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RESEARCH ARTICLE

Estimation of Digoxin in Chloroform extract of *Moringa concanensis* Leaves using Newly Developed and Validated RP-HPLC Method

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Abstract

Cardiac glycosides are the secondary metabolites that are used in atrial fibrillation and atrial flutter, as they bind with the sodium-potassium ATPase pump and increase the force of contraction. There are two types of cardiac glycosides, i.e., cardenolides and bufadienolides. Digoxin is a cardenolide-type of cardiac glycoside. Digoxin is found in a chloroform extract of *Moringa concanensis* leaves. In the present study, the Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated to determine the content of digoxin in a chloroform extract of *M. concanensis* leaves from three different geographical sources. The chromatographic method was developed at 220 nm wavelength with an Acetonitrile: Water:1% Orthophosphoric acid (50:50:0.1 %V/V/V) mobile phase. In this method, the digoxin shows linearity in the range of 10-50 µg/ml with a regression coefficient of 0.995. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were found to be 0.232 µg/ml and 0.703 µg/ml, respectively.

Keywords: Chloroform Extract, *Moringa concanensis*, RP-HPLC Method, Soxhlet Extraction

1. Introduction

Cardiac glycosides are plant metabolites with a steroidal nucleus attached to sugar moiety at the C-3 position. There are two types of cardiac glycosides based on the type of lactone ring attached at the C-17 position i.e. with a 5-membered lactone ring and 6-membered lactone ring known as cardenolides and bufadienolides respectively. Digoxin is a cardenolide type of glycoside with a 5-membered lactone ring attached at the C-17 position and 3 molecules of digitoxose sugar at the C-3 position of the steroidal nucleus[1].

Digoxin is a plant-derived organic constituent that is used for heart failure, atrial fibrillation, and atrial flutter. It blocks the Sodium-Potassium ATPase pump and increases the force of contraction. It is generally sold under the brand name Lanoxin[2].

In the present study, digoxin was isolated from the chloroform extract of *M. concanensis* leaves, which was obtained by successive solvent extraction in the soxhlet apparatus. By using the newly developed RP-HPLC method, the digoxin was quantified in all three specimens collected from different geographical regions[3-11].

2. Materials and Methods

2.1 Collection, Authentication, Extraction and Phytochemical Screening

The *Moringa concanensis* leaves are collected from three different regions: Navsari district, south Gujarat region (Specimen 1), Vadodara district, central Gujarat region (Specimen 2) and Jalgaon district, north Maharashtra region (Specimen 3). All the three specimens are

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authenticated by the Aspee College of Horticulture and Forestry, Navsari Agricultural University, Gujarat, India. After authentication, the dried plant material was subjected to successive solvent extractions, starting with petroleum ether followed by chloroform. Further, the extracts are dried and the yield was calculated. The phytochemical screening of extracts was carried out and results were noted[12,13].

2.2 HPLC Method Development[14-17]

The Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) was developed to qualify as well as quantify the digoxin from chloroform extract. Trials are performed using different mobile phase compositions with different pH at 220 nm wavelengths. After dissolving 1gm of chloroform extract in 100 ml of methanol, it was injected into the chromatographic system. The system details and optimized conditions used for the HPLC analysis are listed as follows:

- HPLC system: Agilent Technology 1220 Infinity LC
- Column: Neclosil-C_{18} (125mm×4.6mm, 5µm particle size)
- The wavelength of measurement: 220 nm
- Detector: PDA (Photodiode Array) Detector
- Flow rate: 0.5 ml/min
- Mobile Phase:Acetonitrile:Water:1% Orthophosphoric Acid (50:50:0.1) (V:V:V)
- The retention time of Digoxin: 12.363 (Specimen 1), 12.380 (Specimen 2), 12.273 (Specimen 3)

2.3 Preparation of Solutions for RP – HPLC Method

2.3.1 Digoxin Stock Solution

The 100 mg of digoxin reference standard was transferred to a 100 ml volumetric flask containing methanol and sonicated for 10 min. Volume was adjusted to the mark with methanol to obtain the concentration of 1000 µg/ml digoxin

