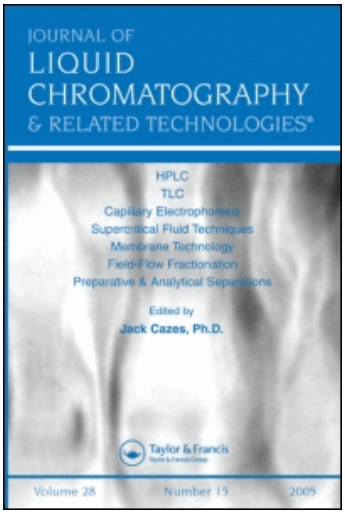

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SIMULTANEOUS DETERMINATION OF DIGOXIN AND DIGITOXIN BY DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and sensitive liquid chromatographic method has been developed for the determination of digoxin and digitoxin by derivatization. The method is based on the derivatization of digoxin and/or digitoxin with derivatizing agent, 1-naphthoyl chloride, in pyridine at 50°C. Under the mild condition, the digoxin and digitoxin were derivatized into their highly sensitive derivatives. After the derivatization reaction, a dimethylamine-acetonitrile solution was added to the reaction mixture to eliminate the interference from the excess derivatizing agent. The derivatives obtained were performed on a reversed-phase C_8 column with 88% acetonitrile as the mobile phase. The parameters affecting the derivatization of digoxin and digitoxin, including reaction temperature, reaction time, and the amount of derivatizing agent, were investigated. The linear ranges of the method for the determination of digoxin and digitoxin were over 1-50 nmol/mL and 5-50 nmol/mL; the detection limit (signal to noise ratio = 5; injection volume, 20 μL) were 0.25 nmol/mL and 0.30 nmol/mL, respectively. Partial application of the method to the analysis of digoxin in commercial tablets has proven to be satisfactory.

INTRODUCTION

Digoxin and digitoxin, obtained from *Digitalis lanata*/*Digitalis purpurea*, are cardiac glycosides prescribed to improve circulation in the patient with congestive heart failure and to slow the ventricular rate in the presence of atrial fibrillation and flutter. A rather low therapeutic plasma concentration in the range of 10-35 ng/mL is recommended for digitoxin, and yet 0.5-2.0 ng/mL is for digoxin administration. Both of the glycosides have comparably narrow safety margin and frequently cause toxic effects which can be very severe even lethal.[1] Therefore, it is quite essential to assure the potency and content uniformity of the cardiac glycosides in the pharmaceutical preparations.

Numerous approaches, including thin-layer chromatography,[2] high performance liquid chromatographic (HPLC) techniques[3-18] and immuno-assays,[19-23] have been reported for the determination of digitalis glycosides in various matrices. The immunoassays are sensitive and convenient; however, the methods would be inappropriate for determination of the cardiac glycosides in the potential presence of degradation or hydrolytic products of the glycosides or impurities.[24,25] Cross-reactions are sometimes noted with the immunoassay kits. Among the analytical methods, the HPLC technique is the most widely used one for the analysis of the cardiac glycosides in various formulations.

Digoxin and digitoxin, shown as in Figure 1, consist of the corresponding aglycone, digoxigenin for the former and digitoxigenin for the latter, with three molecules of digitoxose, joined in glycosidic linkage. For lack of a strong chromophore in the molecule, except for the conjugated system of an α,β-unsaturated lactone ring in the corresponding aglycone residue of the glycosides, direct HPLC methods for the determination of digoxin and digitoxin should be detected in a lower UV region at 218 nm. Strong background absorption from the mobile phase ensued.

The structural formula of digoxin and digitoxin, shown in Figure 1, indicates that each glycoside carries four OH groups of secondary alcohol on the sugar moieties and one OH of tertiary alcohol on the respective aglycone. An additional OH group of secondary alcohol is noted on the aglycone residue of digoxin. In order to enhance the detection sensitivity, chromophoric groups might be introduced onto the OH groups of secondary alcohol in the glycosides with a derivatizing agent under a mild reaction condition that would avoid the hydrolysis or degradation of the molecules.

This paper presents a simple and sensitive HPLC method for the simultaneous determination of digoxin and digitoxin. The method is based on the chemical derivatization of digoxin and digitoxin with 1-naphthoyl chloride. The applicability of the method to the analysis of the digoxin in commercial tablets was also examined.

