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Optimization and validation of an HPLC method for determination of related compounds in enalapril maleate tablets

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ABSTRACT

The possibilityof optimization of an HPLC method for determination of related compounds in Enalapril maleate tablets described in official British Pharmacopeia (BP) was investigated. Columns with different C18 stationary phases were tried out. A full factorial design (2^4) was used to investigate the influence of four variables (pH of the aqueous phase in the mobile phase, acetonitrile fraction, flow rateof the mobile phase and column temperature), as BP recommends extreme column conditions regarding to pH and temperature. Nineresponses were determined in each experiment: retention time (Rt) of enalaprilat (ET) peak, retention time of enalapril diketopiperazine (DKP) peak, resolution factor between the peaks due to ET and DKP, resolution factor between the peaks due to DKP and enalapril,USP tailing of ET and DKP,capacity factor (k') of ET and DKP and retention time of enalapril. The optimal conditions for the chromatographic procedure were determined:pH of the aqueous phase in the mobile phase 2.2, acetonitrile fraction 37.5% v/v, flow rate 1.0 and column temperature 50°C. Method was found to be selective, linear, accurate and precisein the specified ranges. The limits of detection and limits of quantitation were 0.024 μg ml^{-1} and 0.080 μg ml^{-1} for ET, 0.017 μg ml^{-1} and 0.055 μg ml^{-1} for DKPand 0.181 μg ml^{-1} and 0.603 μg $ml^{-1}$for enalapril,respectively. The presented HPLC method is to be implemented in the quality and stability testing of Enalapril maleate tablets 10 mg and 20 mg.

KEYWORDS

Enalapril;
Related compound;
HPLC;
Validation;
Experimental design.

INTRODUCTION

Enalapril is used to treat high blood pressure (hypertension). Lowering high blood pressure helps prevent strokes, heart attacks, and kidney problems. It is also used to treat heart failure. Enalapril is an angiotensin-converting enzyme(ACE) inhibitorand works by relaxing blood vessels so that blood can flow more easily[1].

ACE is a peptidyl dipeptidase that catalyzes the conversion of angiotensin I to the vasoconstrictor substance, angiotensin II. Angiotensin II also stimulates

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aldosterone secretion by the adrenal cortex. The beneficial effects of enalapril in hypertension and heart failure appear to result primarily from suppression of the rennin- angiotensin- aldosterone system. Inhibition of ACE results in decreased plasma angiotensin II, which leads to decreased vasopressor activity and aldosterone secretion[2].

Although the latter decrease in small, it results in small increases of serum potassium. While the mechanism through which enalapril lowers blood pressure is believed to beprimarily suppression of the rennin – angiotensin- aldosterone system, it is antihypertensive even in patients with low – rennin hypertension[2].

Enalapril maleate, is 1-{*N*-[(s)-1-carboxyl-3-phenylpropyl]-L-alanyl-}-L-proline 1-ethyl ester maleatewith the following structural formula:

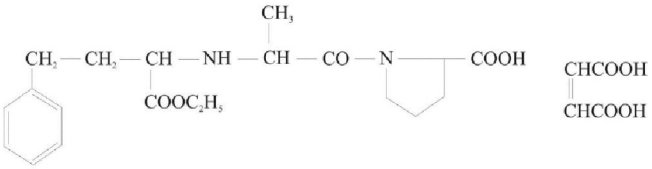

Figure 1 : Structural formula of enalapril maleate

It is reported that enalapril degrades to two major degradation products:diacid enalaprilat, formed by hydrolysisand diketopiperazine, formed by dehydration and cyclization[3-9]. In the solution, the rate and the pathway of degradation of enalapril maleate depend of pH. Below pH 5, the major degradation product isDKP, however, at pH 5 or above, the majordegradation product isET [5]. The structural formulas for these two products areshown below.

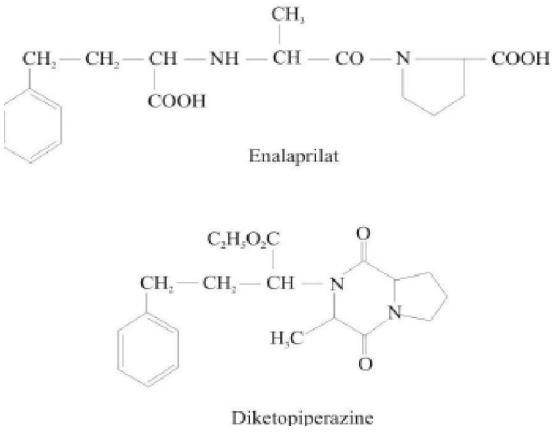

Figure 2 : Structural formulas of enalaprilat (a) and enalapril diketopiperazine (b)

The chromatographic procedure is carried out as per method described in British pharmacopoeia[10]. The major requirements for system suitability are the resolution factor between the peaks due to ET and DKP to be at least 3.0 and the resolution factor between the peaks due to DKP and enalapril to be at least 2.0. In addition, adjustment the ratio of the components of the mobile phase is suggested to attain system suitability. These column conditions are extreme regarding to pH and temperature, as the most silica columns are not recommend to be used at temperature above 60°C and at pH below 2.0.

