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New HPLC method for *in vitro* dissolution study of antihypertensive mixture amlodipine and perindopril using an experimental design

Research Article

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Abstract: A new HPLC method was developed for the determination of amlodipine and perindopril in their binary mixture as a part of a routine control of combined formulations. For the first time an HPLC method was used for an *in vitro* dissolution study of tablets containing the above drugs. The presented method was validated to meet official requirements and this validation included specificity, stability, linearity, precision and accuracy. Chromatography was carried out using a LiChrospher RP-18 column, a mixture containing acetonitrile and phosphate buffer of pH 3.0 (50:50, v/v) as mobile phase and UV detection at 225 nm. The dissolution test was performed using 900 mL of phosphate buffer at pH 5.5 containing 1% cetylpyridini chloride (CPC) at 37°C and 75 rpm, using the paddle method. Robustness procedure was done according to the plan defined by the Plackett-Burman design. The effects of acetonitrile content, pH of the buffer and flow rate of the mobile phase, column temperature, pH and CPC content in the dissolution medium as well as rotation speed of the paddle were considered. After that, both graphical and statistical methods were used for identification of significant and non-significant effects.

Keywords: *HPLC • Experimental Design • Dissolution study • Amlodipine • Perindopril*

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1. Introduction

Although many classes of antihypertensive drugs are now available, only few hypertensive patients can reach their target blood pressure with a single drug. Most hypertensive patients require treatment with two or more antihypertensive agents. The main factor that characterizes a rational drug combination is synergistic action without similar side effects. A one example in the area of hypertension is simultaneously using voltage-dependent calcium channel blockers and angiotensin converting enzyme inhibitors [1], *e.g.* amlodipine and perindopril (Fig. 1).

Formulations combined from the drugs which have complementary properties have the advantage of simplicity, convenience and cost effectiveness. However, as for all chemical mixtures, new analytical methods are then required for different analytical purposes, *e.g.* for the assay or for *in vitro* dissolution tests. It is widely accepted that the results obtained in the dissolution test may be relevant to predicting *in vivo* behavior of pharmaceuticals. Therefore, the dissolution test is a very important tool in the pharmaceutical industry to ensure respective drug quality [2].

As far as concerns the analytical procedures for amlodipine and perindopril, only two HPLC methods are now available for determination of them in such a combination [3,4]. These methods were performed using reversed-phase systems and UV detection, and were described as sufficiently linear, accurate and precise. Additionally, one spectrophotometric method for simultaneous determination of both drugs was found in the literature [5]. However, to the best of our knowledge, any method suitable for the dissolution test of pharmaceuticals containing the mentioned drugs has not been published so far. Thus, the present work has two

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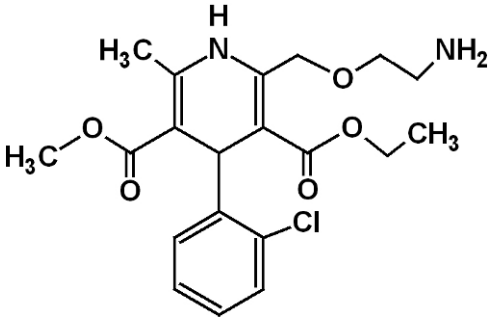
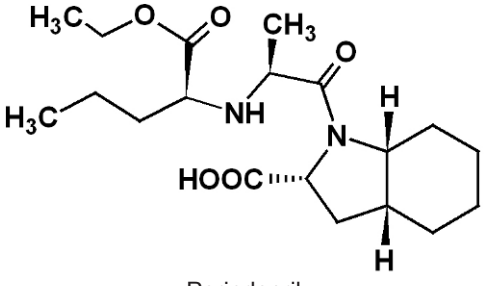


Amlodipine



Perindopril

Figure 1. Chemical structures for the drugs being examined.

main objectives. The first is to report a new reliable and validated HPLC method for simultaneous determination of amlodipine and perindopril. The second is to apply the elaborated method to optimize the dissolution test for commercially available two-component tablets. For better estimation of suitability of our method, the experimental design was proposed as an additive tool.