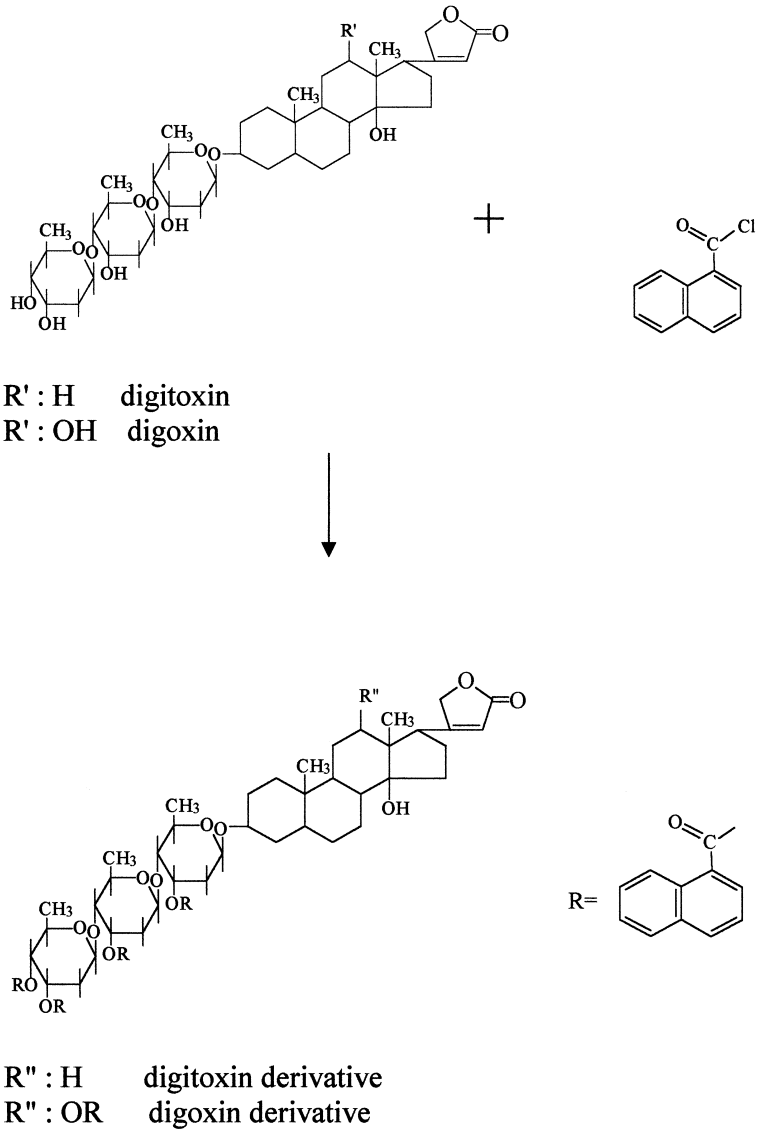

Figure 1. Scheme for the derivatization of digoxin and digitoxin with 1-naphthoyl chloride.

EXPERIMENTAL

Chemicals and Reagents

Digoxin, digitoxin, and vitamin K_1 (Sigma, St. Louis MO, USA), 1-naphthoyl chloride (TCI, Tokyo, Japan), dimethylamine and pyridine (E. Merck, Darmstadt, Germany), acetonitrile, isopropanol, and other reagents were of analytical reagent grade. Solutions of digoxin and digitoxin at various concentrations and 1-naphthoyl chloride were prepared by dissolving a suitable amount of each substance in pyridine. Solutions of vitamin K_1 (internal standard; I.S.) and dimethylamine were prepared in acetonitrile.

HPLC Conditions

A Waters-Millipore LC system with a U6K injector and a Model 486 UV-VIS detector was used. A Nova-Pak C8 column(150 × 3.9 mm I.D., 4 μm) and a mixed solvent of water-acetonitrile (12:88, v/v) at a flow-rate of 1.3 mL/min were used. The column eluate was monitored at 295 nm. The solvent was filtered with filter (Millipore, HVLP, 0.45 μm) under vacuum for degassing before use.

Mass Spectrometry

Mass spectra were obtained on a JEOL JMS-HX 100 mass spectrometer with negative fast atom bombardment (FAB) of xenon as the ionization mode and acceleration energy of 10 kV.