The aim of this work was to investigate the possibility of optimization ofan HPLC method for the determination of related compounds in Enalapril maleate tablets, according to BP. AlthoughHypersil ODS column is recommend, columns with different C18 stationary phases were tested out. In addition,full factorial design, as an efficient statistical method of indicating the relative significance of a number of variables(pH, acetonitrile fraction, flow rate and temperature) and their interactionswas employed in this study.

EXPERIMENTAL

Equipment

Method optimization and validation were carried out on a Agilent HPLC system HP 1200 series equipped with binary pump G1312A, vacuum degasser G1379B, autosampler G1329A, thermostated column compartment G1316A and UV detector G1315D, from Agilent Technologies (Waldbronn, Germany). Data acquisition, data collection and system control provided Chemstation software revision B.03.01-SR1 from the same company.

Hypersil ODS, 5 µm, 250 x 4 mm column purchased from Agilent(New Castle, Delaware, USA)was used for optimization and validation.

The other columns, with the same dimension and particle size, but different stationary phases (TABLE I) were used for optimization.

Other used apparatuses were analytical balance Sartorius MC 1 with precision 0.1 mg (Sartorius AG, Göttingen, Germany), and centrifuge SIGMA 2-**5** (Sartorius AG, Göttingen, Germany).

For experimental design Design-Expert software,

version 7.1.6., Stat-Ease, Inc.(Minneapolis, USA) was used.

Reagents and solutions

HPLC-grade acetonitrile,orthophosphoric acidand potassium dihydrogen phosphatewere purchased from Merck KgaA (Darmstadt, Germany), while the water HPLC-grade wasobtained bySartorius Stedim Biotechwater system (Sartorius AG, Göttingen, Germany).

As standard, in-house working standard of Enalapril maleate provided by Zheijiang Huahai Pharmaceuticals, China, was used, standardized against Enalapril maleateUSP reference standard. Maleic acid USP reference standard, Enalaprilat LGC reference standard and Enalapril diketopiperazine LGC reference standard were used for standard solution preparation. Enalapril 10 mg tablets manufactured by Zdravlje, Actaviswere used forvalidation purposes. One tablet weighted 150 mg and contained 10 mg of enalapril. Solutions 1-5 and solutions A, B and C were prepared according to BP. Exception of this was solution A. In optimization and validation work, pH of this solution was adjusted on 1.8, 2.0 or 2.2.

Enalapril maleate stock solution was prepared as follows: 10.0 mg of enalapril maleate working standard was dissolved in solution A and diluted to 100 ml with the same solvent, c=0.10 mgml^{-1}of enalapril maleate.

Enalapril maleate standard solution was prepared by diluting one volume of stock solution to 50 volumes with solution A, c=2.0 µg ml-1 of enalapril maleate.

For selectivity test, except the solutions described above, the following solutions were prepared:

Placebo solution: 900 mg of excipients (mixture of lactose monohydrate, maize starch, sodium hydrogen carbonate, colloidal anhydrous silica, magnesium stearate and colorants) was shaking with 50 ml of Solution A for 15 minutes and centrifugated. The clear supernatant liquid was filtered through a 0.45 µm membrane filter.

Maleic acid stock solution was prepared as follows: 10.0 mg of maleic acid CRS was dissolved in solution A and diluted to 10 ml with the same solvent, c=1.0 mg ml^{-1} of maleic acid.

Six linearity solutions were prepared by mixing of adequate volumes of Solution B, Solution C and enalapril maleate stock solution and diluting with solution A.

A set of dilute solutions were prepared for determination of limit of detection (LOD) and limit of quantitation (LOQ). After determination LOD and LOQ, six solutions with LOQ concentration were prepared in purpose to check recovery and precision at low concentrations.

RESULTS AND DISCUSSION

Method optimization

The columns were tested out by using the experimental conditions described in BP. Only solution for system suitability (solution 5 in BP) was injected. Except BP requirements for system suitability, resolution between ET peak and maleic acid peak was considered. Results are summarized in TABLE 1.

TABLE 1: Resolution factors at different columns

no	column	Rt (1) Maleic acid	Rt (2) ET	Rt (3) DKP	Rt (4) Enalapril	Resol. (1) - (2)	Resol. (2) - (3)	Resol. (3) - (4)
1	Hypersil ODS	1.64	4.61	6.25	18.11	11.56	6.65	7.21
2	Zorbax ODS	1.97	5.94	11.03	29.45	3.36	5.15	3.75
3	Lichrospher100 RP18	2.33	3.67	13.33	8.65	4.46	6.04[a]	8.08
4	Purospher STAR RP18e	2.01	2.11	9.49	2.74	not resolved	3.08[a]	17.66
5	Zorbax extend C18	2.21	2.21	8.13	2.83	not resolved	2.23[a]	22.89
6	Nucleosil C18	2.54	3.07	10.32	4.51	not resolved	4.25[a]	16.67
7	Luna C18(2)	2.71	2.71	11.39	3.36	not resolved	1.52[a]	8.15
8	Kromasil C18	2.18	2.44	14.11	3.52	not resolved	3.07[a]	28.45
9	Inertsil ODS-3	2.73	2.71	17.75	3.70	not resolved	3.11[a]	31.92

a **Resolution (2) - (4), considering different order of eluation**

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It can be seen that the best results were achieved by recommended ODS Hypersil column.

Critical chromatographic parameters such as the pH of the aqueous phase in the mobile phase, percentage of acetonitrile fraction, flow rate of the mobile phase and column temperature were deliberately varied to test their impact onmethod. Characteristics of chromatogram such as retention time, resolution factor, USP tailing and capacity factorwere used to see the influence of changes mention above. The experimental design and results are shown in TABLE 2.

