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New HPLC Method with Experimental Design and Fluorescence Detection for Analytical Study of Antihypertensive Mixture, Amlodipine and Valsartan

Tadeusz Inglot, Anna Gumieniczek, Paulina Mączka, Ewelina Rutkowska
Department of Medicinal Chemistry, Faculty of Pharmacy, Medical University of Lublin, Lublin, Poland
Email: anna.gumieniczek@umlub.pl

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ABSTRACT

New HPLC method was developed for determination of amlodipine and valsartan in their binary mixture as a part of routine control of combined formulations. The method was validated to meet official requirements including selectivity, stability, linearity, precision and accuracy. Chromatography was carried out using a LiChrospher RP-18 column, a mixture containing acetonitrile, phosphate buffer of pH 3.5 and methanol (45:45:10, *v*/*v*/*v*) and new fluorescence detection at 255 nm for excitation and 448 nm for emission. The effect of methanol content, pH of the buffer, flow rate, detection wavelengths and column temperature was estimated in robustness study, according to a plan defined by the Plackett-Burman design. For identification of significant effects, both graphical and statistical methods were used. Robustness for dissolution test was checked estimating the effects of paddle speed, temperature and pH of dissolution medium. The method was proved to complying with all official guidelines. Therefore, it is suitable for determination of amlodipine and valsartan in their binary mixtures for different analytical and pharmaceutical purposes.

Keywords: HPLC Method; Fluorescence Detection; Experimental Design; Amlodipine and Valsartan; Binary Mixture

1. Introduction

Although many classes of antihypertensive drugs are now available, only few hypertensive patients can reach their target blood pressure with monotherapy. Most of the hypertensive population requires treatment with two or more antihypertensive agents. The main factor that characterizes a rational drug combination is a synergistic action without similar side effects. A one example of synergistic action in this area is the use of voltage-dependent calcium channel blockers and angiotensin II receptor antagonists, e.g. amlodipine (**Figure 1**) and valsartan (**Figure 2**) [1].

Formulations combined from the drugs which have complementary properties have the advantage of simplicity, convenience and cost-effectiveness. On the other hand, as for all chemical mixtures, more sophisticated analytical methods are required for determination and quality control procedures. So far, few spectrometric methods have been developed for determination of amlodipine and valsartan in pharmaceuticals using direct and derivative spectro photometry or spectrofluorimetry [2-6]. HPTLC and HPLC methods were also elaborated and validated in the range of official requirements [4,5,7-10]. Similar chromatographic methods exist for amlodipine and valsartan in triple drug combinations [11-16].

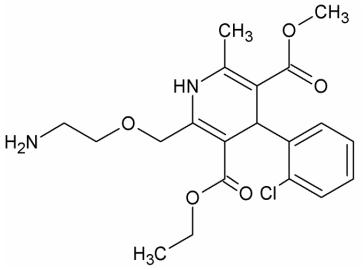


Figure 1. The chemical structure of amlodipine.

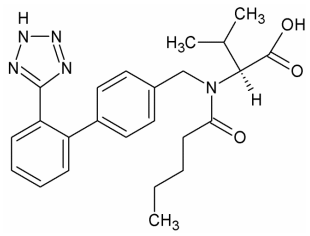


Figure 2. The chemical structure of valsartan.

Drug absorption after oral administration of a solid dosage form depends on the release of the active ingredients from the formulation and their dissolution under physiological conditions and the permeability across the

gastrointestinal tract. Therefore, the dissolution study may be relevant to the prediction of *in vivo* performance. It is therefore widely accepted that *in vitro* dissolution test is very important tool in pharmaceutical industry for providing valuable information to design new products and to ensure respective drug quality [17]. In the literature only one HPLC method was elaborated for the dissolution study of amlodipine and valsartan in their binary mixture using UV detection at 240 nm [10]. During our experiments, the results obtained with UV detection were unsatisfactory, probably due to interferences of respective tablet excipients. Taking into account these results, we decided to increase the specificity of our HPLC method by using fluorescence detection. In the literature concerning amlodipine and valsartan, HPLC with fluorescence detection was previously applied for determination of these drugs in human plasma, using on-line wavelength switching system [18]. Thus, the present work has two main objectives. The first is to report new reliable and validated HPLC method with fluorescence detection for simultaneously determination of amlodipine and valsartan where statistical design of experiments was used for the robustness study. The second is to apply the elaborated assay to determination of amlodipine and valsartan in tablets and to optimization of respective dissolution test.