2. Experimental procedure

2.1. Materials and reagents

Amlodipine (as besylate) from Sigma-Aldrich (USA), perindopril (as arginine) from Les Laboratoires Servier (France) and Co-Prestarium® tablets from Les Laboratoires Servier were used. The declared tablet excipients are: microcrystalline cellulose, colloidal silica anhydrous, magnesium stearate, lactose monohydrate. All solvents were of HPLC grade and were purchased from E. Merck (Germany). Other chemicals were of analytical grade and were supplied by ICN Chemicals (USA), and by Sigma-Aldrich. Phosphate buffers were prepared with 0.067 M KH_2PO_4, 0.067 M Na_2HPO_4 and 85% H_3PO_4.

2.2. Equipment

The HPLC system consisted of an Alliance e2695 separations module, a model 515 isocratic pump and a model 2489 multi wavelength UV-Vis detector from Waters (USA). It was controlled by Empower Pro v.2 software. Separation was carried out on a LiChrospher® 100 RP-18 column (125 × 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 HI9024C from Hanna Instruments (Italy). For the dissolution study, Evolution 6100 bathless dissolution system (Apparatus 2 by European Pharmacopoeia 7th Edition) from Distek Inc. (USA) was used.

2.3. Chromatography

The mobile phase consisted of acetonitrile and phosphate buffer of pH 3.0 (50:50, *v/v*). It was filtered by nylon membrane filters (0.45 µm) and degassed prior to use. The pH value was measured in the buffer solution, not in the final mobile phase. A flow rate of 1.0 mL min^{-1} was used. All chromatographic procedures were conducted at 22°C. Volumes of 20 µL from all solutions were injected onto the column. The peaks were monitored using UV detection at the wavelength of 225 nm.

2.4. Stock solutions

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

2.5. Stability

The stock solutions containing 0.1 mg mL^{-1} of amlodipine and perindopril in methanol were stored at temperature 25°C for 3, 6, 12, 24 and 48 h in tightly capped volumetric flasks. Also, solutions containing 0.1 mg mL^{-1} of amlodipine and perindopril in phosphate buffer of pH 5.5 were heated in a water bath at 37°C for 10, 20, 30, 45 and 60 min. The stability was then checked by respective diluting, analyzing by the above method and checking chromatograms for the presence of some additive peaks. Additionally, recoveries of the drugs were calculated in comparison with respective standards of amlodipine and perindopril.

2.6. Calibration procedure

Series of solutions of amlodipine and perindopril were prepared in 10 mL volumetric flasks by the appropriate dilution of the stock solutions with the mobile phase to reach the concentration ranges from 5 to 30 µg mL^{-1} for amlodipine, and from 10 to 60 µg mL^{-1} for perindopril. Then, five injections onto the column were made for each level and the peak areas were plotted against the corresponding concentrations of the drugs.

2.7. Precision

Precision of the method was evaluated by injecting the solutions at three different concentrations. These solutions contained 8, 16 and 24 µg of amlodipine, and 16, 32 and 48 µg of perindopril in 1 mL and were analyzed three times in the same day. Inter-day precision was assessed by analyzing similar solutions over a period of three days. Finally, the precision was expressed by respective RSD values for the peak areas.

2.8. Accuracy

Accuracy of the method was demonstrated by the standard addition method at three levels. The weighed portions of powdered tablets containing 5 mg of amlodipine and 10 mg of perindopril were transferred to 50 mL flasks, sonicated for 30 min, diluted to the mark and filtered by nylon membrane filters (0.45 µm). Then, 0.25 mL volumes were fortified with 50, 100 or 150% amlodipine and perindopril from the stock solutions, diluted to 10 mL and analyzed. This procedure was repeated three times for each level of addition.

2.9. Dissolution study

Dissolution study of Co-Prestarium® tablets was performed using phosphate buffer of pH 5.5 containing 1% CPC in 900 mL volume at 37°C and 75 rpm. The dissolution medium was degassed by heating, filtering and drawing a vacuum for a short period of time. Volumes of 5.0 mL of each sample were withdrawn after 45 min. The samples were filtered by nylon membrane filters (0.45 µm) 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.

2.10. Assay in tablets

The amounts of Co-Prestarium® tablets equivalent to 5 mg of amlodipine and 10 mg of perindopril were transferred to 50 mL volumetric flasks, sonicated for 30 min, diluted to the mark with methanol, mixed and filtered. Then, 1.8 mL of the filtered solutions were transferred to 10 mL volumetric flasks and diluted with the mobile phase. The assay was repeated six times individually weighing the respective tablet powders.