The number of experiments required for the study depends on the number of independent variables. The responses are measured for each trial and then interactive ($Y=b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 \ldots$) model is fitted by carrying out multiple regression analysis and F-statistics to identify statistically significant terms[11].

TABLE 2 : 2^4full factorial design layout

Run	Factors				Responses								
	A, pH	B, AcCN %	C, Flow ml/min	D, Temp.°C	Rt, ET	Rt DKP	Resol. ET-DKP	Resol. DKP-Enalapril	USP tail. ET	USP tail. DKP	k′ ET	k′ DKP	Rt Enalapril
7	1.8	35.0	0.8	50.0	6.102	12.342	14.07	4.63	1.43	1.05	1.59	4.24	22.34
26	1.8	35.0	0.8	50.0	6.135	12.369	13.74	4.67	1.40	1.05	1.60	4.25	22.64
6	1.8	45.0	0.8	50.0	5.707	6.287	2.46	6.85	1.50	1.03	1.42	1.67	15.31
4	1.8	45.0	0.8	50.0	5.727	6.291	2.40	6.89	1.50	1.02	1.43	1.67	15.40
22	1.8	35.0	1.2	50.0	3.982	8.031	13.51	4.71	1.35	0.99	1.54	4.11	15.26
3	1.8	35.0	1.2	50.0	4.119	8.282	11.93	4.68	1.62	1.05	1.62	4.27	15.61
29	1.8	45.0	1.2	50.0	3.765	4.095	1.48	6.86	1.18	0.98	1.40	1.61	10.20
9	1.8	45.0	1.2	50.0	3.818	4.210	2.10	6.80	1.69	1.02	1.43	1.68	10.45
25	1.8	35.0	0.8	70.0	5.920	11.637	14.50	4.92	1.38	1.09	1.51	3.94	20.95
16	1.8	35.0	0.8	70.0	5.687	11.211	17.81	5.04	1.69	1.06	1.41	3.76	20.70
32	1.8	45.0	0.8	70.0	5.592	6.060	1.63	7.30	1.31	1.05	1.37	1.57	14.52
8	1.8	45.0	0.8	70.0	5.382	5.873	1.80	7.47	1.36	1.03	1.28	1.49	14.27
10	1.8	35.0	1.2	70.0	3.950	7.747	12.28	4.92	1.52	1.11	1.52	3.93	14.24
30	1.8	35.0	1.2	70.0	3.800	7.443	16.68	5.05	1.55	1.06	1.42	3.74	13.93
15	1.8	45.0	1.2	70.0	3.728	4.039	1.37	7.19	1.17	0.97	1.37	1.57	9.78
24	1.8	45.0	1.2	70.0	3.590	3.908	1.58	7.42	1.20	1.03	1.29	1.49	9.57
20	2.2	35.0	0.8	50.0	6.850	12.552	11.85	5.25	1.62	1.01	1.91	4.33	34.08
14	2.2	35.0	0.8	50.0	6.859	12.574	11.88	5.25	1.60	1.01	1.91	4.34	34.13
21	2.2	45.0	0.8	50.0	5.570	6.255	4.25	7.20	1.78	1.02	1.36	1.66	25.84
23	2.2	45.0	0.8	50.0	5.573	6.263	4.31	7.20	1.75	1.02	1.37	1.66	25.87
2	2.2	35.0	1.2	50.0	4.366	8.136	12.92	5.15	1.48	0.96	1.78	4.18	22.45
28	2.2	35.0	1.2	50.0	4.550	8.405	10.94	5.19	1.61	1.00	1.90	4.35	22.78
5	2.2	45.0	1.2	50.0	3.662	4.071	2.59	6.78	1.26	1.00	1.33	1.59	16.77
18	2.2	45.0	1.2	50.0	3.711	4.197	3.96	7.06	1.71	0.99	1.36	1.67	17.23
17	2.2	35.0	0.8	70.0	6.560	11.721	14.79	5.50	1.49	1.04	1.78	3.97	30.30
27	2.2	35.0	0.8	70.0	6.436	11.663	16.12	5.26	1.62	1.06	1.73	3.95	29.87
19	2.2	45.0	0.8	70.0	5.596	6.052	2.24	9.33	1.35	1.07	1.38	1.57	22.75
11	2.2	45.0	0.8	70.0	5.400	5.860	2.29	9.09	1.35	1.07	1.29	1.49	22.14
1	2.2	35.0	1.2	70.0	4.357	7.806	13.62	5.43	1.44	1.06	1.77	3.97	20.17
13	2.2	35.0	1.2	70.0	4.374	7.539	13.60	5.40	1.65	1.09	1.79	3.80	20.28
31	2.2	45.0	1.2	70.0	3.720	4.049	2.15	9.11	1.39	1.07	1.37	1.58	15.15
12	2.2	45.0	1.2	70.0	3.590	3.924	2.27	8.95	1.30	1.05	1.29	1.50	14.76

The estimated effects were interpreted graphically and statistically, to determine their significance. For BP conditions, an overlay plot was created:

