ORIGINAL ARTICLE

THE SIMULTANEOUS DETERMINATION OF CANDESARTAN, AMLODIPINE AND HYDROCHLOROTHIAZIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, FROM A MIXTURE AND PHARMACEUTICAL FORMULATIONS

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Abstract

The aim of this paper consisted in the development and validation of a high performance liquid chromatography (HPLC) method for the determination of three antihypertensive substances from a mixture and pharmaceutical formulations. The three selected antihypertensive substances were candesartan, amlodipine and hydrochlorothiazide. The extraction and quantification of the constituents by HPLC was carried out using an analytical column, C18, and a mobile phase consisting in a mixture of buffer solution pH = 3.5 and methanol (15:85 v/v), at a flow rate of 1 mL/ min. The UV detection was performed at 240 nm for candesartan and amlodipine and at 270 nm for hydrochlorothiazide. The three compounds mentioned above were separated with good resolution, reproducibility and sensitivity under these conditions. The proposed HPLC method was applied to the analysis of the in-house dosage forms.

Rezumat

Obiectul acestei lucrări a constat în dezvoltarea unei metode cromatografice de lichide de înaltă performanţă (HPLC) şi validarea ei, pentru determinarea a trei substanţe antihipertensive dintr-un amestec şi din forme farmaceutice. Cele trei substanţe antihipertensive selectate sunt: candesartan, amlodipină şi hidroclorotiazidă. Extracţia şi cuantificarea constituenţilor prin HPLC a fost realizată folosind o coloană analitică C18 şi o fază mobilă constituită dintr-un amestec de soluţie tampon de pH = 3,5 şi metanol (15:85 v/v) la un debit de 1 mL/min. Detectarea UV a fost la 240 nm pentru candesartan şi amlodipină şi la 270 nm pentru hidroclorotiazidă. Cei trei compuşi menţionaţi mai sus s-au separat cu rezoluţie bună, reproductibilitate şi sensibilitate mărite în aceste condiţii. Metoda HPLC propusă a fost aplicată la analiza formelor de dozare comerciale.

Keywords: candesartan, amlodipine, hydrochlorothiazide, simultaneous assay, HPLC, pharmaceuticals formulations

Introduction

Antihypertensive are a class of drugs which has an important place in the range of medicinal products currently used to treat cardiovascular diseases.

Antihypertensive drugs combine several active ingredients with different mechanisms of action, but with synergistic action, have a better tolerability and an increased effectiveness. Although the first choice for reducing blood pressure is the lifestyle, expressed in diet and exercises, most patients also need drug therapy.

The most commonly used antihypertensive drugs are diuretics, angiotensin-converting enzyme (ACE) inhibitors, angiotensin antagonists and calcium channel blockers, and in some cases is needed the combination of two or three of these [3, 8].

The development of oral formulations containing a combination of a number of therapeutic agents acting synergistically is a necessity, because it is a more effective therapeutic alternative in comparison with the conventional forms.

Such a dosage form reduces the dosing frequency, improves the ratio cost-effectiveness of treatment and patient's compliance [7, 15].

Combined therapy with calcium channel blockers, angiotensin II blockers and diuretics is recommended as one of the most effective and commonly used to treat hypertension [3, 10].

Because of these reasons, we developed a dosed drug containing candesartan (8 mg/tablet), amlodipine (5 mg/tablet) and hydrochlorothiazide (12.5 mg/tablet).

Candesartan cylexethyl (cyclohexyl 1-hydroxy-ethylcarbonate) is a representative of antihypertensive angiotensin II receptor antagonists' class, with long-acting action, thus being a prodrug.

Candesartan cylexethyl is a racemic mixture comprising an ethyl ester group at the chiral centre and after oral administration the active form of candesartan is formed, cyclohexyloxycarbonyloxy, due to the hydrolysis of the ester group [9, 13, 14].

Amlodipine is a dihydropyridine derivative with an amino-ether group, different from the nifedipine, by replacing the group ($-NO_2$) with chlorine, and by an ester group. The ester functions are the ones that influence the selectivity.