Optimization of the Derivatization Procedures

In order to establish the optimum conditions for digoxin and digitoxin analysis, the parameters affecting the derivatization of digoxin and digitoxin, including the amount of derivatizing agent, reaction temperature, and time, were studied. For the investigation, digoxin and digitoxin, each at a concentration of 50 nmol/mL in pyridine, was used. The effects of these parameters were evaluated by the peak-area ratio of the derivatives to vitamin K_1 (I.S.).

Optimized Derivatization Procedures

A 0.1-mL volume of digoxin or digitoxin solution was added to a 10 mL glass-stoppered test tube containing 0.1 mL 1-naphthoyl chloride solution (1.5M). The reaction mixture was shaken mechanically at 50°C in a

thermostated water bath for 1h. At the end of the reaction, a 0.1-mL containing 1 M dimethylamine and 600 μM vitamin K_1 (I.S.) acetonitrile solution was added and mixed well. A 15-μL aliquot of the solution was analyzed by HPLC with UV detection.

Sample Preparation Procedures

Twenty tablets of digoxin from a commercial source were accurately weighed and finely powdered, separately. A quantity of the powder, equivalent to about 50 μg of digoxin, was placed in a 20-mL test tube containing 3.0 mL of distilled water for maceration. A 2-mL aliquot of chloroform-isopropanol (8:1, v/v) was added to the maceration mixture and well mixed. A 0.2-mL aliquot of organic layer was pipetted into a 10-mL glass-stoppered test tube and evaporated to dryness under a slow stream of nitrogen gas. The dried residue was dissolved in 0.1 mL of pyridine and derivatized by the procedure described under the Optimized Derivatization Procedures.

RESULTS AND DISCUSSION

There are four and five OH groups of secondary alcohol in digoxin and digitoxin molecules, respectively. As nucleophiles, the reactivity of alcohols parallels their acidity, that is, in the order as follows: $CH_3OH > 1° > 2° > 3°$. To take account of both the lower reactivity of the secondary alcohol and the lability of unsaturated lactone ring and glycosidic linkage in the glycosides, an acid chloride, rather than an alkyl halide, is a more appropriate derivatizing agent in the present study. A nucleophilic substitution takes place much more readily at an acyl carbon than at saturated carbon under mild condition which can't cause hydrolysis or degradation of the glycosides. Therefore, 1-naphthoyl chloride was utilized as a derivatizing agent in the present work.

Reaction Solvent

Because of the high polarity of cardiac glycosides, the solvent systems commonly employed to solubilize them mostly contain large portions of water and alcohol (especially primary alcohols), and, in this case, those are not suitable for the derivatization. Pyridine can readily solubilize both digoxin and digitoxin, and it has no interference with the derivatization reaction between secondary alcohol and acid chloride. As a weak base ($Kb = 2.3 \times 10^{-9}$), pyridine can neutralize the hydrochloric acid that is generated during derivatization reaction; otherwise the accumulation of hydrochloric acid will bring about hydrolysis of lactone ring moiety and glycosidic linkage of the glycosides. Therefore, pyridine was chosen as the organic solvent for derivatization.

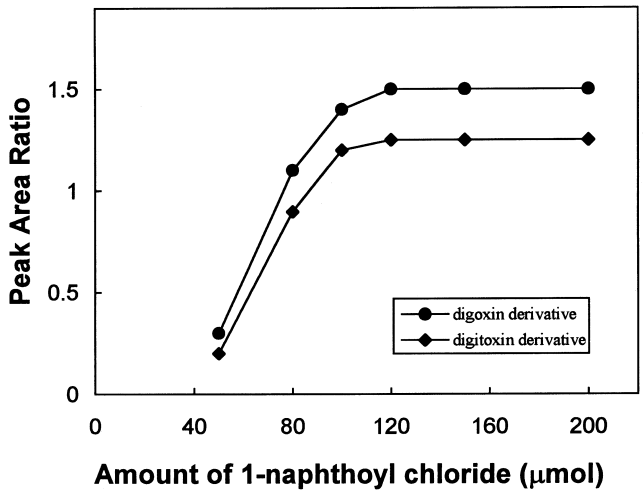

Figure 2. Effect of the amount of 1-naphthoyl chloride on the formation of the derivatives of digoxin and digitoxin.

