method was validated, with respect to specificity, accuracy, precision (repeatability and intermediate precision), linearity and range[26-29]. System suitability features were also assessed.

System suitability for the proposed method was evaluated. A system suitability test can be defined as a test to ensure that the method can generate results of acceptable accuracy and precision. System suitability test results were reported in Table I. According to Table I, the results obtained from the system suitability tests satisfy the USP requirements.

TABLE I
System suitability parameters of the proposed RP-LC method.

Compounds	**AML**	**FSM**
Retention time	3.692	4.279
Capacity factor (k)	0.711	0.983
Selectivity factor (α)	-	1.383
Resolution (R_s)	-	1.756
Theoretical plates	4056	3734
Tailing Factor	1.128	1.090

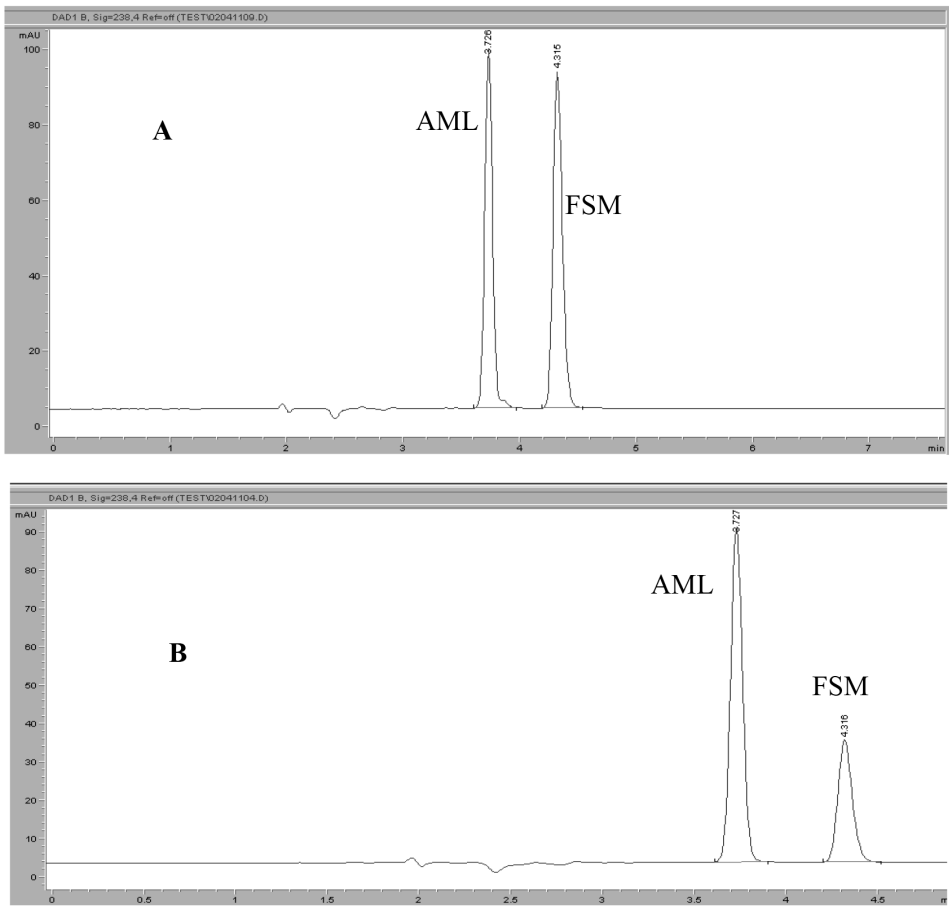


Figure 1
The chromatogram of **A)** Norvasc samples and **B)** Lasix samples taken under optimum conditions.

Linearity

Linearity was established by least squares linear regression analysis of the calibration curve. Linearity of the methods was established by triplicate injections of solutions containing AML and FSM in the range of 1.0–16 μg.mL^{-1} and 0.1–12 μg.mL^{-1}, respectively. Peak area ratios of AML and FSM respect to each other were plotted versus their respective concentrations in the mobile phase and linear regression analysis performed on the resultant curves. The linearity of the calibration plots was confirmed by the high value of the correlation coefficients for both

compounds. Correlation coefficients were higher than 0.999 for both drugs (Table II).