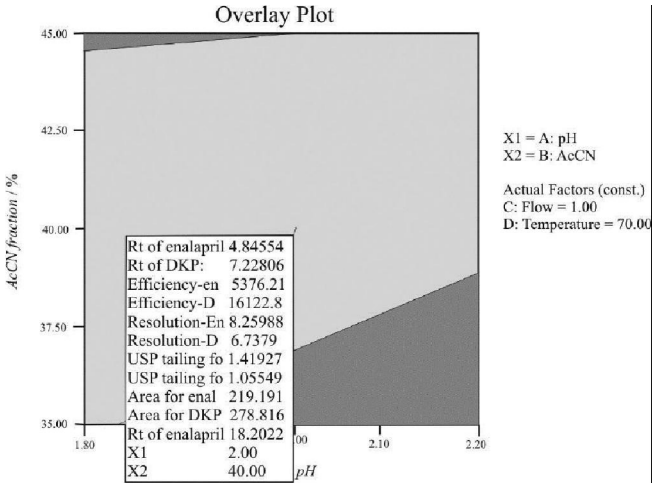

Figure 3 : Overlay plot - BP method

It can be seen that, for the proposed conditions, method is very robust.

The half-normal probability plot was used as a graphical tool to assess significance of the effects. In this plot, the non-significant effects are found on a straight line through zero, while the significant deviate from this line. An example of graphical method is given on Figure 4, showing pH, acetonitrile fraction, temperature, and interaction between pH, acetonitrile fractionan dtemperature as statistically the most significant effectson resolution between peaksof DKPand enalapril.

Interactive statistical first - order complete model was generated to evaluate DKP peak area. Final

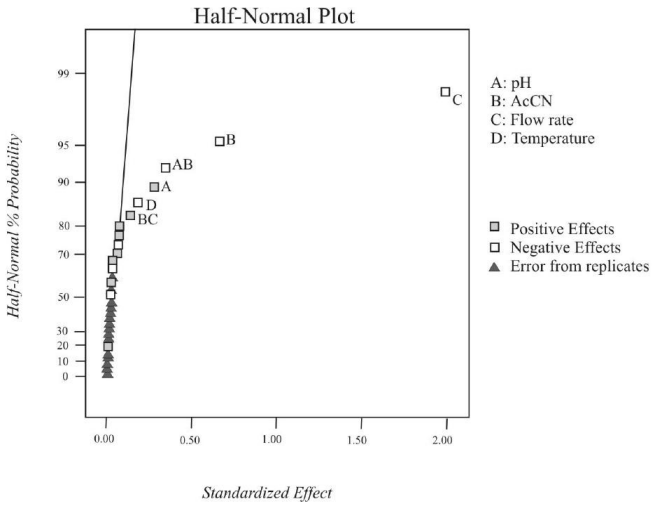

Figure 4 : Half normal probability plot – statistically significant effectson DKP peak area

equation is given in terms of actual factors:

$$1.14 \times A \times B - 0.75 \times A \times D - 0.04 \times B \times D + 0.02 \times A \times B \times D \quad (1)$$

Equations for other responses were also generated. Some examples are given below.

$$Resolution\ ET\ and\ DKP = 56.11 - 22.44 \times A - 1.25 \times B + 0.70 \times D + 0.56 \times A \times B - 0.02 \times B \times D \quad (2)$$

$$Capacity\ factor\ for\ DKP = 16.58 - 0.33 \times B - 0.06 \times D + 1.29 \times B \times D \quad (3)$$

$$USP\ tailing, ET = 1.54 + 0.24 \times A + 0.07 \times B + 0.05 \times D - 0.0013 \times B \times D \quad (4)$$

$$Rt\ of\ ET = 1.40 + 7.08 \times A + 0.19 \times B - 7.61 \times C - 8.80 \times D - 0.16 \times A \times B + 0.07 \times B \times C \quad (5)$$

Where are:

A - pH

B - Acetonitrile fraction / %

C - Flow rate / ml min^{-1}

D - Temperature / °C

After building, model was interpreted graphically, by drawing 2D contour plot. A 2D contour plot shows the isoresponse lines as a function of two levels of two factors. As an example, Figure 5 is graphical representation of USP tailing for peak of ET in a function of acetonitrile fraction and temperature.

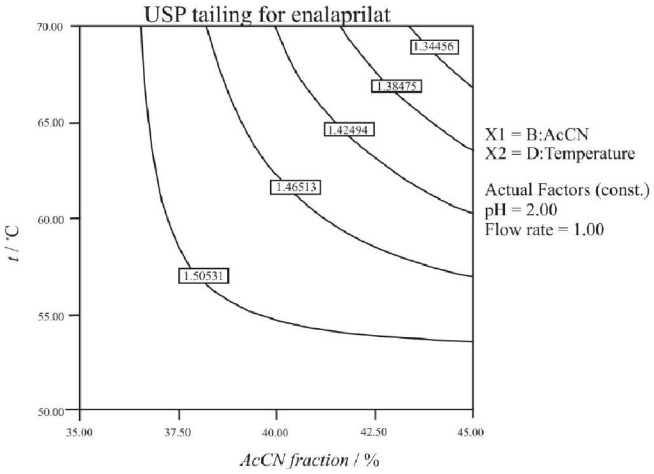

Figure 5 : 2D contour plot - number of theoretical plates

Statistically the most significant factors and interactions were obtainedby statistical analysis. This is summarized in TABLE 3.