Hydrochlorothiazide is a thiazide sulphonamide, saluretic diuretic drug, with an average efficiency because it increases elimination of Na^+, K^+, Mg^{2+}, Cl^- ions, and, used on long term, reduces Ca^{2+} removal [4, 12].

In the scientific literature, there are only few methods [1, 2, 5, 6, 11, 16] for the simultaneous assay of candesartan and hydrochlorothiazide, but there is no method for the simultaneous determination of the combined three antihypertensive substances: candesartan, amlodipine and hydrochlorothiazide.

The aim of this paper consisted in the development and validation of a HPLC method for the determination of the three antihypertensive substances from a mixture and pharmaceutical doses. The three selected antihypertensive substances are Candesartan, Amlodipine and Hydrochlorothiazide.

This study is part of a larger project that investigated possible interactions between the selected active substances and excipients. The experimental determinations were done in order to conduct the process of pre-formulating a triple-layered tablet with modified release and containing this fixed combination.

Materials and Methods

Equipments. It was used a HPLC apparatus 1200 series with data handling system ChemStation B 04.02 version, producer Agilent Technologies.

Reagents. The following reagents were used: candesartan cylexethyl (Aurobindo, Hungary); amlodipine besylate (Hetero Drugs Limited, India); hydrochlorothiazide (Changzhon Pharmaceutical, China); microcrystalline cellulose (Comprecel M102, Mingtai China); sodium croscarmellose SD-711 (AcDiSol, FMC Biopolymer Irlanda); magnesium stearate (Mosselman, Belgium); colloidal silicon dioxide, anhydrous (Aerosil 200, Evonik Degussa China); pigment blend red (Colorcon UK); methanol HPLC grade (VWR Chemicals); sodium acetate (VWR Chemicals); glacial acetic acid (Sigma Aldrich, Germany); purified water (Milli-Q grade).

Method description. The following chromatographic conditions were used: HPLC Column: Zorbax SB C18, 250 mm X 4,6 mm, ID 5 µm; column temperature: 25°C, flow rate: 1mL/min, UV detection: 240 and 270 nm, injection volume: 20 µL, runtime: 10 minutes.

Solutions. Mobile phase solution: 15 volumes of buffer solution pH 3.5 mixed with 85 volumes of methanol; buffer solution pH 3.5: a sodium acetate 0.01 M solution adjusted at pH 3.5 using glacial acetic acid; diluent: mobile phase.

Candesartan stock solution was prepared weighing about 4.00 mg of candesartan standard and transferred into a 50 mL volumetric flask. It was dissolved in 20 mL of methanol, sonicated for about 2 minutes and diluted to 50 mL with methanol.

Amlodipine stock solution was prepared weighing about 2.50 mg of amlodipine standard and transferred into a 50 mL volumetric flask. It was dissolved in 20 mL of methanol, sonicated for about 2 minutes and diluted to 50 mL with methanol.

Hydrochlorothiazide stock solution. Was prepared weighing about 6.25 mg of hydrochlorothiazide standard and transferred into a 50 mL volumetric flask. It was dissolved in 20 mL of methanol, sonicated for about 2 minutes and diluted to 50 mL with methanol.

The standard solution of candesartan 0.008 mg/mL, amlodipine 0.005 mg/mL and hydrochlorothiazide 0.0125 mg/mL were prepared by diluting 10.0 mL of each stock solution to 100 mL volumetric flask with diluent.

The test solution was prepared by weighing about 240 mg of powder obtained by crushing 20 in-house tablets (Figure 1) into a 50 mL volumetric flask. The powder was dissolved into 30 mL of methanol, sonicated for about 15 minutes to disperse the content, diluted to volume with methanol and properly mixed.

Further 1.0 mL of this solution was diluted into a 10 ml volumetric flask and filled up to volume with diluent.

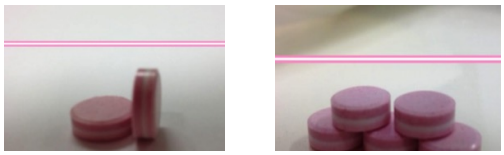

Figure 1.
Candesartan-Amlodipine-Hydrochlorothiazide in-house tablets

Placebo solution was prepared in the same way like test solution, using an excipients mixture.