The limit of detection (LOD) and limit of quantitation (LOQ) of method were determined by calculating the standard deviation of the response of lowest standard on the calibration curve and the slope of calibration curve of analyte. LOD and LOQ were calculated by LOD = 3.3σ/S and LOQ = 10σ/S respectively, (σ = the standard deviation of the response, S = the slope of the calibration curve). The LOQ that produced the requisite precision and accuracy was found to be 2.139 $\mu g.mL^{-1}$ for AML and 0.031 $\mu g.mL^{-1}$ for FSM, respectively.

Precision and Accuracy

The intra-day, inter-day precision and accuracy of the developed method were assessed by using standard solutions prepared to produce solutions of different concentrations of each drug in the mobile phase. The intra- and inter-day variability or precision data are summarized in Table III.

Repeatability and reproducibility were characterized for different concentrations and given by mean recovery and RSD%. Based on these results, there was no significant difference for the assay, as tested by within-day (repeatability) and between days (reproducibility). Repeatability or intra-day precision was investigated by injecting three replicate assays of the samples. Inter-day precision were assessed by injecting the samples over three consecutive days.

Assay

Currently, more than ten different AML (as besylate) containing tablets (5.0 and 10.0 mg) and two different FSM containing tablets are commercially available in the Turkish drug market. Once validated, the developed method was applied to the simultaneous assay of AML and FSM in commercial dosage forms. The results, corresponding to the tablet dosage form of AML and FSM, are shown in Table IV.

Proposed RP-HPLC method can be used for the simultaneous determination of AML and FSM in the use of each other as internal standard and without prior separation of the excipients. Each tablet contains the active ingredients 10.0 mg of AML as amlodipine besylate, 40.0 mg FSM

TABLE II
Statistical evaluation of the calibration data of AML and FSM by RP–HPLC.

Compounds	AML	FSM
Linearity Range (μg.mL^{-1})	1.0-16.00 (n=6)	0.1-12.00 (n=7)
Slope	0.176	1.610
Intercept	-0.040	0.052
SE of slope	0.003	0.023
SE of intercept	0.025	0.130
Correlation coefficient	0.9995	0.9995
Detection limit (μg.mL^{-1})	0.642	0.010
Quantitation limit (μg.mL^{-1})	2.139	0.031

TABLE III
Summary of repeatability (intra-day) and reproducibility (inter-day) precision data for AML and FSM (n=3).

Concentration (μg.mL $^{-1}$)		Intra-day	Inter-day
		Mean Recovery ± RSD	Mean Recovery ± RSD
Amlodipine	2.0	99.152 ± 0.832	98.768 ± 0.542
	12.0	100.968 ± 0.947	99.240 ± 0.761
Furosemide	0.5	99.697 ± 0.669	100.298 ± 0.771
	4.0	100.360 ± 0.548	100.168 ± 0.493

TABLE IV
Summary of the analysis of amlodipine and furosemide in commercially available pharmaceutical dosage forms

	Norvasc (AML)	Dilapin (AML)	Monovasc (AML)	Penvasc (AML)	Lasix (FSM)	Desal (FSM)
Label claim (mg)	10.0	10.0	10.0	10.0	40.0	40.0
Amount found (mg)	9.99	9.96	9.92	10.00	40.66	39.86
RSD (%)	0.51	1.42	0.84	0.19	2.36	1.39
Bias (%)	-0.11	-0.39	-0.78	0.03	1.66	-0.36

and the inactive ingredients. Removal of the excipients before analysis was found to be unnecessary. Figure 2 shows a typical chromatogram obtained by the analysis of AML and FSM in tablets. The substances were eluted, forming well shaped, symmetrical single peaks, well separated from the solvent front. No interfering peaks were obtained in the chromatogram due to tablet excipients. The labeled amount of drugs indicates that the active ingredients in samples were present at a level included within the requirements with respect to the label claimed by the manufacturer. The utility of all of the proposed method was verified by means of replicate estimations of pharmaceutical preparations and results obtained were evaluated statistically.

Conclusions

The proposed RP-HPLC method for simultaneous assay of AML and FSM in tablets was simple, precise, specific and highly accurate and less time consuming. So, it can definitely be employed for the routine analysis. Hence this RP-HPLC method is suitable for quality control of raw materials and formulations. The intra-run and inter-run variability and accuracy results were also in acceptable limit. In addition, this method has the potential application to clinical research of drug combination, interactions studies and multi-drug pharmacokinetics.

