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An HPLC method for the determination of digoxin in dissolution samples

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Abstract: An HPLC method for digoxin quantification in dissolution samples obtained as per the official British Pharmacopeia (BP) method is presented in this paper. The chromatography was performed at 20 °C on a Symmetry C18; 3.5 μm, 75 mm×4.6 mm column with water–acetonitrile (72:28, v/v), as the mobile phase and UV detection at 220 nm. The method was found to be selective, linear, accurate and precise in the specified ranges. The *LOD* and *LOQ* were 0.015 and 0.050 μg mL^{-1}, respectively. Robustness testing was conducted to evaluate the impact of minor changes in the chromatographic parameters (*i.e.*, acetonitrile fraction, flow rate of the mobile phase, column temperature and column length) on the characteristics of the digoxin peak. A full factorial design (2^4) was used to investigate the influence of the four variables The presented HPLC method was applied in quality and stability testing of digoxin tablets 0.25 mg.

Keywords: digoxin; tablets; dissolution testing; HPLC; validation; experimental design.

INTRODUCTION

Digoxin is a cardiac glycoside that is widely used in the treatment of congestive heart failure and certain cardiac arrhythmias. This drug is characterized by a narrow therapeutic range (typically 0.5–2.0 ng mL^{-1} in serum), wide individual variability in dosage requirements and complex metabolic pathways.[1]

As is known, dissolution testing is a routine quality control procedure in good manufacturing practice (GMP), and the dissolution data are a substantial parameter for the estimation of the bioavailability of solid oral dosage forms.

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Numerous high performance liquid chromatographic (HPLC) methods have been reported for the determination of digoxin.[2–13] There are some methods reported for digoxin determination after a dissolution test.[14–16]

In the United States Pharmacopoeia (USP), 0.1 M hydochloric acid (HCl) and in the Japanese Pharmacopoeia (JP), diluted HCl is used as the dissolution medium,[17,18] while distilled water is employed as the dissolution medium in the British Pharmacopeia (BP).[19] As some degradation could occur by acid-catalyzed hydrolysis of digoxin[16], dissolution testing with water as the dissolution medium was used in this study. Quantification of digoxin in the dissolution samples is, as per all three compendia (*i.e.*, the BP, USP and JP), based on measurement of fluorescence induced by exposing digoxin to drastic conditions (*i.e.*, ascorbic acid–methanol solution and hydrochloric acid, followed by hydrogen peroxide–methanol solution over a period of 2 h). Here, a fast and simple HPLC method is applied for digoxin determination after dissolution and filtration without any derivatization.

The Pharmacopoeias state that digoxin (Fig. 1) is practically insoluble in water. It can also be found on the web that the solubility of digoxin is 0.07 and 0.0648 mg mL^{-1}.[20,21] When the described BP dissolution test conditions are applied to 0.25 mg digoxin tablets, the resulting concentration should be about 0.42 μg mL^{-1} when the whole dose is dissolved (*i.e.*, 0.25 mg in 600 mL). This concentration can be determined by the HPLC method without any additional sample treatment, except filtration, when an aliquot of 100 μl is injected.

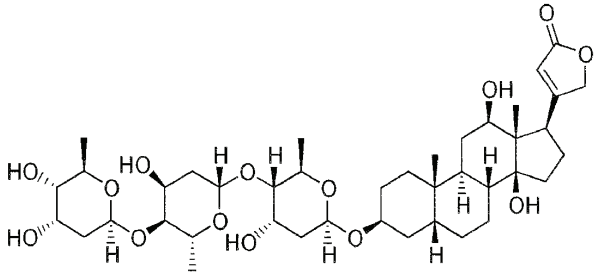

Fig. 1. Structural formula of digoxin.

A robustness test was performed to indicate factors that could affect the results. Evaluating robustness according to the International Conference on Harmonization (ICH) definition is possible by two approaches: one variable at a time or experimental design. For several reasons (too many experiments, no interactions terms, *etc.*), the univariate approach, although often performed and described in the literature, is not recommended for robustness testing.[22]. Full factorial design, as a powerful statistical tool, was employed in this study.