2.11. Statistical analysis

Statistical analysis and graphical enhancement of the designed experiments were performed using Statistica v. 10.0 as well as R statistics software. Statistical significance was set at a significance level α=0.05.

3. Results and discussion

3.1. Chromatography optimization

The chromatographic conditions were optimized to achieve the best resolution and peak shapes for amlodipine and perindopril. Different mobile phases containing acetonitrile in phosphate buffer were examined. With an acetonitrile content of less than 50%, the retention for amlodipine was too long (retention time >6 min). However, with increasing acetonitrile content above 50%, resolution for the drugs failed. Phosphate buffers of pH 3.0-7.0 were tried but the peak shapes were sufficiently symmetrical only at pHs below 4. Finally, the mobile phase containing acetonitrile and phosphate buffer at pH 3.0 (50:50, *v/v*) was chosen as optimal for obtaining well defined and resolved peaks with mean retention times of ca. 5.9 and 3.7 min, for amlodipine and perindopril, respectively (Fig. 2).

3.2. Specificity

The chromatograms obtained from the standard solutions of amlodipine and perindopril were almost identical to those obtained from commercially available two-component tablets.

3.3. Robustness of the method by an experimental design

The variables evaluated in this study included the percentage content of acetonitrile, pH, flow rate of the mobile phase, column temperature, pH and percentage content of cetylpyridine chloride (CPC) in the dissolution medium as well as rotation speed of the paddle in the apparatus. These factors and their lower, upper and nominal values are shown in Table 1. As the most important parameter, the percentage recovery of the both drugs was selected for further analysis (Table 2).

A graphical approach in combination with the algorithm of Dong was used to identify significant effects [6,7]. The 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. After that, the s_1 value was used to calculate a margin of error (ME) and a simultaneous margin of error (SME) which are the critical limits (Table 3). The rankits of Table 3 were used to build the half-normal plot presented in Fig. 3 where the ME and SME limits are included. From the plot obtained for amlodipine it was seen that the CPC content in our dissolution medium significantly affected the recovery of the drug. Therefore, a non-