To find optimal conditions, with respect to experimental results, it was considered that response factors should be:

- Retention time of ET:minimum, of DKP: minimum.
- Resolution between ET and DKP: in range 5 – 17.81,
- Resolution between DKP and enalapril: in range 2 – 9.33

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TABLE 3 : Statistically significant factors

Response	Individual factors	Interactions
Retention time ofET	A, B, C, D	AB, BC
Retention time ofDKP	B, C, D	BC,BD
Resolution ET and DKP	B	AB, BD
Resolution DKP and enalapril	A, B, D	AB, AD, BD, ABD
USP tailing of ET	B, D	BD
USP tailing of DKP	B,D	AB, AD
k' of ET	A, B, D	AB
k' of DKP	B, D	BD
Retention time of enalapril	A, B, C, D	AB, AC, AD, BC,BD,CD,ACD

- USP tailing of ET: in range 0.8 –2.0, of DKP: in range 0.8 – 1.5.
- k' of ET: maximize, of DKP: maximize
- Retention time of enalapril: in range 9.57 – 34.13.

From many solutions, proposed by software, two were chosen:

1 pH of the aqueous phase in the mobile phase 2.2, acetonitrile fraction 37.84% v/v, flow rate 0.98 and column temperature 50°C - by numerical optimization.

2 pH of the aqueous phase in the mobile phase 2.0, acetonitrile fraction 38.5% v/v, flow rate 1.0 and column temperature 50°C - by graphical optimization.

System suitability test was performed at suggested conditionsand at BP conditions. Chromatograms are shown in Figure 6.

Results obtained statistically and experimentally are summarizedin TABLE 4.

Relatively good agreement can be observed between statistical and experimental results. Only lower value forresolution between ET and DKP at BP conditions was obtained. One of reason for this could be column affected by temperature. Based on results above, the optimal conditions for the chromatographic procedure were determined:pH of the aqueous phase in the mobile phase 2.2, acetonitrile fraction 37.5% v/v, flow rate 1.0 and column temperature 50°C.

Method validation

The parameters to be validated for the HPLC assay were according to the ICH guidelines (International

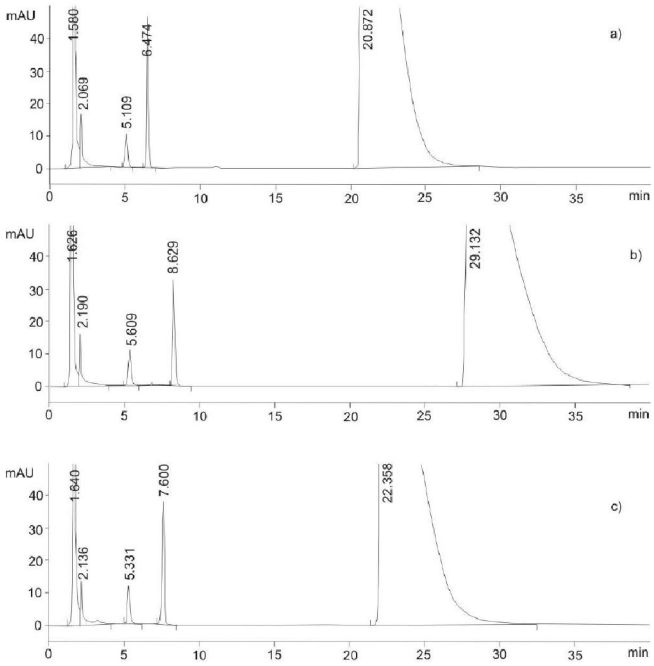

Figure 6 : Chromatograms of system suitability solution at conditions of BP (a), statistically obtained (b) and obtained through overlay plot (c)

TABLE 4 : Results at different conditions, statistically and experimentally

	Rt ET	Rt DKP	Resol. ET-DKP	Resol. DKP-Enalapril	USP tailing ET	USP tailing DKP	k' ET	k' DKP	Rt Enalapril
BP conditions Statistic	4.85	7.23	8.26	6.74	1.42	1.06	1.47	2.68	18.2
BP results Experimental	5.11	6.47	3.51	7.93	1.21	1.16	1.71	2.44	20.9
Optimal conditions 1 Statistic	5.47	9.16	9.63	5.73	1.57	0.99	1.72	3.51	27.0
Optimal conditions 1 Experimental	5.61	8.63	7.57	7.56	1.57	1.08	1.92	3.49	29.1
Optimal conditions 2 Statistic	5.12	8.61	9.21	5.65	1.52	1.01	1.61	3.34	21.3
Optimal conditions 2 Experimental	5.33	7.60	5.67	6.89	1.53	1.16	1.83	3.03	22.4

Conference on Harmonization)[12].

Selectivity

In order to determine the selectivityof the method, placebo solution, solutions1 – 5 and standard solution of maleic acid were filtrated and injected into HPLC system. Representative chromatograms are shown below (Figure 7).

In placebo solutions there were no interfering peaks observed at the expected retention time of the active ingredient and main impurities. In addition, there are no interference between maleic acid, ET, DKP and enalapril.