EXPERIMENTAL

Equipment

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

A Symmetry C18, 3.5 µm, 75 mm×4.6 mm column purchased from Waters (Wexford, Ireland) was used.

The dissolution tests were performed using an Erweka DT700LH Dissolution apparatus (USP I) (Heusenstamm, Germany). The dissolution medium was filtered through Whatman 25 mm, blue ribbon glass microfibre filters (Maidstone, UK).

Other equipment used was a Sartorius MC 1 analytical balance with a precision of 0.1 mg (Göttingen, Germany) and a Bandelin Sonorex ultrasonic bath (Berlin, Germany).

Design-Expert software, version 7.1.6, Stat-Ease Inc. (Minneapolis, USA), was used for the experimental design of the robustness test.

Reagents and solutions

HPLC-grade acetonitrile and HPLC-grade methanol were purchased from Merck KgaA (Darmstadt, Germany), while the HPLC-grade water was obtained from a Sartorius Stedim Biotech water system (Göttingen, Germany).

As the standard, an in-house working standard provided by Roche Diagnostics GmbH, Mannheim, Germany, (assay calculated on “as is” substance, 96.46 %) was used and standardised against digoxin EPCRS (assay calculated on “as is” substance, 98.50 %) as reference. Digoxin tablets (dilacor 0.25 mg tablets) manufactured by Zdravlje Actavis, Leskovac, Serbia, were used for validation purposes. One tablet weighed 120 mg and contained 0.250 mg of digoxin.

A digoxin stock solution was prepared by dissolving 21.0 mg of reference substance digoxin in 90 m of a mixture of methanol–acetonitrile–water (20:20:60, v/v) and diluted to 100.0 mL with the same solvent mixture, c = 0.21 mg mL^{-1}. A standard solution of digoxin was prepared by diluting 1 volume of stock solution to 500 volume with water, c = 0.42 µg mL^{-1}.

Sample solutions were prepared by performing the dissolution test.

For the selectivity test the following samples were prepared in 600 mL of the dissolution medium:

– Excipients (mixture of lactose monohydrate, maize starch, soluble starch, colloidal anhydrous silica and magnesium stearate), in the same amount as present in one tablet.

– Digoxin, in the same amount as present in one tablet.

– Digoxin and excipients in the same amount as present in one tablet.

The linearity stock solution was prepared by diluting 1.0 mL of digoxin stock solution to 50 mL with water, c = 4.2 µg mL^{-1}. Six linearity solutions were prepared by diluting of linearity stock solution.

A set of dilute solutions were prepared for the determination of the limit of detection (*LOD*) and limit of quantification (*LOQ*). After determination of the *LOD* and *LOQ*, one solution with the *LOD* concentration and six solutions with *LOQ* concentration were prepared by diluting the standard solution.

Experimental conditions

The experimental conditions described in the BP were used for the dissolution tests. For dissolution experiments, digoxin tablets were weighed and placed in the dissolution apparatus in batches of six. Sample aliquots (*ca*. 10 mL) were withdrawn at 5, 10, 15, 20, 30, 45 and 60 min and filtered through Whatman 25 mm, blue ribbon filters, whereby the first portion of the filtrate was discarded. No additional pretreatment was required prior to HPLC analysis.

HPLC Chromatography was performed with a column temperature of 20 °C and the mobile phase (mixture of water and acetonitrile, 72:28 % v/v) was pumped at a flow rate of 0.8 mL min^{-1}.[2] The run time cycle was completed in 10 min, while for the robustness test, it was set on 20 min. Peak areas registered at 220 nm were used for digoxin quantification.

RESULTS AND DISCUSSION

Method validation

The parameters to be validated for the HPLC assay were according to the ICH guidelines.[24]

System suitability. The USP states the tailing should be in the interval 0.8––1.5; the *RSD* derived from six injections of standard solution should be less than 2.0 % and number of theoretical plates per column for the digoxin peak should be not less than 2000. 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 a second analyst are given in Table I.