The blank, standard and test solutions were filtered through a 0.45 µm membrane filter using a plastic

syringe. The blank, standard in replicate (6 injections) and test preparation in duplicate were injected into the chromatographic system. The system suitability parameters from the standard chromatogram were evaluated as follows: %RSD of peak area response for each active from the standard preparation (six injections) not more than 2.0%; USP tailing for each active peak not more than 1.2.

The identification of the active ingredients was made based on the retention times which were about 3 minutes at 240 nm for amlodipine, about 7 minutes at 240 nm for candesartan and about 2.3 minutes at 270 nm for hydrochlorothiazide.

Method validation parameters

For the analytical method validation, the following parameters were evaluated: specificity, linearity, accuracy, precision and robustness.

Results and Discussion

Specificity was evaluated in terms of the potential interferences produced by the diluent used during sample preparation and by the placebo. For the evaluation of specificity the following solutions were prepared according to the test procedure and injected in the HPLC system: diluent, *placebo*, standard solution and test solution.

The obtained results are summarised in Table I.

Table I

Determined chromatographic parameter related with specificity

Retention time, min.	Amlodipine	Candesartan	Hydrochlorothiazide
Diluent	-	-	-
Placebo	-	-	-
Standard solution	2.964	6.678	2.291
Test solution	2.972	6.689	2.287

The interference of the diluent and placebo was considered insignificant, because the chromatogram of the diluent and placebo shows no peak at the retention time of active ingredients as shown in Figures 2, 3, 4 and 5.