2. Experimental

2.1. Materials and Reagents

Amlodipine (as besylate) from Sigma-Aldrich (USA), valsartan from Topharman (China), and Exforge® tablets from Novartis Pharma AG (Switzerland) containing 160 mg of valsartan and 10 mg of amlodipine were used (the declared excipients are: microcrystalline cellulose, type A crospovidone, colloidal silica, magnesium stearate, hypromellose, macrogol 4000, talc, titanium dioxide, yellow and red ferric dioxides). All solvents were of HPLC grade and were purchased from E. Merck (Germany). All chemicals were of analytical grade and were supplied by ICN Chemicals (Irvine, CA), and by Sigma Chemicals Co. (St. Louis, MO). Stock Solutions

Amlodipine and valsartan stock solutions were prepared by dissolving 10 mg of these compounds in methanol to obtain the concentration of 1 $mg \cdot mL^{-1}$ and then by diluting in methanol 10 times (to obtain the concentration of 0.1 $mg \cdot mL^{-1}$).

2.2. Equipment

HPLC system consisted of Alliance e2695 separations module, a model 515 isocratic pump and a model 2475 multi wavelength fluorescence detector from Waters (Milford, MA). It was controlled by Empower Pro v.2 software. Separation was carried out on a Li Chrospher® 100 RP-18 column (125 mm × 4.0 mm i.d., with a particle size of 5 μm) from E. Merck. All pH measurements were performed with a pH-meter, model HI 9024 C from Hanna Instruments (Italy). For dissolution study, evolution 6100 bathless dissolution system from Distek Inc. (North Brunswick, NJ) was used.

2.3. Chromatography

The mobile phase consisted of acetonitrile, phosphate buffer of pH 3.5 and methanol (45:45:10, *v*/*v*/*v*). It was filtered by nylon membrane filters (0.45 μm) and degassed prior to use. Buffer solution of pH 3.5 was prepared with 0.067 M KH_2PO_4, 0.067 M Na_2HPO_4 and 85% H_3PO_4. The pH value was measured in the buffer solution, not in the final mobile phase. A flow rate of 1.0 $mL \cdot min^{-1}$ was used. All chromatographic procedures were conducted at 25˚C. Volumes 20 μL from all solutions were injected onto the column. The fluorescent peaks were monitored at wavelength of 255 nm for excitation and 448 nm for emission, respectively.

2.4. Stability

The stock solutions of amlodipine and valsartan were stored at temperature 25˚C for 3, 6, 12, 24 and 48 h in tightly capped volumetric flasks. Additionally, samples of amlodipine and valsartan in phosphate buffer of pH 5.5 (dissolution medium) were heated in a water bath at 37˚C for 10, 20, 30, 40, 50 and 60 min. Respective samples were diluted with the mobile phase to gain the concentration over the linearity range and analyzed by the proposed HPLC method in term of the presence of some additional peaks and recoveries.

2.5. Linearity

Series of solutions of amlodipine and valsartan were prepared in 25 mL volumetric flasks by the appropriate dilution of the stock solutions with the mobile phase to reach the concentration ranges from 0.8 to 5.6 $\mu g \cdot mL^{-1}$ for amlodipine, and from 12 - 84 $\mu g \cdot mL^{-1}$ for valsartan. Six injections were made for each solution at each level and the peak area was plotted against the corresponding concentration of the drug.

2.6. Precision

Precision of the assay was evaluated by injecting the series of solutions at three different concentrations: the solutions containing 1.2, 2.8 and 4.4 μg of amlodipine, and 19.2, 44.8 and 70.4 μg of valsartan in 1 mL were analyzed five times in the same day. Inter day precision was assessed by analyzing similar solutions three times over a period of three days. Finally, precision was expressed by respective RSD values.