TABLE I. Results for system suitability test

Run	Analyst 1			Analyst 2		
	Area of digoxin peak	USP Tailing	Theoretical plates	Area of digoxin peak	USP Tailing	Theoretical plates
1	52.6	1.028	6952	46.1	1.073	6877
2	52.4	1.054	6861	45.9	1.084	6838
3	52.2	1.064	7069	45.1	1.092	6836
4	54.1	1.101	6867	44.9	1.100	6748
5	54.5	1.032	6730	44.6	1.104	6659
6	52.7	1.021	6883	43.8	1.112	6671
Mean	53.08	1.05	6894	45.1	1.094	6772
SD	0.97	0.03	112.09	0.85	0.014	92.76
RSD / %	1.82	2.84	1.63	1.89	1.296	1.37

Selectivity. In order to determine the selectivity of the dissolution medium, placebo solutions, sample and standard solutions were filtrated and injected into HPLC system. Representative chromatograms are shown in Fig. 2.

In the placebo solutions, no interfering peaks were observed at the expected retention time of the active ingredient.

Linearity. The linearity of digoxin (area of peak against concentration) was verified within the range 20–120 % of the reference solution, which corresponds to concentrations 0.084–0.504 μg mL^{-1} of digoxin. The best-fit line through un-

weighted least squares linear regression was generated. Digoxin gave a linear response over the tested range and the linear regression equation was obtained:

$$y = 129.69x / 0.0867 \quad (1)$$

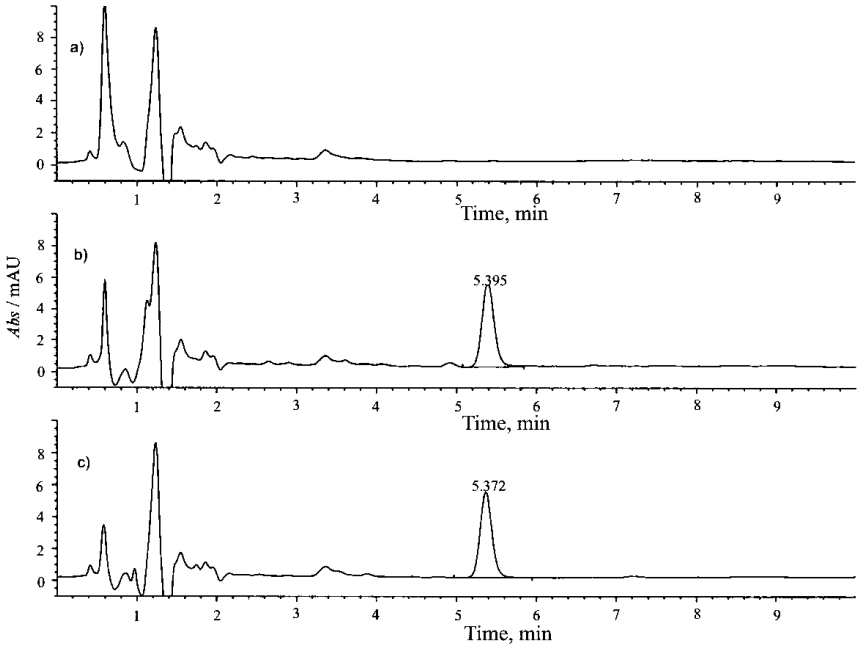

Fig. 2. Chromatograms of placebo solution (a), standard solution (b) and sample solution (c) under the described conditions.

The raw data for the best line calculation is given in Table II as well as the calculated peak areas of the best line, the peak area residual values and the relative peak area residual values.

TABLE II. Linearity of the response for digoxin (peak area residuals)

c / μg mL^{-1}	Area, mAU s	Best line peak area	Peak area residuals	Relative peak area residuals
0.084	12.1	10.98	1.12	10.19
0.168	21.2	21.88	–0.68	–3.09
0.252	32.3	32.77	–0.47	–1.43
0.336	43.3	43.66	–0.36	–0.83
0.420	53.8	54.56	–0.76	–1.39
0.504	66.6	65.45	1.15	1.75

The correlation coefficient (r) was 0.999. No apparent non-linearity was observed. This indicates functional linearity between the concentration of analyte and the digoxin peak area.