2.4 Calibration Curve for Digoxin

The 10 ml of digoxin stock solution (1000 µg/ml) was taken in a 100 ml volumetric flask and the volume was adjusted to the mark with methanol (100 µg/ml). From the above stock solution (100 µg/ml) further, 1, 2, 3, 4, and 5 ml aliquots were transferred to a 10 ml volumetric flask, and methanol was added up to the mark to obtain a concentration range of 10, 20, 30, 40, 50 µg/ml.

The above standard solution range was injected sequentially into the chromatographic system through a 10 µl loop and chromatograms were developed using a 0.5 ml/min flow rate at 220 nm wavelength. The calibration curve was plotted as the average peak area against concentration and the regression equation was calculated.

2.5 Assay of Digoxin in Chloroform Extract of all Three Specimens

In a 100 ml volumetric flask, 1g of dried chloroform extract was transferred, then methanol was added, sonicated for 10 minutes, and then diluted to the mark using the same solvent. The resulting solution was filtered. From the above solution, 1 ml was transferred into a 10 ml volumetric flask and diluted to the mark with the same solvent to obtain the solution for measurement. The same procedure was performed for all three extracts from 3 different specimens. This Test solution was injected and a chromatogram was recorded for the same. The amount of digoxin was calculated.

2.6 Validation Parameters

2.6.1 Linearity and Range

The linearity was determined in the range of 10-50 µg/ml digoxin. The above solutions were injected three times into a chromatographic system to obtain a peak area. The mean of each peak area was calculated and the plot of the mean peak area against concentration was plotted and the regression coefficient was calculated.

2.6.2 Accuracy Study

The recovery studies were performed by the addition of digoxin stock solution at 3 different concentration levels (80%, 100%, and 120%) to the sample solution, i.e., 10 µg/ml digoxin solution. The above-spiked solutions were analysed 3 times each and the results were calculated.

2.6.3 Precision

2.6.3.1 Repeatability Study

The repeatability study was performed on standard solutions of 20, 30 and 40 µg/ml digoxin. The mean peak area was measured for the same concentration solution six times and the %RSD was calculated.

2.6.3.2 Intra-day Precision

The standard stock solutions containing 20, 30, and 40 µg/ml digoxin were analysed thrice a day, and %RSD was calculated.

2.6.3.3 Inter-day Precision

The standard stock solutions containing 20, 30, and 40 µg/ml digoxin were analysed on three different days, and %RSD was calculated.

2.6.4 Limit of Detection (LOD)

The standard deviation of intercepts from the calibration curve was calculated. The limit of detection (LOD) of the drug was calculated by using the following equation:

$$LOD = 3.3 \times Intercept/Slope$$

2.6.5 Limit of Quantitation (LOQ)

The limit of quantitation is the minimum amount of components that can be practically quantified. The LOQ for digoxin was calculated using the following equation:

$$LOQ = 10 \times Intercept/Slope$$

2.6.6 Robustness

The robustness of any analytical method means the resulting data remains unchanged by making minor variations in method parameters. The following parameters are changed slightly to check the robustness of the developed method;

- the pH of the mobile phase
- Changing the mobile phase composition
- Flow rate

The effects of the changes were observed, and the %RSD was calculated.

3. Results

The % yield of the successive solvent extractions by the Soxhlet method is shown in Table 1. Phytochemical screening of different extracts was performed using conventional methods and the results are shown in Table 2. After the phytochemical screening, it is confirmed that chloroform extract shows the presence of cardiac glycosides and further confirmatory tests confirm the cardenolide type of cardiac glycosides.