Linearity

The linear dependence of peak areaagainst concentration forET, DKP and enalaprilwere verified within the range 20-120% specification, which corresponds to concentrations3.0 – 18.0 μg ml^{-1} for ET, 1.0 – 6.0 for DKPand 0.6 – 3.6 μg ml^{-1} for enalapril. The best-fit lines through least squares linear regression were generated. The main components gave linear response over the tested range, and linear regression equations were obtained:

$$Y = 27.6097 \times X + 0.5827 \text{ For ET} \quad (6)$$

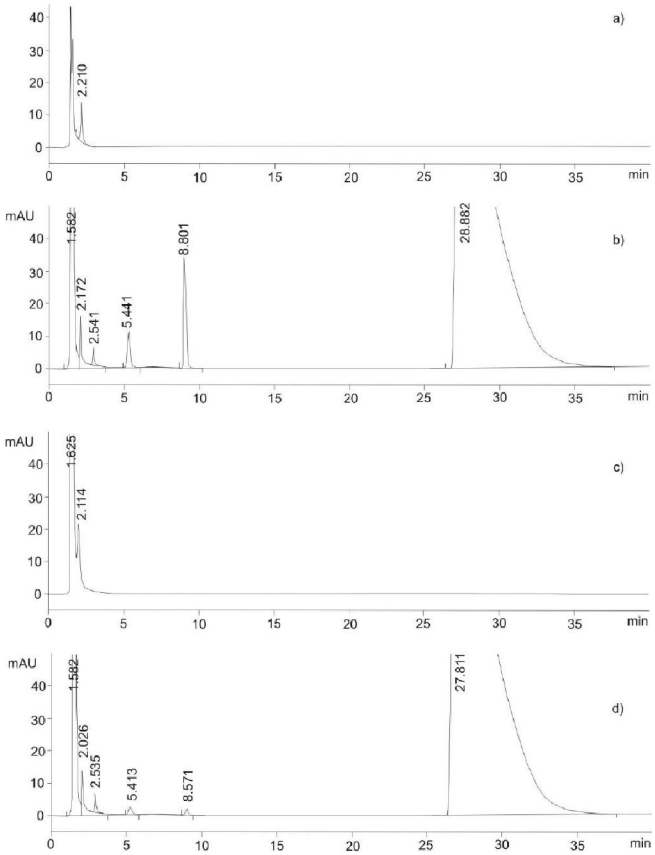

Figure 7 : Chromatograms of placebo solution(a), system suitability solution (b) maleic acid standard solution (c) and test solution (d)

TABLE 5 : Linearity Of Response For ET, DKP And Enalapril (Peak Area Residuals)

	Conc. / ug ml^{-1}	Area / mAU*s	Best line peak area	Peak area residuals	Relative peak area residuals
Enalaprilat	3.00	83.01	83.41	-0.40	-0.48
	6.00	165.38	166.24	-0.86	-0.52
	9.00	252.80	249.07	3.73	1.50
	12.00	329.90	331.90	-2.00	-0.60
	15.00	413.01	414.73	-1.72	-0.41
	18.00	498.82	497.56	1.26	0.25
Diketopiperazine	1.00	28.54	27.65	0.89	3.22
	2.00	59.15	55.68	3.47	6.23
	3.00	77.63	83.71	-6.09	-7.27
	4.00	110.97	111.75	-0.78	-0.69
	5.00	141.28	139.78	1.50	1.07
	6.00	168.82	167.81	1.01	0.60
Enalapril	0.60	11.40	11.45	-0.06	-0.50
	1.20	24.25	24.02	0.23	0.94
	1.80	37.31	36.60	0.71	1.95
	2.40	48.13	49.17	-1.04	-2.11
	3.00	61.07	61.74	-0.67	-1.09
	3.60	75.15	74.31	0.83	1.12

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$Y = 28.0336 \times X - 0.3883$ For DKP (7)

$Y = 20.9538 \times X - 1.1197$ For enalapril (8)

In TABLE 5 the raw data for the best line calculation is given as well as the calculated peak areas of the best line, the peak area residual values and the relative peak area residual values.

The correlation coefficients (r) were0.9998 for ET,0.9961 for DKPand 0.9990 for enalapril. No apparent non-linearity was observed. This indicates functional linearity between the concentration of analyte and the corresponding peak area.

Limit of detection and limit of quantitation.

TheLOD and LOQ were determined based on the S/N criteria i. e. S/N = 3 for LOD and S/N = 10 (12), and were found to be:

0.024 μgml^{-1}and 0.080μgml^{-1}, for ET

0.017 μg ml^{-1} and 0.055 μg ml^{-1}, for DKP

0.181 μg ml^{-1} and 0.603 μg ml^{-1}, for enalapril

Six LOQ solutions were tested against standard solutions. Obtained *RSD*of6,08%, 8.85 %, 6.48 % and mean recoveries of 102.90, 101.56, 93.2 for ET, DKP and enalapril, respectively, show that acceptable accuracy and precision were obtained.

Precision

In order to determine repeatability, test was performed as per method described above, on six samples. To determine the intermediate precision of the method, a second analyst performed the repeatability determination on the same batch of 10 mgEnalapril tablets on different day, using a different HPLC system (TABLE 6).

Accuracy

Accuracy was tested as recovery of ET, DKP and enalapril at levels 80, 100 and 120% of specification, with 100% content of placebo. Appropriate amount of ET, DKP and enalapril maleate were added in the form of standard solutionsto placebo and dilute with solution A to obtain adequate test samples. Accurate concentrations ofsamples were:11.76, 14.70 and 17.64 μg ml^{-1}for ET, 4.0, 5.0 and 6.0 for DKP and 1.52, 1.90 and 2.28 μg ml^{-1} for enalapril. These samples were testing as per method.