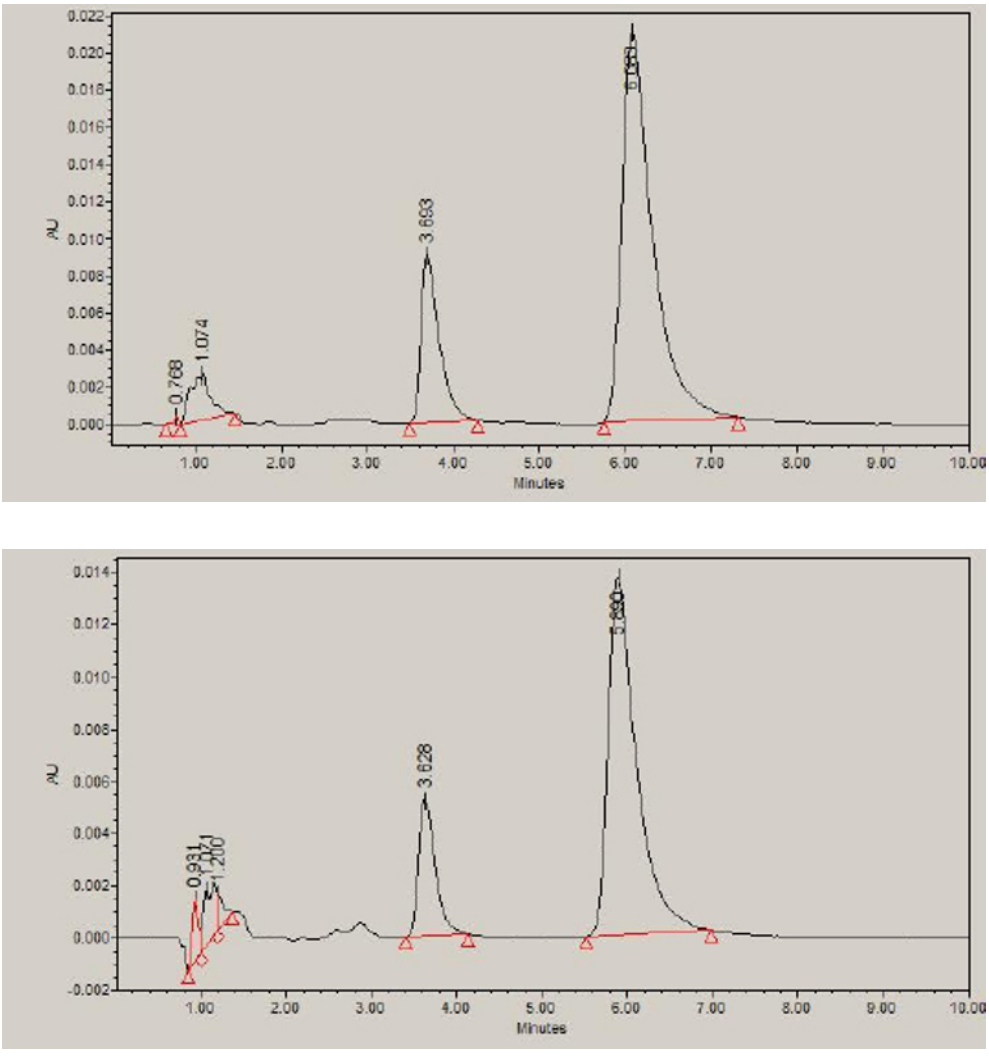


Figure 2. Representative chromatograms of amlodipine (t_R~5.9) and perindopril (t_R~3.7) from the standard solution (a) and from the dissolution test (b).

Table 1. The variables used for the robustness testing.

Variable		Lower value (-1)	Nominal value (0)	Upper value (1)
A	**Flow rate/mobile phase (ml min^{-1})**	0.9	1.0	1.1
B	**Buffer pH/mobile phase**	2.7	3.0	3.3
C	**Column temperature (˚C)**	20	22	24
D	**% Acetonitrile/mobile phase**	45	50	55
E	**Buffer pH/dissolution medium**	5.0	5.5	6.0
F	**Rotation speed/apparatus (rpm)**	70	75	80
G	**% CPC/dissolution medium**	0.8	1.0	1.2

significance interval for this significant effect was determined using respective formula [7]. This interval was determined as 0.85-1.15% CPC in the dissolution medium. In the case of perindopril, any significant effect was not observed. This could be explained by different chemical structures of the mentioned drugs and their different solubility. It was seen that for slightly soluble substances like amlodipine, properties of each dissolution medium should be controlled very carefully.

Table 2. The plan of Plackett-Burman design (A-G-variables are described in Table 1) and mean recoveries of amlodipine and perindopril (n=3).

Run	A	B	C	D	E	F	G	% Recovery of amlodipine	% Recovery of perindopril
4	1	1	-1	1	-1	-1	-1	78.71	81.90
2	1	-1	-1	-1	-1	1	1	79.10	82.31
7	-1	1	1	-1	-1	1	-1	78.61	81.73
3	-1	1	-1	-1	1	-1	1	80.82	81.81
1	-1	-1	-1	1	1	1	-1	78.24	82.45
5	-1	-1	1	1	-1	-1	1	80.40	81.82
8	1	1	1	1	1	1	1	80.11	82.71
6	1	-1	1	-1	1	-1	-1	79.23	81.60

Table 3. The obtained factor effects (A-amlodipine, P-perindopril, 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				
	A	P	A	P		A	P
B	0.46	0.09	0.33	0.00	s_0	0.57	0.30
C	0.66	0.27	0.38	0.15	$2.5xs_0$	1.425	0.75
A	0.27	0.46	0.22	0.20	S_1	0.420496	0.277102
E	0.90	0.66	0.38	0.20	ME	1.041568	1.016134
G	1.71	0.90	1.43	0.25	tME	2.477	2.365
D	0.09	1.21	0.08	0.35	SME	1.639092	1.016134
F	1.21	1.71	0.78	0.50	tSME	3.898	3.667