The derivatizing agent, 1-naphthoyl chloride, was believed to react with the secondary alcohol in present study by the tetrahedral mechanism, and pyridine serves not only a reaction medium but also a nucleophilic catalyst for the derivatization reaction.[26]

Effect of Amount of Derivatizing Agent

To optimize the amount of derivatizing agent for the derivatization of 5 nmol of each of the glycosides, different amounts of 1-naphthoyl chloride over a range 50-200 μmol were examined. As shown in Figure 2, the formation of the derivatives of digoxin and digitoxin increased with the increasing amount of derivatizing agent. It's not until 120 μmol of derivatizing agent was added that the plateau formation of the derivatives of digoxin and digitoxin were attained. An excess amount of 1-naphthoyl chloride about 150 μmol was used to compensate for possible consumption of the derivatizing agent by other components of the glycoside tablet in the further applications.

Elimination of the Excess Derivatizing Agent

A large excess of derivatizing agent was used to speed up the derivatization reaction and achieve the plateau formation of the derivatives. Unfortunately, a broad tailing peak from the excess of 1-naphthoyl chloride was

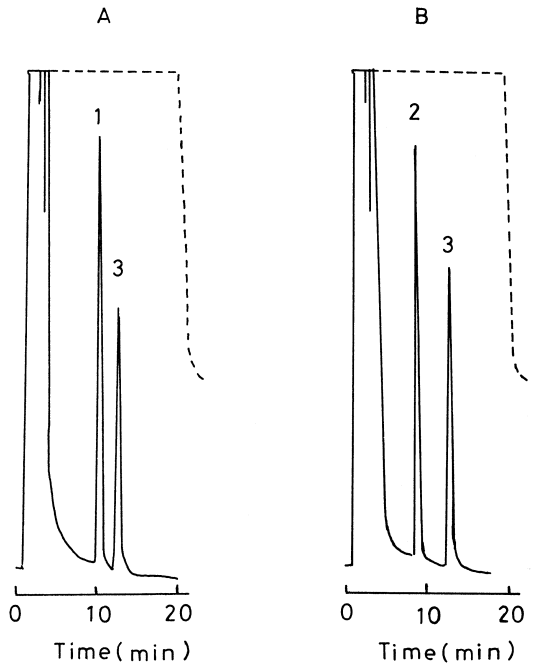

Figure 3. Composite chromatograms of digoxin and digitoxin derivatized with 1-naphthoyl chloride: (A) digoxin standard and (B) digitoxin standard, with (solid line) and without (dashed line) dimethylamine treatment, after derivatization. Peaks: 1 = the derivative of digoxin; 2 = the derivative of digitoxin and 3 = vitamin K_1 (I.S.).

observed to overlap those of the derivatives in the chromatogram and brought about the interference with the determination of the glycosides, shown as in Figure 3. Various bases, including ammonia water, potassium bicarbonate, dimethylaminopyridine, and dimethylamine were examined to eliminate the interference from the excess derivatizing agent after derivatization. Among bases mentioned above, dimethylamine was found to be the best one in this study. A more polar compound than the derivatizing agent was formed during the treatment of the reaction mixture with dimethylamine acetonitrile solution at the end of the derivatization. The resulting product of 1-naphthoyl chloride and dimethylamine, with its more polar character, was eluted completely from the reversed-phase column prior to the derivatives of digoxin and digitoxin.

Effects of Reaction Time and Reaction Temperature

The effects of reaction time at 50°C and 70°C on the derivatization of digoxin and digitoxin are shown in Figure 4. For derivatization at 50°C and

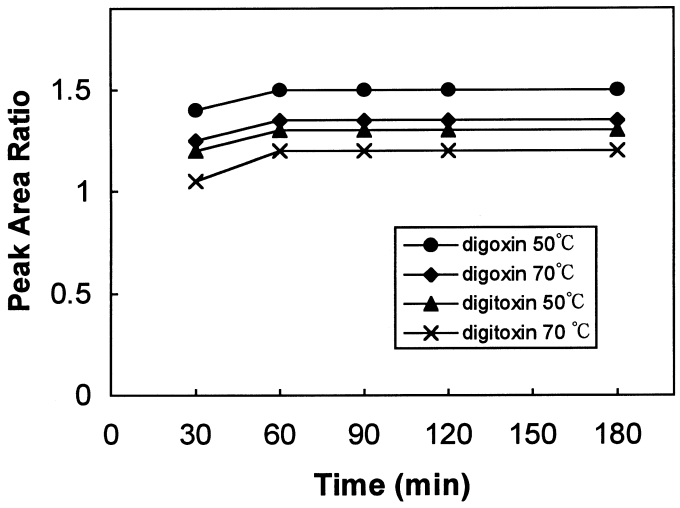

Figure 4. Effects of reaction temperature and reaction time on the formation of the derivatives of digoxin and digitoxin.