The accuracy results for ET, DKP and enalapril maleate in all samples showed good recovery and are summarized in TABLE 7.

Stability of solutions.

The stability of ET and DKP in standard during the period for 144 hours was determined. Solution B, solution C, solution 3 and solution 4were injected into HPLC system at zero point, after 24, 48, 96 and 144 hours. The areas of ET and DKP in standard solutions, at initial time point were compared to the areas of ET and DKP in same solutions at time points. Solutions were stored in autosampler vials at ambient temperature. The results show that the solutions are stable when stored in autosampler over a period of 144 h, since measured

TABLE 6 : Repeatability and intermediate precision results

Sample	ET		DKP		Impurity at RT= 3,9	
	Analyst 1, day 1	Analyst 2, day 2	Analyst 1, day 1	Analyst 2 day 2	Analyst 1, day 1	Analyst 2 day 2
1	0.0836	0.0940	0.0627	0.0660	0.0368	0.0322
2	0.0847	0.1026	0.0588	0.0735	0.0363	0.0351
3	0.0864	0.1034	0.0597	0.0742	0.0317	0.0375
4	0.0853	0.1006	0.0595	0.0734	0.0359	0.0340
5	0.0880	0.1050	0.0602	0.0743	0.0313	0.0369
6	0.0838	0.0898	0.0613	0.0645	0.0384	0.0327
Mean	0.0853	0.0992	0.0604	0.0710	0.0351	0.0347
SD	0.0017	0.0060	0.0014	0.0045	0.0029	0.0022
RSD / %	1.98	6.06	2.34	6.35	8.27	6.26
95% confid. intervals	0.0840 – 0.0867	0.0944 – 0.1040	0.0592 - 0.0615	0.0674 – 0.0746	0.0327 – 0.0374	0.0330 – 0.0365
RSD / % of 12 samples	9,10		9,73		7,02	

TABLE 7 : Accuracy results ET, DKP and enalapril

	n/a	Sample	1	2	3	Mean	SD	*RSD* / %
Enalaprilat	80% level	Determ. conc. / μg ml^{-1}	11.33	11.32	11.57	11.41	0.1411	1.24
		Recovery	96.33	96.28	98.38	97.00	1.2001	1.24
	100% level	Determ. conc. / μg ml^{-1}	14.01	14.02	13.91	13.98	0.0593	0.42
		Recovery	95.28	95.34	94.61	95.08	0.4031	0.42
	120% level	Determ. conc. / μg ml^{-1}	17.05	17.09	16.56	16.90	0.2972	1.76
		Recovery	96.66	96.86	93.85	95.79	1.6845	1.76
Diketopiperazine	80% level	Determ. conc. / μg ml^{-1}	4.00	4.01	4.01	4.01	0.0063	0.16
		Recovery	100.06	100.32	100.35	100.25	0.1580	0.16
	100% level	Determ. conc. / μg ml^{-1}	5.16	5.16	5.16	5.16	0.0025	0.05
		Recovery	103.12	103.19	103.22	103.18	0.0507	0.05
	120% level	Determ. conc. / μg ml^{-1}	6.20	6.19	6.17	6.19	0.0156	0.25
		Recovery	103.34	103.16	102.82	103.11	0.2601	0.25
Enalapril	80% level	Determ. conc. / μg ml^{-1}	1.53	1.54	1.53	1.53	0.0085	0.55
		Recovery	100.35	101.44	100.68	100.83	0.5568	0.55
	100% level	Determ. conc. / μg ml^{-1}	1.83	1.82	1.72	1.79	0.0631	3.52
		Recovery	96.55	95.68	90.42	94.22	3.3187	3.52
	120% level	Determ. conc. / μg ml^{-1}	2.20	2.23	2.25	2.23	0.0276	1.24
		Recovery	96.36	97.70	98.77	97.61	1.2088	1.24

areas in stored solutions differs by NMT 2.0% from initially measured areas.

System suitability.

System suitability acceptance criteria are set according to results obtained through optimization and validation work, and considering BP:

a) In solution 5, the resolution factor between the peaks due to ET and DKP to be at least 3.0 and the resolution factor between the peaks due to DKP and enalapril to be at least 2.0.

b) *RSD* derived from five injections of solution 5 should be less than 5.0% for ET and DKP peak areas

c) In solution 5, USP tailing should be in interval 0.8-1.5 for DKP and 0.8-2.0 for ET.

This parameter was demonstrated throughout the validation work. The results obtained in the beginning, as well as the results obtained when performing the intermediate precision by the second analyst, are shown in TABLE 8 below.

Stress testing

Stress testing showed that enalapril maleate is the most susceptible to degradation at hydrolytic conditions.