2.7. Accuracy

Accuracy of the method was proved by determination of amlodipine and valsartan by the standard addition method at three levels. The weighed portions of powdered tablets containing 2.27 mg of amlodipine and 36.34 mg of valsartan were transferred to 25 mL flasks, sonicated for 10 min, diluted to the mark and filtered by nylon membrane filters (0.45 μm). Then, 0.5 mL volumes were fortified with 50%, 100% or 150% respective drug from the stock solutions, diluted to 25 mL and analyzed. These procedures were repeated three times for each level of addition.

2.8. Assay in Tablets

The amounts of tablet powders equivalent to 2.27 mg of amlodipine and 36.34 mg of valsartan were transferred to 25 mL volumetric flasks, sonicated for 10 min, diluted to the mark with methanol and filtered. Then, 1.0 mL of the filtered solutions were transferred to 25 mL volumetric flasks and diluted with the mobile phase. Volumes 20 μL from these solutions were injected onto the column. The assay was repeated six times individually weighing the respective tablet powders.

2.9. Dissolution Test

Dissolution study of Exforge® tablets was performed using 900 mL of phosphate buffer of pH 5.5 at 75 rpm and 37.0˚C ± 0.2˚C. Buffer solution of pH 5.5 was prepared according to European Pharmacopoeia 7thedition (PhE 7). The dissolution medium was degassed by heating, filtering and by drawing a vacuum for a short period of time. Five mL of each sample was withdrawn at the time intervals of 10, 20, 30, 40, 50 and 60 min. The samples were filtered by nylon membrane filters (0.45 μm). Then, 1.0 mL volumes were diluted to 10 mL with mobile phase and analyzed by the proposed HPLC method. The above procedure was repeated three times and the mean recoveries were calculated from the linear regression equations. Later as acceptance criteria were altered, respective samples were withdrawn at 30 and 45 min and treated by the above procedure.

2.10. Statistics

Statistical analysis and graphical enhancement of the designed experiments were performed using Statistica software v. 10.0. All statistical calculations were done at a significance level $\alpha = 0.05$.

3. Results and Discussion

3.1. Chromatography Optimization

The chromatographic conditions were optimized to achieve the best resolution and peak shape for amlodipine and valsartan. Different mobile phases containing acetonitrile in phosphate buffer were examined. With acetonitrile content 50% or more, the retention time for valsartan was weak (retention time < 2 min). On the other hand, with decreasing of acetonitrile, the retention of amlodipine was too strong and its retention time was increased up to 10 min. Phosphate buffers of pH 3.0 - 7.0 were tried but the peak shapes for the drugs were sufficiently symmetrical only for pH value below 4. Therefore, the addition of methanol was decided and finally, the mobile phase containing acetonitrile, phosphate buffer at pH 3.5 and methanol (45:45:10, *v*/*v*/*v*) was selected as optimal for obtaining well defined and resolved peaks with mean retention times of ca. 5.3 and 2.2 min, for amlodipine and valsartan, respectively.

The chromatograms obtained from standard solutions were almost identical to those obtained from tablets solutions containing equivalent concentration of amlodipine and valsartan as well as for solutions in our dissolution medium (phosphate buffer of pH 5.5) confirming sufficient selectivity.

During our developing study, amlodipine and valsartan were monitored using UV detection. However, the results were unsatisfactory probably due to some interference from tablet excipients. Taking into account the above results, we decided to use a fluorescence detector to increase the specificity of our HPLC method. The optimal wavelengths for excitation and emission were set at 255 or 448 nm, at which the best detector responses for two drugs were obtained. In effect, much better precision and accuracy were achieved for amlodipine as compared with the results obtained using classical UV detection. The fluorescence properties of valsartan were lower, however much higher quantity of valsartan in existing commercial tablets allowed the simultaneous determination of both from one sample.

3.2. Experimental Design

The variables evaluated in this study and their lower, upper and nominal values are given in **Table 1**.

Because six real factors were chosen, one dummy variable was added to set the Plackett-Burman design for $n = 8$. As the most important parameter, the resolution factor between amlodipine and valsartan (R_s) was selected for further analysis. Respective data are showed in **Table 2**.