Table 1. Extraction details of *M. concanensis* leaves

Sr. No.	Solvent	Colour	Consistency	% Yield w/w		
				ML01	ML02	ML03
1	Petroleum ether	Greyish green	Sticky	16%	12%	13%
2	Chloroform	Dark green	Sticky	3%	2.8%	2.6%

Table 2. Phytochemical screening of extracts

Sr. No.	Phytoconstituents	Types of Extract	
		Petroleum Ether	Chloroform
1	Carbohydrates	-	-
2	Proteins	-	-
3	Terpenoids	-	-
4	Steroids	-	+
5	Cardiac Glycosides (Kedde's test, Legal's test)	-	+ + +
6	Anthraquinone Glycosides	-	-
7	Flavanoids	-	-
8	Tannins and phenols	-	-
9	Alkaloids	-	-

The RP-HPLC method was developed for the estimation of digoxin in chloroform extract. Different solvent systems and wavelengths were tried. Figure 1 shows the structure of digoxin. Figures 2, 3 and 4 show the chromatogram of chloroform extract, showing the digoxin in all 3 specimens having an approximate purity of 83%. Figure 5 is a chromatogram of the methanol blank solvent run.

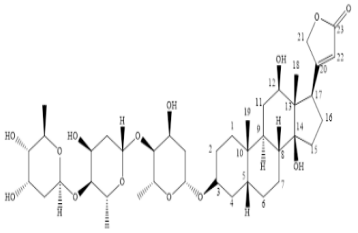

Figure 1. Structure of digoxin.

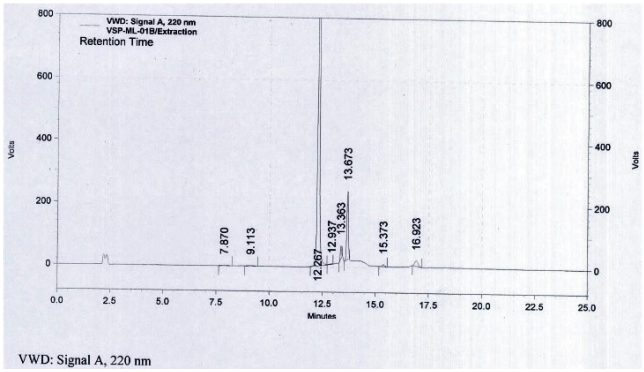

Figure 2. HPLC chromatogram of chloroform extract of *M. concanensis* leaves (Specimen 1).

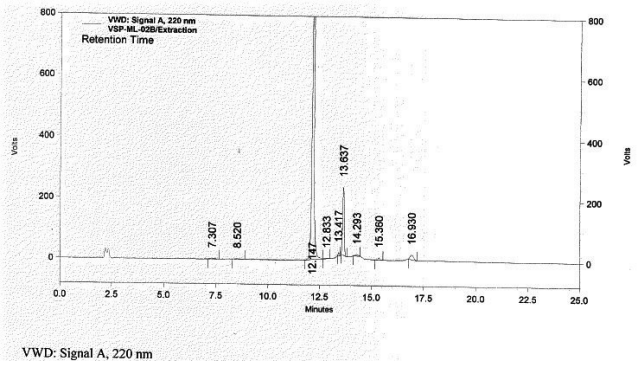

Figure 3. HPLC chromatogram of chloroform extract of *M. concanensis* leaves (Specimen 2).

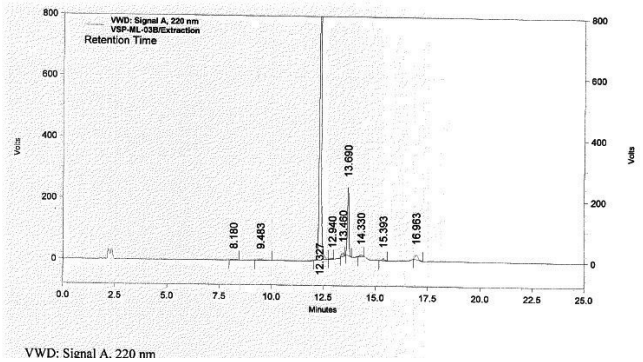

Figure 4. HPLC chromatogram of chloroform extract of *M. concanensis* leaves (Specimen 3).