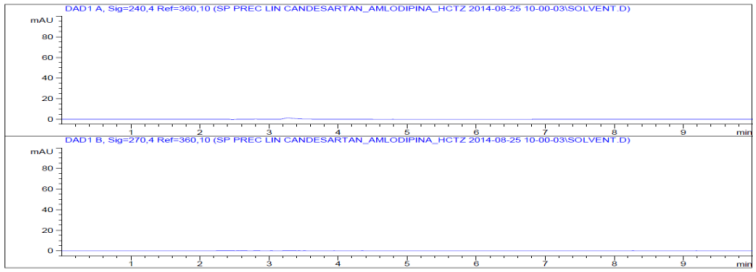

Figure 2.
Diluent chromatogram

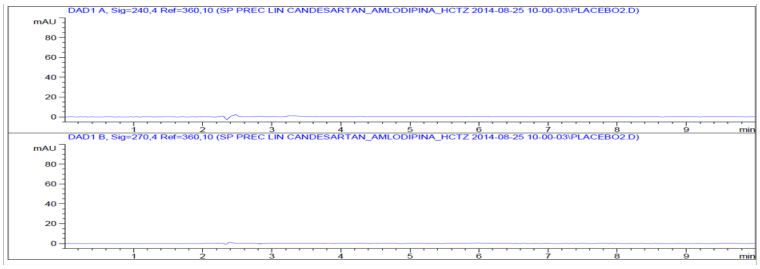

Figure 3.
Placebo chromatogram

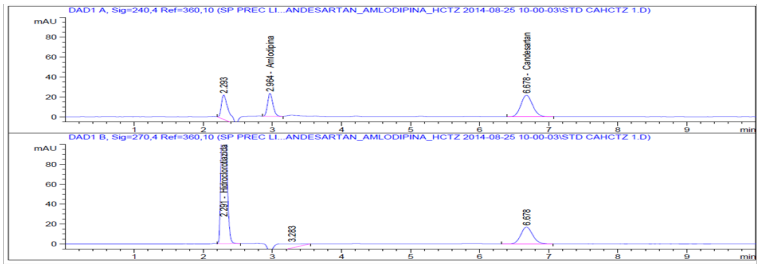

Figure 4.
Standard solution chromatogram

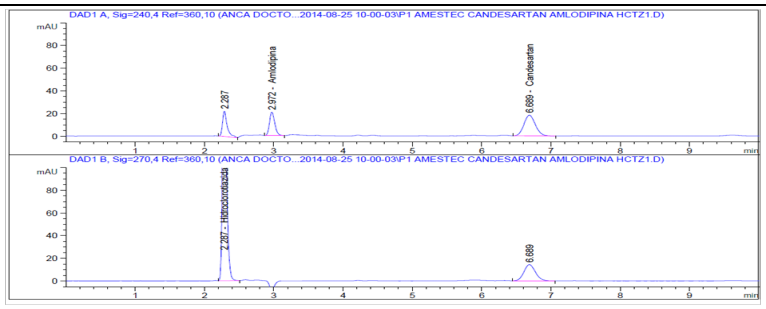

Figure 5.
Test solution chromatogram

For the evaluation of linearity, a series of standard solutions were prepared by dilution from actives ingredients stock solutions, the concentrations ranging from 70% to 120% of the targeted one (70%, 80%, 90%, 100%, and 120%). The slope of the regression, the y-intercept of the regression equation and the residual sum of squares were also calculated.
The study was performed by injecting three replicate standard solutions for each level into the HPLC system.

The correlation coefficients (r) for amlodipine, candesartan and hydrochlorothiazide were 0.998, higher than the accepted value which was 0.99 [17]. The linearity and the equation of calibration are represented in figures 6, 7, 8, 9.
Linearity graphs representing amlodipine, candesartan and hydrochlorothiazide concentration *versus* peak area for the levels: 70 %, 80%, 90 %, 100% and 120% showed that the method is linear in the prescribed range.