70°C, 1 h was needed to reach the plateau formation of the derivatives of both digoxin and digitoxin. The yield of the derivatives of both glycosides at 50°C was found higher than that at 70°C. This indicates that the derivatization of the glycosides with 1-naphthoyl chloride can be an exothermic reaction resembling the reaction between acyl chlorides and amines.[27] Therefore, the reaction temperature and reaction time for the derivatization of digoxin and digitoxin were set at 50°C for 1 h.

Analytical Calibration

On the basis of the optimized conditions, we formulated the analytical procedure for digoxin and digitoxin determination as described in Experimental Section. To validate the quantitative application of the method, five different concentrations of digoxin and digitoxin over the range 1-50 nmol/mL and 5-50 nmol/mL, respectively, were evaluated. The calibration graphs were established with the peak-area ratio of the derivatives to I.S. as ordinate (y) vs. the amount of cardiac glycoside in nmol as abscissa (x). The linear regression equations were obtained as follows: for digoxin assay, $y = (-0.026 \pm 0.007) + (0.032 \pm 0.008)\ x$ for intra-day ($n = 6$, $r = 0.999$) and $y = (-0.019 \pm 0.011) + (0.030 \pm 0.009)\ x$ for inter-day ($n = 8$, $r = 0.999$); for digitoxin assay, $y = (-0.015 \pm 0.002)$

Table 1

Precision and Recovery for the Analysis of Digoxin and Digitoxin

Concentration Known (nmol/mL)	Concentration Found (nmol/mL)	R.S.D. (%)	Recovery (%)
	Digoxin		
Intra-Day* (n=6)			
50	50.52 ± 0.23	0.46	101
10	9.90 ± 0.19	1.92	99
5	5.06 ± 0.06	1.19	101
Inter-Day* (n=8)			
50	49.80 ± 0.21	0.42	100
10	9.94 ± 0.13	1.31	99
5	5.05 ± 0.04	1.00	101
	Digitoxin		
Intra-Day* (n=6)			
50	50.31 ± 0.32	0.64	101
10	10.11 ± 0.15	1.49	101
5	5.07 ± 0.07	1.38	101
Inter-Day* (n=8)			
50	50.28 ± 0.26	0.52	101
10	10.14 ± 0.19	1.87	101
5	5.06 ± 0.05	0.98	101

* Intra-day data were based on six replicate analyses and inter-day data were from eight consecutive days.

+ (0.022 ± 0.003) x for intra-day (n = 6, r = 0.999) and y = (-0.013 ± 0.005) + (0.020 ± 0.002) x for inter-day (n = 8, r = 0.999). The data indicate good linearity of the proposed method. The detection limit for digoxin and digitoxin (signal to noise ratio = 5; injection volume, 20 μL) were 0.25 nmol/mL and 0.30 nmol/mL, respectively.

The reproducibility and reliability of the proposed method were assessed at three different concentrations of digoxin and digitoxin and evaluated as relative standard deviation (R.S.D.) and relative recovery, respectively. As shown in Table 1, the precision of the method for digoxin and digitoxin are all less than 2% R.S.D. for both intra-day and inter-day analyses.

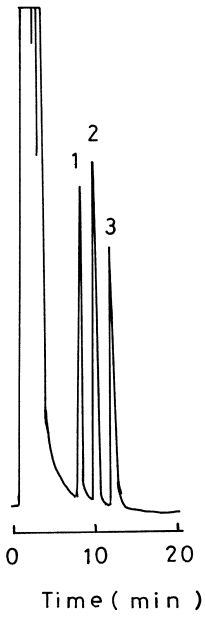

Figure 5. HPLC chromatogram for simultaneous determination of digoxin and digitoxin. Peaks: 1 = the derivative of digitoxin; 2 = the derivative of digoxin and 3 = vitamin K_1 (I.S.).