A graphical approach in combination with the algorithm of Dong was used to identify significant effects [19]. First step was the calculation of factor's effects for each factor (E_i). Then, the algorithm of Dong was used for further calculations. An initial estimation of the error on an effect (s_0) as well as the final estimation of the error on an effect (s_1) was obtained. It was stated that

Table 1. The variables used for the robustness testing.

	Variable	Lower value (−1)	Nominal value (0)	Upper value (1)
A	Flow rate (mL^{-1})	0.9	1.0	1.1
B	Methanol content (%)	7	10	13
C	pH of the buffer (pH unit)	3.4	3.5	3.6
D	Temperature (˚C)	22	25	28
E	Excitation wavelength (nm)	222	225	228
F	Emission wavelength (nm)	445	448	451
G	Dummy 1	−1	0	1

Table 2. The plan of Plackett-Burman design (A-G-variables described in Table 1, R_s: resolution factor).

Run	A	B	C	D	E	F	G	R_s
7	−1	1	1	−1	−1	1	−1	3.029
4	1	1	−1	1	−1	−1	−1	2.954
8	1	1	1	1	1	1	1	2.949
2	1	−1	−1	−1	−1	1	1	2.828
5	−1	−1	1	1	−1	−1	1	2.989
6	1	−1	1	−1	1	−1	−1	2.861
1	−1	−1	−1	1	1	1	−1	3.009
3	−1	1	−1	−1	1	−1	1	2.954

any effect did not exceed the value 2.5 × s_0. After that the s1 value was used to calculate a margin of error (ME) and a simultaneously margin of error (SME) which are the critical limits (**Table 3**).

The rankits of **Table 3** were used to build the half-normal plot where the ME and SME limits are included (**Figure 3**).

From the plot it is clearly seen that all our effects are non-significant. They tend to fall on a straight line through zero. In addition, any effect is not larger than our ME and SME values confirming non-significant influence of our factors on the resolution factor between amlodipine and valsartan.

3.3. Stability

The drugs were stable in methanolic solutions stored at temperature 25˚C for 48 h and no additional peaks were observed on the chromatograms. Also, the samples in dissolution medium, heated at 37˚C for 60 min did not show any significant changes. Recoveries of the both drugs from the stored solutions in comparison with respective standards were sufficient.

Table 3. The obtained factor effects (E_i: The value of effect *i*; ME: The margin of error; SME: The simultaneous margin of error; s_0: The initial estimation of the error; s_1: The final estimation of the error).

	Rankit	E_i	ME	SME
E	0.09	0.00675	0.116531	0.180685
F	0.27	0.01425	t (0.975.7) = 2.365	t (0.996.7) = 3.667
C	0.46	0.02075		
G	0.66	0.03325	median	0.033250
B	0.9	0.04975	s_0	0.049875
D	1.21	0.05725	2.5 × s_0	0.124688
A	1.71	0.09725	s_1	0.049273

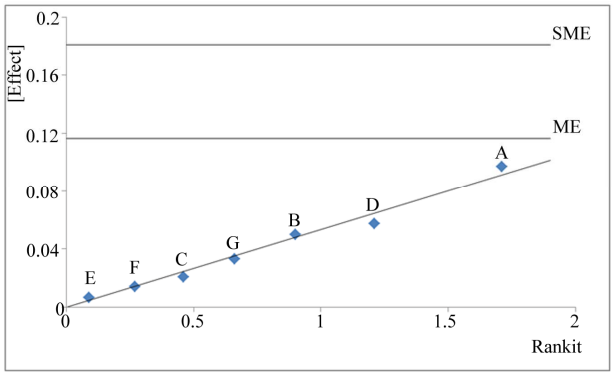


Figure 3. The half normal probability plot for the effects with identification of the critical effects ME and SME (A-G-variables described in Table 1).