Summary

The aim of this study was to develop a new, fast, reliable and validated RP-HPLC method for the simultaneous determination of amlodipine and furosemide in tablet dosage forms. A C_{18} column (Fortis™ 250x4.60 mm 5 μm) which was heated at 45°C during the analysis, was used for the separation and quantification of these drugs. The mobile phase consisted of water (15 mM o-phosphoric acid, pH 5.0) and acetonitrile (50:50 v/v). Analyses were run at a flow rate 1.0 mL.min^{-1} and UV detector was set at 238 nm. The injection volume was 20 μL and total run time for an assay was approximately 5 min. The developed method was validated according to the ICH guideline. For the application of the proposed RP-HPLC

method, commercially available four different AML containing tablets (one reference (Norvasc) and three generic (Dilapin, Monovasc, Penvasc) tablets) and commercially available two different FSM containing tablets (one reference (Lasix) and one generic (Desal) tablet) were obtained from the market and analyzed for their drug content. Under the given chromatographic conditions, AML and FSM were eluted at 4.28 and 3.68 min., respectively. The method was linear in the concentration range of 1.0 to 16.0 $\mu g.mL^{-1}$ and 0.1 to 12.0 $\mu g.mL^{-1}$ for AML and FSM, respectively with a correlation coefficient >0.999. LOD and LOQ were 0.642 $\mu g.mL^{-1}$ and 2.139 $\mu g.mL^{-1}$ for AML 0.010 $\mu g.mL^{-1}$ and 0.031 $\mu g.mL^{-1}$ for FSM, respectively. Under the conditions used, the analysis completely fulfilled the system suitability test limits suggested by FDA for the quantitative chromatographic methods. The method was successfully applied for the analysis of these drugs in commercially available tablets.

Keywords: Amlodipine, Furosemide, HPLC, Drug Analysis.

Özet

Amlodipin ve Furosemidin Ticari Tablet Dozaj Şekillerinden Eş Zamanlı Tayini İçin Valide Edilmiş Bir RP-HPLC Metodun Geliştirilmesi

Bu çalışmanın amacı, tablet dozaj formlarından amlodipin (AML) ve furosemidin (FSM) eş zamanlı tayini için, hızlı, güvenilir ve valide edilmiş yeni bir HPLC metodunun geliştirilmesidir. Bu ilaçların ayrımı ve miktar tayini için, analiz sırasında 45°C'ye ısıtılan bir C_{18} kolon (Fortis™ 250x4.60 mm 5 μm) kullanılmıştır. Mobil faz, su (15 mM o-fosforik asit, pH 5.0) ve asetonitril (50:50 h/h)'den oluşmaktadır. Analizler 1.0 $mL.dk^{-1}$ akış hızında, UV dedektör 238 nm'ye ayarlanarak yapılmıştır. Her bir analizin toplam süresi yaklaşık 5 dakika ve injeksiyon hacmi 20 μL'dir. Geliştirilen metod ICH kılavuzuna göre valide edilmiştir. Önerilen metodun uygulaması için, ticari olarak bulunabilen dört farklı AML (1 referans, Norvasc üç jenerik, Dilapin, Norvasc, Penvasc) içeren tablet ve iki farklı FSM (bir referans Lasix, bir jenerik Desal) içeren tablet piyasadan sağlanmış ve ilaç içerikleri tayin edilmiştir. Belirlenen kromatografik koşullar altında, AML ve FSM sırasıyla 4.28. ve 3.68. dakikalarda ayrılmışlardır. Metod AML ve FSM için sırasıyla 1.0-16.0 $\mu g.mL^{-1}$ ve 0.1-12.0

μg.mL^{-1} konsantrasyon aralığında 0.999 korelasyon katsayısı ile doğrusal bulunmuştur. AML için tayin alt sınırı (LOD) ve ölçülebilir alt sınır (LOQ) 0.642 μg.mL^{-1} ve 2.139 μg.mL^{-1} ve FSM için tayin alt sınırı (LOD) ve ölçülebilir alt sınır (LOQ) 0.010 μg.mL^{-1} ve 0.031 μg.mL^{-1}'dir. Belirlenen koşullar, FDA tarafından kantitatif kromatografik metodlar için önerilen system uygunluk limitlerine tamamen uymaktadır. Metod, bu ilaçların ticari preparatlarından tayini için başarıyla uygulanmıştır.

Anahtar kelimeler: Amlodipin, Furosemid, HPLC, İlaç Analizleri.

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