TABLE 8 : Results For System Suitability Test

Run	Resol. ET-DKP	Resol. DKP-Enalapril	USP tail. ET	USP tail. DKP	Peak area ET	Peak area DKP	Resol. ET-DKP	Resol. DKP-Enalapril	USP tail. ET	USP tail. DKP	Peak area ET	Peak area DKP
	Analyst 1						Analyst 2					
1	10.78	7.17	1.63	1.22	171.3	160.9	10.80	7.06	1.56	1.22	184.1	182.4
2	10.86	7.20	1.62	1.23	170.3	160.9	10.81	7.07	1.56	1.22	180.3	180.9
3	10.74	7.19	1.62	1.23	171.1	161.4	10.81	7.04	1.57	1.22	185.0	182.8
4	10.89	7.20	1.62	1.22	169.7	160.4	10.80	7.03	1.57	1.22	180.8	182.9
5	10.83	7.19	1.61	1.22	167.0	161.2	10.85	7.07	1.58	1.23	185.1	183.1
Mean	10.82	7.19	1.62	1.22	169.9	161.0	10.81	7.05	1.57	1.22	183.1	182.4
SD	0.060	0.012	0.007	0.005	1.733	0.378	0.021	0.018	0.008	0.004	2.331	0.887
RSD,%	0.56	0.17	0.44	0.45	1.02	0.23	0.19	0.26	0.53	0.37	1.27	0.49

Full Paper

Under alkaline conditions, the major degradation product was ET, under neutral conditions there were ET and DKP. Under acidic conditions, beside the DKP as major degradation product, ET and also two peaks at retention times 2.03 minute and 11.36 minute were observed. Examination showed that enalapril is resistance to hydrogen peroxide. Also, no decomposition was observed at dry heat at 80°C after 15 and 30 days. As an example, chromatogram obtained under acidic conditions is shown on Figgure 8.

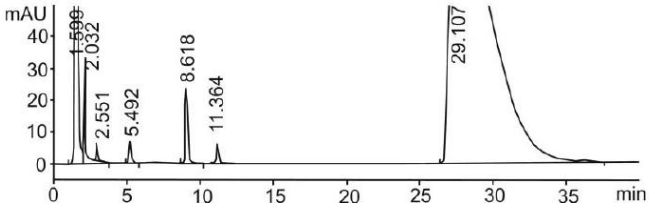

Figure 8 : Chromatogram of sample treated with 0.1M HCl

For evaluation of Photostability the solution 1 was exposing with overall illumination of 1.2 million lux hours and anultraviolet (UV) energy of 200 Wh/m2. The samples were pulling in regular intervals and tested. Slight degradation of enalapril to DKP and ET was noticed, and equations for degradation were determined:

UV light exposure:

$$C_{ET}, \% = 0.0003 \times l + 0.0929 \quad (9)$$

$$C_{DKP}, \% = 0.0009 \times l + 0.0500 \quad (10)$$

Visible light exposure:

$$C_{ET}, \% = 0.0333 \times l + 0.0933 \quad (11)$$

$$C_{DKP}, \% = 0.1179 \times l + 0.0476 \quad (12)$$

CONCLUSION

By application of experimental design, an HPLC method for the determination of related compounds in Enalapril maleate tablets, according to British Pharmacopoeia, was optimized. Small changes of four method variableslead to changes of responses of the system.

Comparing optimized to the BP method, it can be noticed that BP method has a little shorter time of analysis and better USP tailing of ET. On the other side, better resolution factors and capacity factors were achieved with the optimized method. In addition, a longer life time of column can be provided, as optimal conditions are not so extreme regarding to temperature and pH.

Validation of the HPLC method under optimal conditions provided good selectivity, sensitivity, linearity, precision and accuracy.

Stress study showed DKP and ET as main degradation products.

Testing of the samples showed that the proposed method can be successfully applied in quality and stability testing of Enalapril maleate tablets.

REFERENCES

[1] http://www.rxlist.com/vasotec-drug.htm (accessed 15 June, 2012)

[2] http://razak-labs.ir/Products/humanProducts/Cardiovascular/enalapril.aspx?lang=en-US (accessed 15 June, 2012)

[3] M.De Diego, G.Godoy, S.Mennickent, R.Godoy; Chemical stability of enalapril maleate drug substance and tablets by a stability-indicating liquid chromatographic method, Quim.Nova, **34(3)**, 450-454 **(2011)**.

[4] S.Bhardwaj, S.Singh; Study of forced degradation behavior of enalapril maleate by LC and LC-MS and development of a validated stability-indicating assay method, J.Pharm.Biomed.Anal., **46**, 113-120 **(2008)**.

[5] B.Stanisz; Evaluation of stability of enalapril maleate in solid phase, J.Pharm.Biomed.Anal., **31**, 375-380 **(2003)**.

[6] M.Al-Omari, M.Abdelah, A.Badwan, A.Jaber; Effect of the drug-matrix on the stability of enalapril maleate in tablet formulations, J.Pharm.Biomed.Anal., **25**, 893-902 **(2001)**.

[7] B.Stanisz; Kinetics of degradation of enalapril maleate in dosage forms, Acta Pol.Pharm., **61**, 415-418 **(2004)**.

[8] D.Lima, L.Dos Santos, E.Lima; Stability and in vitro release profile of enalapril maleate from different commercially available tablets: Possible therapeutic implications, J.Pharm.Biomed.Anal., **47**, 934-937 **(2008)**.

[9] R.Roskar, Z.Simoncic, A.Gartner, V.Kmetec; Stability of new potential ACE inhibitor in the aqueous solutions of different pH, J.Pharm.Biomed. Anal., **49**, 295-303 **(2009)**.

[10] British Pharmacopoeia 2011, Specific Monographs

[11] M.C.Gohel, A.F.Amin; Formulation optimization of controlled release diclofenac sodium microspheres using factorial design, J.Controlled Release, **51**, 115-122 **(1998)**.

[12] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (I. C. H.), Q2/ R1 Validation of analytical procedures. Text and methodology, **(1995)**.