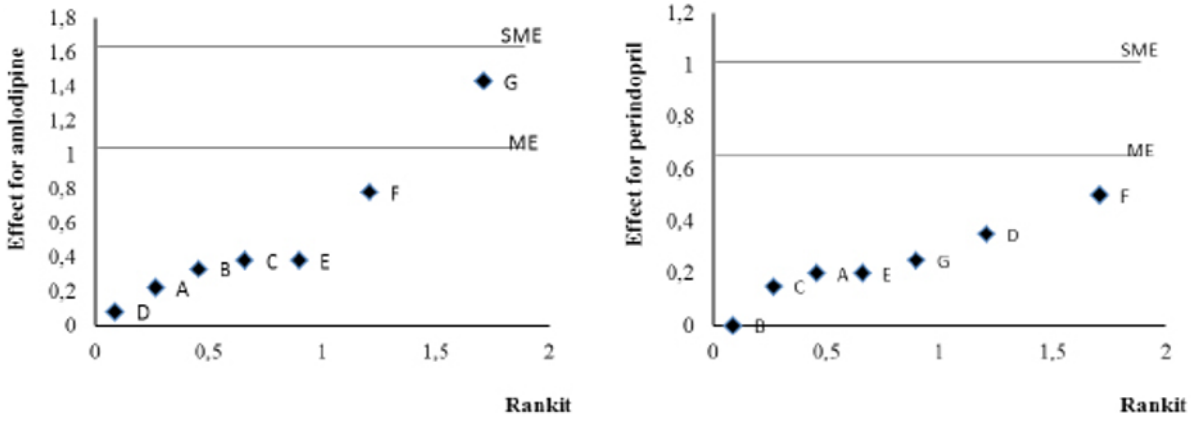


Figure 3. The half normal probability plot for the effects with identification of the critical effects ME and SME (A-G-variables are described in Table 1).

3.4. Stability

The drugs resolved in methanol were stable when stored at a temperature of 25ºC for 48 h. Also, the samples of amlodipine and perindopril resolved in the dissolution medium and when heated at 37°C for 45 min did not show any significant changes (the lack of additive peaks). Recovery of the drugs from the stored solutions in comparison with respective standards were sufficient.

3.5. Linearity

For calibration, five independent determinations were performed at each of six levels for both drugs. The relationships were constructed between the peak area of the respective drug and the corresponding concentration, by a linear regression equation. The method was tested for linearity by means of the Mandel's and the Lack-of-fit tests as well as by the analysis of residuals. Finally, straight lines were considered

Table 4. Statistical evaluation of calibration data for amlodipine (A) and perindopril (P) (n=5 for each concentration).

Drug	y=ax+b	RSD a	RSD b	r	p	Mandel's F	Mandel's p	Lack-of-fit F	Lack-of-fit p
A	y=30059x-9735	0.83	-68.40	0.9993	0.0	0.0002	0.9987	2.4008	0.07804
P	y=6353x-5931	1.14	-60.20	0.9992	0.0	0.1977	0.6602	0.3262	0.8576

Table 5. Precision in the standard solutions.

Drug concentration	Intra-day precision (n=3)		Inter-day precision (n=3)	
($\mu g\ mL^{-1}$)	Peak area mean±SD	RSD	Peak area mean±SD	RSD
Amlodipine				
8	221717±1246	0.56	211561±1890	0.85
16	430000±2220	0.51	442314±2025	0.46
24	677972±618	0.09	677510±497	0.07
Perindopril				
16	94519±219	0.23	94352±433	0.46
32	191935±201	0.10	191607±1258	0.66
48	309092±469	0.15	299778±1200	0.46

adequate to describe the relationships between the peak areas and the concentrations of each compound (Table 4).

3.6. Precision

The data obtained from the precision experiments are shown in Table 5. The inter-day precision for amlodipine expressed as RSD was 0.85 and 0.07% for the lowest and the highest concentration. The respective values for perindopril were in the range 0.66-0.46%. Taking together, all RSD values for intra-day as well as for inter-day precision were far below 2%, confirming that the method was sufficiently precise.

3.7. Accuracy

Accuracy of the method (Table 6) was demonstrated by determination of amlodipine and perindopril in the fortified samples at three levels of addition (50, 100 and 150%). In the amlodipine assay, the recovery ranged from 98.79 to 100.88% for the lowest and the highest concentration of the drug with mean RSD 1.37%. For perindopril, the mean recovery was 98.45% with RSD value 0.76%. Therefore, it was stated that the recovery of the actives from the matrix was correct and the proposed HPLC method was sufficiently accurate.