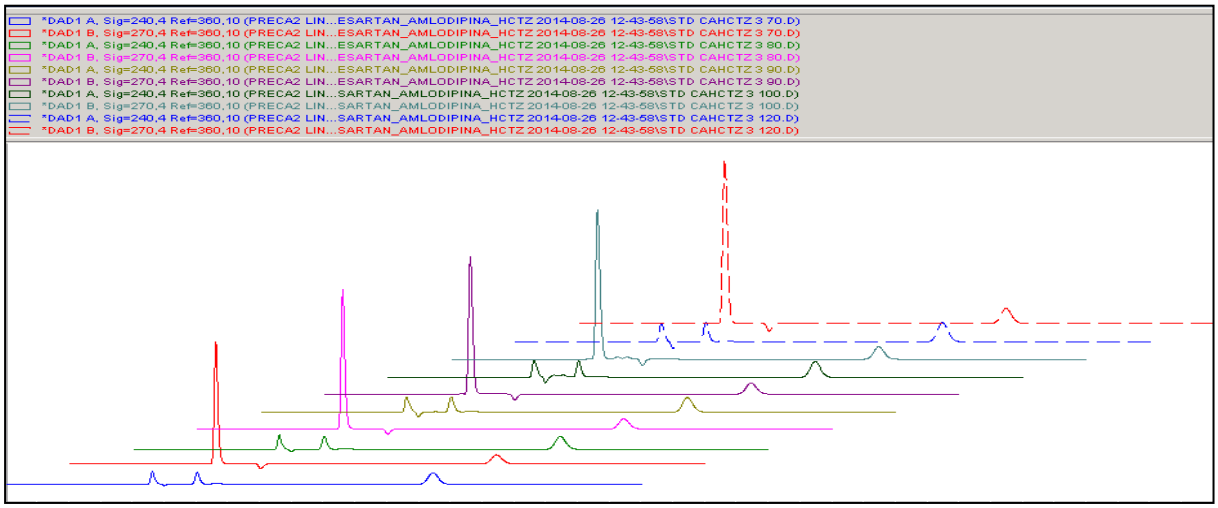

Figure 6.
Linearity - 3D chromatogram

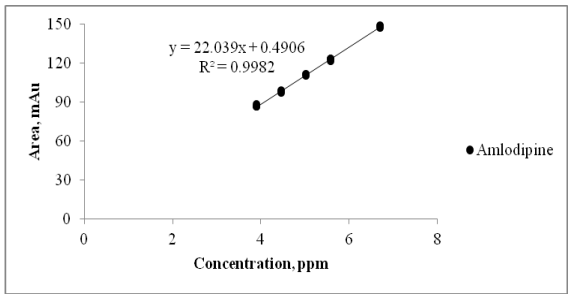

Figure 7.
Amlodipine linearity

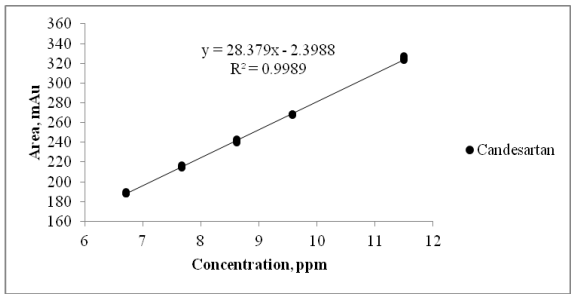

Figure 8.
Candesartan linearity

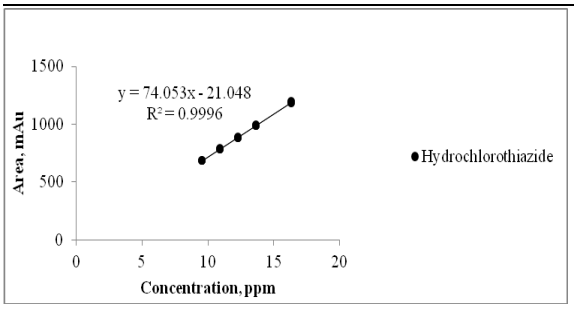

Figure 9.
Hydrochlorothiazide linearity

For the active ingredients, accuracy was assessed on spiked *placebo* solutions with candesartan, amlodipine and hydrochlorothiazide at 70%, 80%, 90%, 100% and 120% level of the target concentration.
Six standard solutions at 100% specification level and three spiked placebos at each level were injected. The average recoveries for the candesartan was between 100.02 and 101.68%, for amlodipine was between 98.12 and 99.44% and for hydrochlorothiazide was between 100.57 and 101.66%, and were framed in the prescribed range (which was 98.0 and 102%). The accuracy data are presented in Table II and the calibration curves for accuracy are shown in Figures 10-12.

Table II
Accuracy results

Concentration level, %	Average recovery, %		
	Candesartan	Amlodipine	Hydrochlorothiazide
70%	100.68	99.41	100.60
80%	100.02	99.11	101.07
90%	101.03	99.44	100.57
100%	101.20	98.12	101.66
120%	100.63	99.06	100.97