Structural Identification of the Derivatives

The typical HPLC chromatograms from determination of digoxin and digitoxin, each at 50 nmol/mL in pyridine, are presented in Figure 3(A) and 3(B). Peaks 1, 2, and 3 represent the digoxin derivative, digitoxin derivative, and the I.S., respectively. As shown in Figure 5, the derivatives of digoxin and digitoxin could be resolved from each other in this study. It indicates the proposed method can be used for the determination of digoxin and digitoxin, simultaneously. The structures of digoxin and digitoxin derivatives of peak 1 and 2 in Figure 3, were identified as naphthoyl derivatives by comparing the retention time with that of authentic sample, which were synthesized by a procedure similar to that described in the Optimized Derivatization Procedure section.

The resulting products were analyzed by negative FAB mass spectrometry. Quasi-molecular ions of the derivatives were found at m/z = 1552 $(M\text{-}H)^+$ and m/z = 1380 $(M\text{-}H)^+$ for digoxin and digitoxin, respectively. This indicates that all of the hydroxyl groups of secondary alcohol moieties of the cardiac glycosides, but not that of the tertiary alcohol moieties, were acylated.

Table 2

Analytical Results for Content Uniformity of Digoxin Tablets Obtained from Commercial Source

Capsule[a]	Amount Found[b] (μg)	% of Claimed Content[c]
1	230.1 ± 2.6	92.0
2	235.9 ± 6.3	94.4
3	227.5 ± 1.8	91.0
4	236.5 ± 5.2	94.6
5	229.1 ± 2.3	91.6
6	229.4 ± 0.4	91.8
7	226.9 ± 4.6	90.8
8	228.3 ± 7.7	91.2
9	237.8 ± 8.6	95.1
10	232.4 ± 8.9	93.0
	Mean (%):	92.6
	S.D.:	1.6

[a] Labeled amount of digoxin in each tablet is 250 μg. [b] Mean ± S.D. of three replicate analyses. [c] Content uniformity test is used to check the variation of digoxin in each tablet.

Table 3

Assay Results of Digoxin in Tablet Obtained from Commercial Source

Sample[a] No.	Amount Found[b] (μg)	% of Claimed Content
1	230.0 ± 2.9	92.0
2	235.0 ± 2.5	94.0
3	230.1 ± 2.1	92.0
4	229.3 ± 2.2	91.7
5	232.4 ± 5.9	93.0
6	232.2 ± 3.2	92.9
7	228.3 ± 3.4	91.3
8	229.7 ± 2.3	91.9

[a] Labeled amount of digoxin in each tablet is 250 μg. [b] Mean ± S.D. of three replicate analyses.

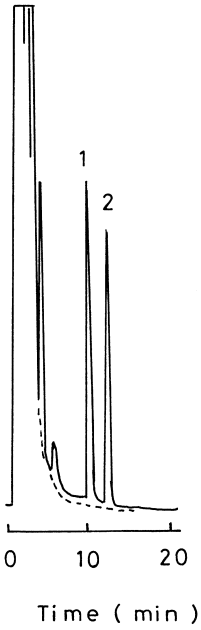

Figure 6. Composite HPLC chromatogram for the determination of digoxin in tablet (solid line) and reagent blank (dashed line). Peaks: 1 = the derivative of digoxin; 2 = vitamin K_1 (I.S.).

The stability of the derivatives of digoxin and digitoxin after derivatization were studied over a period of 10 h, no significant change of the peak area ratio was found. This indicates the favorable stability of the derivatives for digoxin and digitoxin analyses.

Application

The proposed method was applied to determination of digoxin in commercial tablets. The results of analysis of digoxin tablets are shown in Tables 2 and Table 3.

All the analytical values for digoxin tablets fell within the range of 91-94% of the labelled content, which are acceptable by Pharmacopoeia for tablet formulation. The chromatogram for analysis of digoxin in tablet is shown in Figure 6.

CONCLUSIONS

A simple and sensitive HPLC method, based on the derivatization of all secondary hydroxyl groups of digoxin and digitoxin with 1-naphthoyl chloride in pyridine, has been established and optimized. The large excess of derivatizing agent, 1-naphthoyl chloride, was removed by adding dimethylamine solution after the derivatization reaction.

Validation of the method for quantitation of digoxin and digitoxin showed that the method has excellent precision, accuracy, and reproducibility. The application of the method to commercial digoxin tablets has proven satisfactory.

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