Limit of detection and limit of quantification. The *LOD* and *LOQ* values were determined based on the *S*/*N* criteria and were found to be 0.015 μg mL^{-1} (*S*/*N* = 3) and 0.050 μg mL^{-1} (*S*/*N* = 10), respectively. The *LOQ* solutions were tested against the standard solution (c = 0.42 μg mL^{-1}). The *RSD* value of 8.9 % and a mean recovery of 103.4% show that acceptable accuracy and precision were obtained.

Precision. In order to determine the repeatability, the dissolution test was performed as previously described. To determine the intermediate precision of the method, a second analyst performed the repeatability determination on the same batches of 0.25 mg digoxin tablets on a different day, using a different dissolution apparatus and a different HPLC system. The results of the precision testing are given in Table III.

TABLE III. Repeatability and intermediate precision results for digoxin

Sample	Digoxin dissolved, %	
	Analyst 1, day 1	Analyst 2, day 2
1	100.07	101.63
2	101.96	99.66
3	98.75	98.76
4	104.42	98.40
5	101.20	98.04
6	106.69	104.50
Mean	102.18	100.17
SD	2.92	2.48
RSD / %	2.86	2.48
RSD / % of 12 samples	2.76	
95 % confidence intervals	99.85–104.52	98.18–102.15

Accuracy. The accuracy was tested as the recovery of digoxin at levels 80, 100 and 120 % of the declared content of digoxin, with 100 % content of placebo. Placebo (120 mg) and the appropriate amount of digoxin, *i.e.*, 0.200, 0.250 or 0.300 mg of digoxin, were added in the form of a solution of concentration 0.10 mg mL^{-1} (*i.e.*, 2.0, 2.5 or 3.0 mL, respectively) into dissolution vessels filled with 600 mL of dissolution medium (three replicates for each concentration level) and the dissolution testing was performed as per the method.

The accuracy results for digoxin in all samples showed good recovery and are summarized in Table IV.

Stability of solution. The stability of digoxin in the test solution and the stability of digoxin in the standard solution during a period of 48 h were determined. Three test solutions representing 100 % of the 0.25 mg digoxin tablets were used to determine the stability of the test solutions. The initial concentration of digoxin was compared to the concentration of digoxin in same solutions after 24 and 48 h. The test solutions and standard solution were stored in autosampler

vials at ambient temperature. The results show that the solutions were stable when stored in autosampler for a period of 48 h, since the measured content of digoxin in the stored solutions differed by not more than 2.0 % from the initially measured content.

TABLE IV. Accuracy results for digoxin (accurate concentrations of digoxin were 0.336, 0.420 and 0.504 μg mL^{-1}, respectively)

Sample	Determined concentration μg mL^{-1}	Recovery	Determined concentration μg mL^{-1}	Recovery	Determined concentration μg mL^{-1}	Recovery
	80 % level		100 % level		120 % level	
1	0.327	97.32	0.415	98.81	0.509	100.99
2	0.321	95.54	0.419	99.76	0.499	99.01
3	0.321	95.54	0.422	100.48	0.491	97.42
Mean	0.323	96.13	0.419	99.68	0.500	99.14
SD	0.003	1.031	0.004	0.836	0.009	1.789
RSD / %	1.072	1.072	0.839	0.839	1.805	1.805
95 % Confidence interval	0.319–0.327	94.96–97.30	0.415–0.423	98.74––100.6	0.489–0.510	97.12––101.2

Robustness. Critical chromatographic parameters, such as the percentage of acetonitrile in the mobile phase, flowrate of the mobile phase, column temperature and column length, were deliberately varied to test their impact on the digoxin determination. The characteristics of the digoxin peak, such as peak area, retention time, USP tailing, symmetry, number of theoretical plates per column (half-width method) and capacity factor, were used to observe the influence of these changes in the method. The experimental results of the robustness study are summarized in Table V.