3.4. Linearity

For calibration, six independent determinations were performed at each of six levels. The relationships were constructed between the peak area of the respective drug and the corresponding concentration by a linear regression equation. The residuals of regression did not show significant heteroscedascity (investigated by Bartlett test) so the use of weighed regression was not necessary. The method was tested for linearity by means of the Mandel's fitting test with quadratic equation as the alternative fitting. Also, the significance of quadratic term (t value) was investigated. The Shapiro-Wilk test did not reject the hypothesis that residuals were normally distributed (**Table 4**).

3.5. Precision

The data obtained from precision experiments are given in **Table 5**. The interday precision for amlodipine expressed as RSD was 0.54% and 0.34% for the lowest and the highest concentration. The respective values for valsartan ranged from 1.02% to 0.49%.

3.6. Accuracy

Accuracy of the method (**Table 6**) was proved by determination of amlodipine and valsartan in the fortified samples at three levels of addition (50%, 100% and 150%).

Therefore, the recovery of the actives from the matrix was correct and the proposed analytical method was sufficiently accurate.

3.7. Assay in Tablets

Total recovery (mean ± RSD) from tablets was found to be 99.69% ± 1.20% and 100.89% ± 0.42%, for amlodipine and valsartan, respectively. All results were homogenic and t student test did not show significant differences between them and the declared contents. The results were also estimated by calculating the 95% confidence intervals and checking if the declared amounts were inside them. The declared contents were in the confidence intervals so our determinations in tablets were sufficiently accurate (**Table 7**).

3.8. Dissolution Test

During our developing study, three phosphate buffers of pH 4.5, 5.5 and 6.8 were examined as dissolution media. The choice of optimal pH value was difficult due to significant differences in chemical properties of amlodipine and valsartan. According to official requirements (PhE 7) no less than 80% of the active ingredients of the labeled claim should be dissolved within 30 - 45 min. The average percentage of drugs released after 10, 20, 30, 40, 50 and 60 min in different pH values are depicted in **Figure 4**. After 30 min, the results obtained in pH 4.5 were satisfactory for amlodipine but not for valsartan, while in pH 6.8, both the drugs showed worse recovery than in pH 5.5. Therefore, the phosphate buffer of pH 5.5 was chosen as optimal for both the drugs and used finally, instead of previously described phosphate buffer of pH 3.6 [10]. After 45 min, at pH 5.5, with a paddle speed 75 rpm, the dissolution at the level 80% was achieved. Mean values for amlodipine and valsartan were 87.49% and 80.70%, respectively (**Table 7**). Robustness of the method, checked after deliberate alterations in paddle speed, temperature and pH of dissolution medium showed that small changes of these operational parameters did not lead to essential changes in term of recovery for the peaks of interest. The effect of a single factor at

Table 4. Statistical evaluation of calibration data for amlodipine (A) and valsartan (V) (n = 6).

Drug	Linear equation $y = ax + b$	RSD a (%)	RSD b (%)	r	p	Mandel's test	
						F	p
A	$y = 1050084x - 58902$	1.57	76.23	0.9999	0.0	2.3416	0.1340
V	$y = 3664782x + 17541751$	1.35	9.28	0.9998	0.0	1.1120	0.2981

Table 5. Precision in the standard solutions.

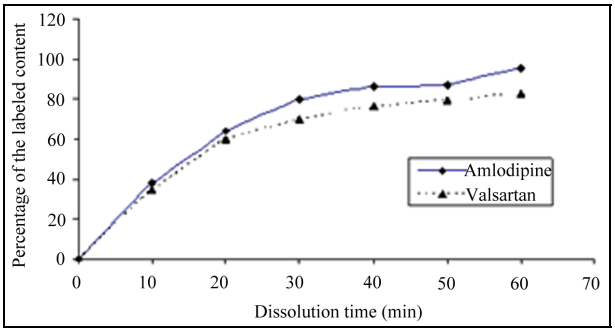
Drug concentration ($\mu g \cdot mL^{-1}$)	Intraday precision (n = 5)		Interday precision (n = 3)	
	Recovery mean ± SD	RSD %	Recovery mean ± SD	RSD %
Amlodipine				
1.2	100.86 ± 0.96	0.95	100.87 ± 0.54	0.54
2.8	99.75 ± 0.52	0.52	99.96 ± 0.48	0.48
4.4	100.60 ± 0.64	0.63	100.17 ± 0.34	0.34
Valsartan				
19.2	99.73 ± 1.05	1.05	99.40 ± 1.01	1.02
44.8	100.25 ± 1.18	1.17	100.43 ± 0.72	0.71
70.4	100.88 ± 0.83	0.82	100.34 ± 0.49	0.49

Table 6. Accuracy of the method in the fortified samples of amlodipine (A) and valsartan (V).