3.8. Dissolution study

The choice of optimal pH for dissolution medium was difficult due to differences in chemical structures and low water solubility of the drugs, especially amlodipine. After many initial tests, phosphate buffer of pH 5.5 was chosen as a compromise for both drugs. Tablets were treated with 900 mL of the buffer at 75 rpm as the paddle speed and temperature of 37°C. The samples were withdrawn at time intervals of 15, 30 and 45 min, filtered and analyzed by the HPLC method. According to European Pharmacopoeia 7th edition, no less than 80%, the label claims, should be dissolved within 30-45 min. However, after 45 min in the buffer at pH 5.5, a dissolution of 80% was not achieved. Therefore, two different surfactants, anionic sodium dodecyl sulfate (SDS) and cationic CPC in two concentrations (0.5 and 1.0%) were tried [8]. The results obtained with 0.5% of both surfactants were almost satisfactory for perindopril but not for amlodipine. However, while 1% CPC in the dissolution medium was used, the level of 80% for both drugs was achieved (Fig. 4). Finally, six independent tablets were analyzed (900 mL of the buffer at pH 5.5 containing 1% CPC, 75 rpm, 37°C, 45 min) and mean values for amlodipine and perindopril were 81.67 and 84.75% (Table 7).

Table 6. Accuracy of the method in the fortified samples.

	Level of Addition (%)	Amount expected ($\mu g\ mL^{-1}$)	Recovery Mean±SD (n=3)	RSD (n=3)	Recovery (n=9)	RSD (n=9)
Amlodipine	50	12.5	98.79±0.51	0.52		
	100	15.0	101.50±1.29	1.28	100.26±1.37	1.37
	150	17.5	100.88±0.85	0.84		
Perindopril	50	25.0	98.67±0.83	0.84		
	100	30.0	98.29±0.32	0.33	98.45±0.75	0.76
	150	35.0	98.38±1.16	1.18		

Table 7. Statistical evaluation of the results obtained for amlodipine (A) and perindopril (P) in tablets and in dissolution study (n=6).

Assay		Declared amounts ($\mu g\ mL^{-1}$)	Recovery Mean±SD (%)	95% Confidence interval	RSD
Tablet assay	A	18.0	99.20±0.54	98.63-99.76	0.54
	P	36.0	99.01±0.09	98.91-99.10	0.09
Dissolution test	A	22.0	81.67±0.35	81.30-82.08	0.42
	P	44.0	84.75±0.01	84.73-84.76	0.01

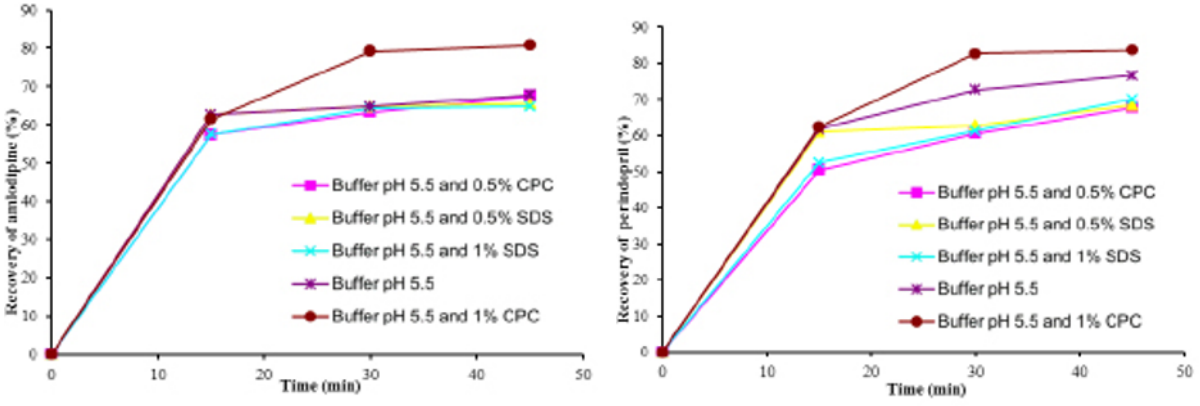


Figure 4. Dissolution profiles in different dissolution media (CPC-cetylpirydini chloride, SDS-sodium dodecyl sulfate).

3.9. Assay in tablets

The proposed method was used for the assay of amlodipine and perindopril in tablets. All obtained results were homogenic and the t Student test did not show significant differences between them and the declared contents of the drugs. The results were also estimated by calculating the 95% confidence intervals and checking if the determined amounts were inside them. For the both drugs, almost all contents were in the confidence intervals so our determinations in tablets were sufficiently accurate (Table 7).

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