The number of experiments required for a study depends on the number of independent variables. The responses are measured for each trial and then the interactive model ($Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + \cdots$) is fitted by performing multiple regression analysis and *F*-statistics to identify the statistically significant terms.[25]

The estimated effects were graphically and statistically interpreted to determine their significance. 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 the graphical method is given in Fig. 3, which shows that changing the flow rate has statistically the most significant effect on the area of the digoxin peak.

The interactive statistical first-order model was generated completely to evaluate the digoxin peak area. The final equation was given in terms of the actual factors:

TABLE V. Robustness – 2^4 full factorial design layout

No	Variable level in actual form				Responses					
	Acetonitrile fraction, %	*t* °C	Flow rate mL min^{-1}	Column length mm	Area mAU s	Retention time min	USP Tailing	Symmetry	Theoretic plates per column	*k*'
1	25.0	18.0	0.6	75.0	69.6	12.0	1.11	0.87	8317	8.64
2	25.0	22.0	0.6	75.0	67.8	12.9	1.10	0.87	8672	9.37
3	25.0	18.0	1.0	75.0	44.1	7.5	1.07	0.91	6439	9.06
4	25.0	22.0	1.0	75.0	41.6	8.1	1.09	0.90	7009	9.83
5	31.0	18.0	0.6	75.0	69.2	6.2	1.19	0.86	15612	4.00
6	31.0	22.0	0.6	75.0	78.5	4.4	1.21	0.82	5946	2.55
7	31.0	18.0	1.0	75.0	48.2	2.6	1.21	0.81	4660	2.49
8	31.0	22.0	1.0	75.0	46.7	2.7	1.06	0.83	4831	2.72
9	25.0	18.0	0.6	100.0	72.5	17.5	0.98	1.02	11297	9.51
10	25.0	22.0	0.6	100.0	69.7	18.7	0.95	1.01	11920	10.26
11	25.0	18.0	1.0	100.0	44.3	9.4	1.00	1.00	9086	8.48
12	25.0	22.0	1.0	100.0	43.7	10.2	1.00	0.97	9424	9.20
13	31.0	18.0	0.6	100.0	70.6	4.8	1.05	0.94	10082	1.88
14	31.0	22.0	0.6	100.0	71.9	4.9	1.06	0.94	9907	1.99
15	31.0	18.0	1.0	100.0	44.4	2.9	1.07	0.92	6742	1.95
16	31.0	22.0	1.0	100.0	41.1	3.1	1.05	0.94	7108	2.07

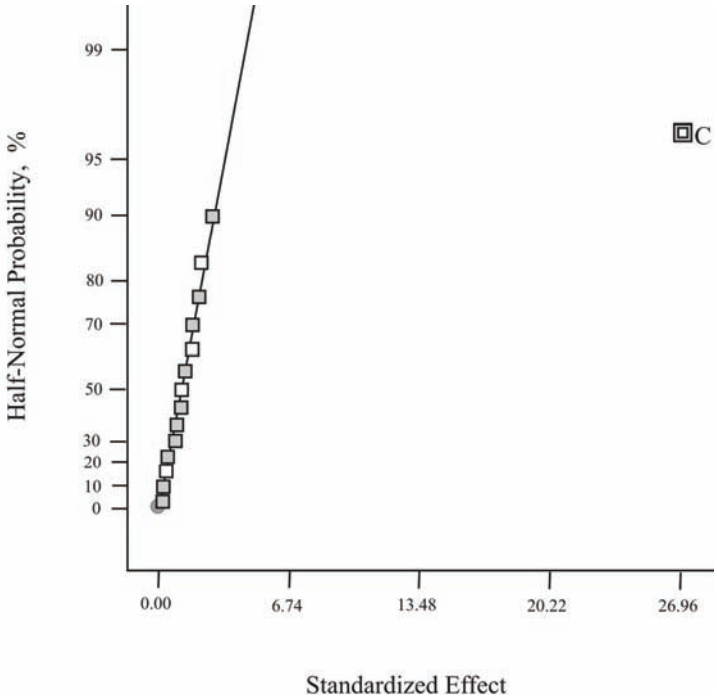

Fig. 3. Half-normal probability plot – effect of flow rate on the digoxin peak area.