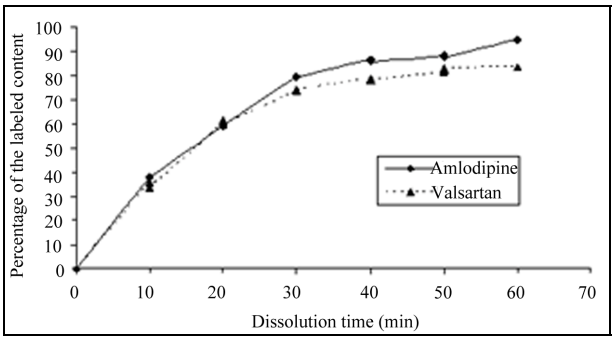
Drug	Level of addition (%)	Amount expected ($\mu g \cdot mL^{-1}$)	Recovery mean ± SD (%, n = 3)	RSD (%, n = 3)	Recovery (%, n = 9)	RSD (%, n = 9)
A	50	2.29	100.93 ± 0.96	0.96		
	100	2.69	102.17 ± 0.30	0.31	101.27	1.09
	150	3.09	100.70 ± 1.40	1.21		
V	50	38.3	101.84 ± 0.49	0.49		
	100	46.3	101.22 ± 0.34	0.34	101.05	0.86
	150	54.3	100.10 ± 0.55	0.55		

Table 7. Statistical evaluation of the results obtained for amlodipine (A) and valsartan (V) assay in tablets and dissolution medium (n = 6)

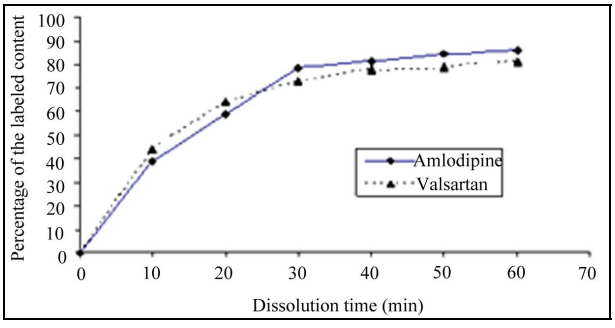
Assay		Declared amounts (μg·mL^{-1})	Recovery mean ± SD (%)	95% Confidence interval	RSD (%)	t student	
						t	p
Tablets	A	3.63	99.69 ± 1.20	98.26 - 101.35	1.20	0.00000	1.00000
	V	58.15	100.83 ± 0.42	100.45 - 101.33	0.41	0.008774	0.99258
Dissolution 30 min	A	1.11	79.56 ± 0.23	79.32 - 79.81	0.29	0.01755	0.98668
	V	17.78	74.09 ± 0.34	73.74 - 74.45	0.46	0.024110	0.98169
Dissolution 45 min	A	1.11	87.49 ± 0.15	87.33 - 87.64	0.17	−0.083276	0.93686
	V	17.78	80.70 ± 0.62	80.05 - 81.35	0.77	0.006600	0.99499



pH 4.5



pH 5.5



pH 6.8

Figure 4. The dissolution profiles of amlodipine and valsartan in different pH values.

three levels, estimated with F test, indicated that percentage recoveries remained unaffected by small variations of these parameters (data not shown).

4. Conclusion

We conclude that our method with fluorescence detection may be successfully applied as an alternative technique to HPLC with classical UV detection. Taking into account the above results, it can be stated that our method complies with all official guidelines, indicating suitability for determination of amlodipine and valsartan in binary mixtures for different analytical and pharmaceutical purposes, e.g. determination in pharmaceuticals as well as for respective *in vitro* dissolution tests.

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