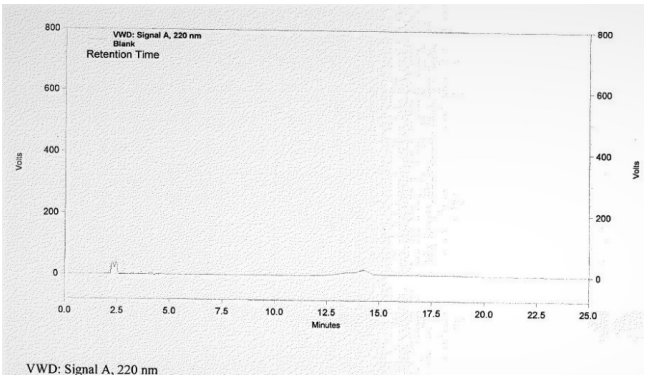

Figure 5. HPLC chromatogram of blank solvent methanol at 220 nm.

The calibration curve of standard digoxin solutions, i.e., 10-50 µg/ml was performed (Table 3), with the above-developed method showing linearity with a regression coefficient of 0.9948 (Figure 6).

The amount of digoxin present in the individual extracts of all three specimens was determined using a digoxin assay, and the % yield was calculated (Table 4).

The validation parameters are determined to show the capability and reliability of the developed method for the estimation of digoxin in the given plant extract. The different validation parameters like; linearity as previously shown the linearity between the range of 10-50 µg/ml had a regression coefficient of 0.9948 (Figure 6, Table 3), accuracy, precision, the limit of detection, the limit of quantitation and robustness and their % Relative Standard Deviation (%RSD) were calculated (Tables 5, 6, 7, 8, 9 and 10).

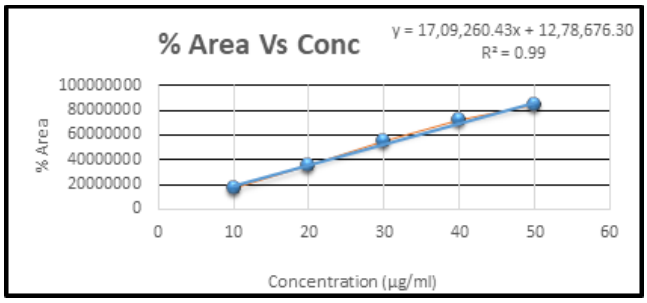

Figure 6. Plot of % area vs. concentration of digoxin.

Table 3. Calibration curve of digoxin standard solutions

(n = 5)		
Conc. (µg/ml)	Mean Area ± S.D	% RSD
10	17027819 ± 5531.43	0.032485
20	35338663 ± 8355.551	0.023644
30	54450456 ± 9156.036	0.016815
40	71624863 ± 7635.745	0.010661
50	84354312 ± 9715.109	0.011517

Table 4. Assay of digoxin in chloroform extract

Specimen	% Area	Amount of digoxin in solution (µg/ml)	Amount of digoxin in 1 gm of extract (D.F. 1000) (mg)	Amount of digoxin in total amount of extract (mg)	Amount of digoxin (mg) in 100 gm leaf powder	% yield (%W/W)
ML01	119230560	69.0075	69.0075	103.5113	207.0226	0.207 %
ML02	119526805	69.9289	69.9289	104.8934	209.7868	0.209 %
ML03	116537777	68.1802	68.1802	102.2703	204.5407	0.204 %

Table 5. Recovery study of digoxin in developed method

Target Conc., (µg/ml)	Spiked Conc., (µg/ml)	Final Conc., (µg/ml)	Conc., Obtained	% Recovery	S.D.	Average	%RSD
10	8	18	17.9482	99.7122	0.4389	99.80	0.4389
			18.05003	100.278			
			17.89446	99.4137			
10	10	20	20.0646	100.323	0.2707	100.60	0.2707
			20.12812	100.641			
			20.17235	100.862			
10	12	22	21.94714	99.7597	0.2536	99.90	0.2536
			21.99262	99.9665			
			22.05813	100.264			