$$\text{Dioxin peak area} = 111.67 - 67.41c \quad (2)$$

Equations for other responses were also generated:

$$\text{Retention time} = -161.24 + 5.72A + 1.49B + 133.98C + 2.57D - \\ -0.05AB - 4.73AC - 0.09AD - 2.22CD + 0.07ACD \quad (3)$$

$$\text{USP Tailing} = 1.11 + 0.01A - 4.4\times10^{-3}D \quad (4)$$

$$\text{Symmetry} = 0.086 - 0.01A + 4.15\times10^{-3}D \quad (5)$$

$$\text{Theoretic plates} = -2.05\times10^{6} + 82476.83A + 94770.67B + \\ +2.01\times10^{6} + 20173.27D - 3821.04AB - 81230.83AC - \\ -808.69AD - 92704.17BC - 923.86BD - 19611.22CD + \\ +3751.04ABC + 37.36ABD + 792.12ACD + 903.75BCD - \\ -36.65ABCD \quad (6)$$

$$\text{Capacity factor} = 37.58 - 1.13A \quad (7)$$

where A is the % acetonitrile, B is the temperature (°C), C is the flow rate (mL min^{-1}) and D is the column length (mm).

From the generated equations, it can be observed:

– statistically, the most significant factors that have an influence on the USP tailing and symmetry, are the % acetonitrile and the column length;

– statistically, the most significant factor which has an influence on the capacity factor is the % acetonitrile;

– statistically, all factors have a significant influence on the number of theoretical plates (N) and the retention time.

After construction, model was interpreted graphically by drawing 2D contour plots. A 2D contour plot shows the iso-response lines as a function of two levels of two factors. As an example, the graphical representation of the dependence of the number of theoretical plates on the % acetonitrile and temperature is shown in Fig. 4.

Finally, the robustness of the method can be seen in Fig. 5.

For the construction of the overlay plot, it was taken that the response factors were peak area above 50; retention time less than 10 min; symmetry, min 0.85; USP tailing, 0.8–1.2; number of theoretical plates (N) more than 2000 and capacity factor, 2–10.

In addition, the temperature and column length were chosen to be constant.

From the figure, it can be seen that the chosen experimental conditions are in the middle of the graph.

Dissolution profile. Method described above was used to analyse commercial batches of digoxin tablets. As per the applied BP dissolution test method, the requirement for released digoxin is a minimum of 75 % in 60 minutes. The dissolution profile of one of the batches with error bars is shown in Fig. 6.

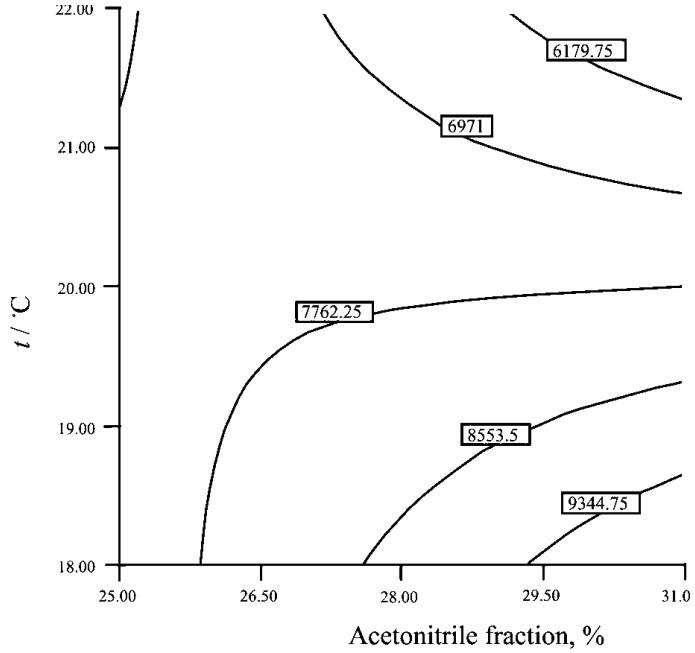

Fig. 4. 2D Contour plot – number of theoretical plates.