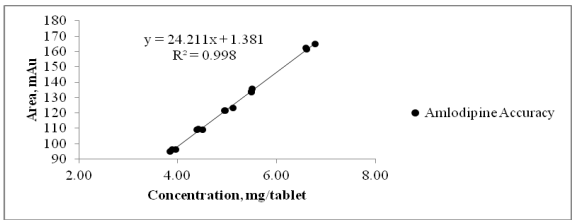

Figure 10.
Accuracy evaluation of the tested method for amlodipine

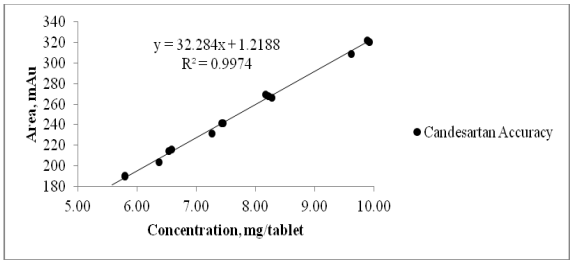

Figure 11.
Accuracy evaluation of the tested method for candesartan

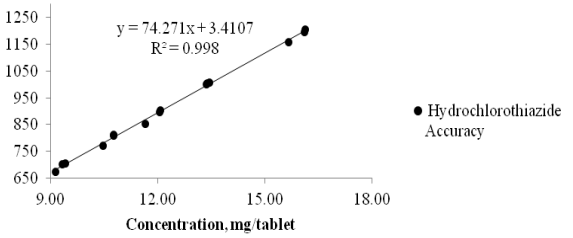

Figure 12.
Accuracy evaluation of the analysis method for hydrochlorothiazide

Precision was evaluated as method precision and intermediate precision.
The method precision was evaluated by analysing six injections of sample solution prepared as per the test method.
For the intermediate precision, a second analyst tested the same batch on a different day, in order to assess the impact of the complete analytical procedure, from sample preparation to final result, in the same laboratory.
Candesartan content was between 7.91 mg and 8.08 mg for the first analyst and between 7.68 mg and 7.92 mg for the second analyst and was in the prescribed range (7.60 - 8.40) mg.
Amlodipine content was between 4.80 mg and 5.19 mg for the first analyst and between 4.79 mg and 5.15 mg for the second analyst and was in the prescribed range (4.75 - 5.25) mg. Hydrochlorothiazide content was between 11.88 mg and 12.13 mg for the first analyst and between 11.88 mg and 12.19 mg for the second analyst and was in the prescribed range (11.88 - 13.13) mg.
The robustness of the assay method was evaluated by making small changes to the parameters: mobile phase flow rate, column temperature and different columns with regard to the method specific parameters.
The influence of the mobile phase flow rate on the method performance was tested using flow rates of 0.9 mL/min and 1.1 mL/min, respectively, instead of 1.0 mL/min as per the test method.
The influence of the column temperature variation on the method performance was tested using column temperature of 23°C and 27°C, respectively, instead of 25°C as per the test method and the column

influence was evaluated using a Kromasil 250 x 4.6 mm, 5 µm column.

The established acceptance criteria (variations on retention times not more than ± 15%, %RSD of peak area response for each active ingredient from the standard preparation (six injections) not more than 2.0% and USP tailing for each active peak not more than 1.2) were met as is shown in Table III.

Table III

Evaluation of the separation method for amlodipine, candesartan and hydrochlorothiazide

Parameters	Retention time (minutes)/Area (mAu)/%RSD (averages)								
	Amlodipine			Candesartan			Hydrochlorothiazide		
23°C	3.01	135.72	0.12	6.66	260.10	0.10	2.29	971.70	0.04
25°C	**2.92**	**137.35**	**0.09**	**6.29**	**263.52**	**0.22**	**2.28**	**980.50**	**0.04**
27°C	2.97	136.87	0.15	6.40	260.26	0.04	2.28	969.23	0.09
0.9 mL/min	3.25	153.66	0.06	7.00	308.38	0.15	2.53	1145.58	0.05
1 mL/min	**2.92**	**138.23**	**0.15**	**6.29**	**308.38**	**0.15**	**2.28**	**1145.58**	**0.05**
1.1 mL/min	2.66	138.23	0.57	5.75	254.47	0.73	2.07	940.57	0.07
Zorbax column	**2.92**	**138.23**	**0.15**	**6.29**	**275.05**	**0.10**	**2,28**	**1029.12**	**0.08**
Kromasil column	2.94	136.64	0.09	6.34	277.01	0.22	2.28	1022.90	0.10

The method robustness was determined by injecting amlodipine, candesartan and hydrochlorothiazide solutions under various flow and temperature conditions and changing the column. The obtained data reveal that the method for the quantitative determination of the three drugs is reliable and manifest constant validity.

Because the literature describes some methods [5, 6, 14] for the simultaneous determination of candesartan and hydrochlorothiazide, we attempted an adaptation of such a method for a combination which additionally has amlodipine. For this purpose two wavelengths were selected because hydrochlorothiazide has a maximum of absorption at 270 nm, whereas amlodipine shows a maximum of absorption at 237 nm and the maximum absorption of candesartan is 202 nm.

Conclusions

The presented method has been validated and may be used for the simultaneous determination of candesartan, amlodipine and hydrochlorothiazide from pharmaceutical drug formulations.

Hence it is recommended to be used in routine testing, release and stability samples testing because the analysis time is also relatively short.

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