Table 6. Repeatability study

Concentration (µg/ml)	% Area ± S.D. (n = 6)	% RSD
20	35377669.67 ± 103758.0003	0.293287
30	54455757 ± 60440.98273	0.110991
40	71546269.33 ± 105035.5637	0.146808

Table 7. Intra-day precision study

Concentration (µg/ml)	% Area ± S.D. (n = 3)	% RSD
20	35328151 ± 104518.6361	0.295851
30	54553226.67 ± 89902.35134	0.164797
40	71666570.67 ± 103922.2594	0.145008

Table 8. Inter-day precision study

Concentration (µg/ml)	% Area ± S.D. (n = 3)	% RSD
20	35370694.67 ± 162914.0798	0.460591
30	54465753 ± 175541.8144	0.322298
40	71644403.67 ± 175897.8847	0.245515

Table 9. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Drug	LOD (µg/ml)	LOQ (µg/ml)
Digoxin	0.232093504	0.70331365

Table 10. Robustness study

Sr. no.	Digoxin (30 µg/ml)					
	pH		Flow rate		Mobile phase	
	+ 0.2 units	-0.2 units	+0.2 units	-0.2 units	+2.0 %	-2.0 %
1	54449781	54125698	54189632	54495682	54426985	54358463
2	54598632	54386945	54231586	54598421	54584632	54600251
3	54345885	54265421	54494852	54369854	54396517	54465123
Mean	54464766	54259355	54305357	54487986	54469378	54474612
S.D	127038.1	130729.11	165443	114477.7	100968.75	121173
% R.S.D	0.233248	0.2409338	0.304653	0.210097	0.1853679	0.2224394

4. Discussion

The current study was designed to meet the requirements for the determination and estimation of digoxin using the RP-HPLC method, as well as a comparison of the variability of digoxin content in *Moringa concanensis* leaves on a geographical basis.

The successive extraction with two solvents, including petroleum ether and chloroform, was carried out in the soxhlet apparatus. Further, phytochemical studies show that cardiac glycosides are present in chloroform extract. The RP-HPLC method was developed for the chloroform extract to obtain the optimised chromatographic method having Acetonitrile:Water:1% Orthophosphoric acid (50:50:0.1 % V/V/V), mobile phase with 0.5 ml/min flow rate at 220 nm wavelength. In the concentration range of 10-50 µg/ml, the standard digoxin shows linearity with a regression coefficient of 0.9948. An assay of digoxin was done by the developed RP-HPLC method and the percentage of digoxin found in specimens 1, 2 and 3 of *Moringa concanensis* is 0.207, 0.209 and 0.204, respectively. The major criteria for the validation of any analytical method are the determined data having a % relative standard deviation (%RSD) of ≤ 2%. In the recovery studies, on three different spiked concentration levels, i.e., 80%, 100% and 120% of 10 µg/ml standard digoxin solution the % recovery is found between 99.80%-100.60% with %RSD of 0.25-0.43. The precision study involves repeatability, intra-day precision and inter-day precision study on the three different concentration ranges i.e. 20, 30 and 40 µg/ml standard digoxin concentrations and the %RSD is found between 0.110-0.460. Further, the limit of detection and quantitation is found to be 0.232 and 0.703 µg/ml respectively. The robustness of any method means the intactness of the data results after changing the conditions of the method slightly. In the present study, the conditions like flow rate, pH and mobile phase compositions are slightly changed by +0.2 and -0.2 units and the %RSD observed between 0.210-0.304.

5. Conclusion

From the present study, the conclusion drawn is that the developed RP-HPLC method is suitable for the estimation of digoxin in *Moringa concanensis* leaves. This study also shows the slight geographical variability of the digoxin

content in all three specimens collected from the 3 different regions. In the future, this study might be useful for the estimation of digoxin from other plant extracts as well as different pharmaceutical formulations.

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