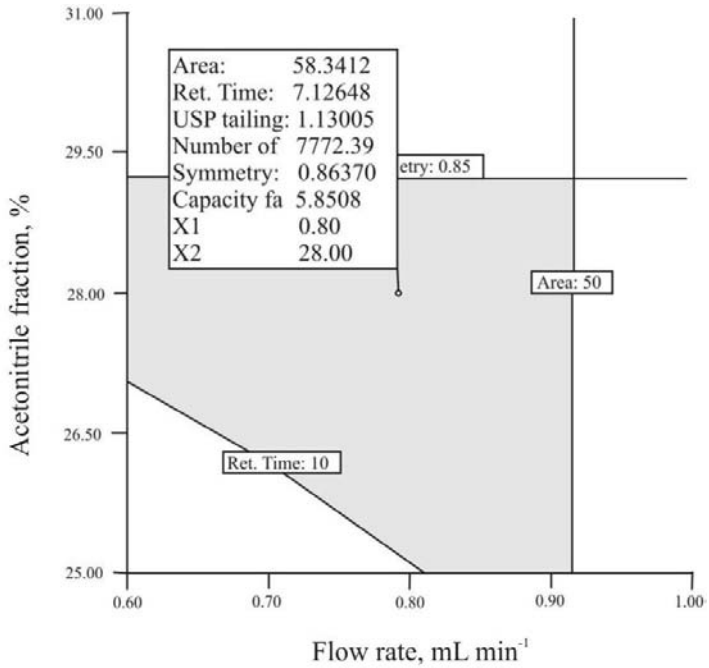

Fig. 5. Overlay plot – robustness of the method.

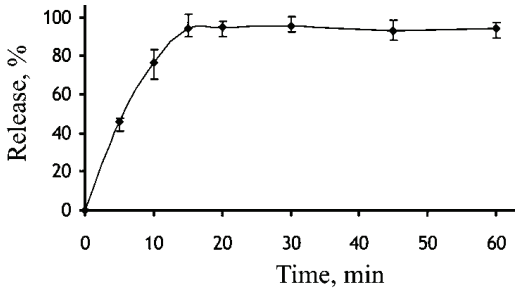

Fig. 6. Dissolution profile of digoxin.

CONCLUSIONS

In this work, an HPLC method for the evaluation of the dissolution of digoxin tablets in water is presented and validated by the modification of an existing in-house HPLC method. By this method, using the BP dissolution conditions, it is possible, simply and exactly, to determine digoxin in the dissolution medium without fluorescence detection or any additional sample pre-treatment. The acquired validation parameters indicate that this method is selective, accurate, precise and linear. In addition, by 2^4 full factorial design, it was shown that the method is robust and hence suitable for routine analyse of the dissolution of digoxin tablet.

И З В О Д

HPLC МЕТОДА ЗА ОДРЕЂИВАЊЕ ДИГОКСИНА У РАСТВОРЕНИМ УЗОРЦИМА

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У овом раду представљена је HPLC метода за одређивање дигоксина у узорцима након теста растворљивости, по Британској фармакопеји (БФ). Хроматографија је изведена на 20 °C, на Symmetry C18 колони, 3,5 µm, 75 mm ×4,6 mm, са мобилном фазом вода–ацетонитрил (72:28, v/v) и УВ детекцијом на 220 nm. Установљено је да је метода селективна, линеарна, тачна и прецизна у специфицираном опсегу. *LOD* и *LOQ* вредности износиле су 0,015 µg mL^{-1} и 0,050 µg mL^{-1}. Тест робусности је такође изведен да би показао ефекат минорних промена у хроматографским параметрима (тј. у уделу ацетонитрила, протоку мобилне фазе, температури колоне и дужини колоне) на карактеристике пика дигоксин система. Потпуни факторијелни дизајн (2^4) је употребљен да би се показао утицај четири променљиве. Представљена метода је примењена у контроли квалитета и у тестовима стабилности дигоксин таблета.

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