DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR DETERMINATION OF β-ACETYLDIGOXIN

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ABSTRACT

**Objective:** The objective of the present study was to develop and validate a novel stability indicating reverse phase-high performance liquid chromatography (RP-HPLC) method for determination of β-acetyldigoxin, an active pharmaceutical ingredient (API).

**Methods:** The chromatographic separation was carried out on Agilent Technologies 1200 series HPLC system equipped with photo diode array detector and C-18 (4.6x250 mm, 5 µ) column. The mobile phase consisted of water: acetonitrile (65:35 v/v), delivered at a flow rate of 1.5 ml/min and eluents were monitored at 225 nm.

**Results:** The retention time of β-acetyldigoxin was 9.2 min. The method was found to be linear (R² = 0.9995) in the range of 3.125-500 µg/ml. The accuracy studies showed the mean percent recovery of 101.02%, LOD and LOQ were observed to be 0.289 µg/ml and 0.965 µg/ml respectively. The method was found to be robust and system suitability testing was also performed. Forced degradation analysis was carried out under acidic, alkaline, oxidative and photolytic stress conditions. Significant degradation was observed under stressed conditions, except for oxidative condition. The method was able to separate all the degradation products within runtime of 20 min and was able to determine β-acetyldigoxin unequivocally in presence of degradation products.

**Conclusion:** The novel, economic, rapid and simple method for analysis of β-acetyldigoxin is reported. The developed method is suitable for routine quality control and its determination as API, and in pharmaceutical formulations and stability study samples.

**Keywords:** β-Acetyldigoxin, HPLC, Stability indicating assay method

INTRODUCTION

β-Acetyldigoxin

β-Acetyldigoxin, 3β-[4-O-acetyl-2,6-Dideoxy-β-D-ribo-hexopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl]oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (fig. 1) [1], is a prodrug of digoxin, a well-established agent for managing heart failure and controlling rapid ventricular rate in atrial fibrillation [2, 3].

β-Acetyldigoxin-Need for novel stability indicating assay method

Stability indicating assay method is a validated analytical procedure that is able to measure unequivocally the drug substance in presence of degradation products [13, 14]. Previously reported HPLC methods for analytical determination of β-acetyldigoxin simultaneously with related cardiac glycosides, indicate its retention time at 17.3 min [15] and 23.4 min [16], respectively. Since elution is quite late, these methods aren’t suitable for routine quality control. Also, the European Pharmacopoeia HPLC assay method [1] for β-acetyldigoxin utilizes gradient elution. There are various disadvantages associated with gradient elution such as poor robustness, longer equilibration periods and complexity of method development. The column life is also shortened and method transfer is difficult [17]. No stability indicating assay method for forced degradation studies, as per regulatory guidelines, has been reported for the determination of β-acetyldigoxin.

The present report describes development and validation of a novel and rapid isocratic stability indicating assay method for determination of β-acetyldigoxin.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade β-acetyldigoxin was purchased from Clearsynth Labs, Mumbai. HPLC grade solvents (water, methanol, acetonitrile) were procured from Loba Chemie and Fischer Scientific. All chemicals were analytical grade and were used as supplied.

Instrumentation

The RP-HPLC method was developed on Agilent Technologies 1200 series HPLC system. It was equipped with Agela Technologies C-18 (4.6x250 mm, 5µ) column, binary pump, photo diode array detector, auto sampler and EnChrom Elite software. Digital temperature controlled oven (Relitech) and UV chamber (Inco) were used for forced degradation studies.

Digoxin affects cardiac rate, rhythm and the force of cardiac contraction. It increases vaga activity and slows down conduction in the atrioventricular (AV) node. Digoxin binds to the K+-binding site of Na+K+ ATPase pump and increases the force of contraction [4]. The acetyl group of β-acetyldigoxin is cleaved on passage through intestinal wall and active digoxin reaches the heart [5]. β-Acetyldigoxin is a substrate of p-glycoprotein and has a similar transport mechanism as digoxin [6]. β-Acetyldigoxin is reported to have better absorption [7], efficacy and tolerance [8], as compared to parent drug, digoxin. Like digoxin, β-acetyldigoxin also belongs to the category of narrow therapeutic index drugs [9, 10]. Digoxin and β-acetyldigoxin are two of the most important marketed cardiac glycosides [11, 12].
Preparation of stock solution
Accurately weighed β-acetyldigoxin was transferred to volumetric flask and volume was made up with methanol: acetonitrile (50:50 v/v) to obtain a 1000 µg/ml stock solution.

Optimization of chromatographic conditions
The detection wavelength for β-acetyldigoxin was selected according to European Pharmacopoeia method [1]. The chromatographic conditions were optimized to get a sharp and symmetric peak. Initial experiments, beginning with water: acetonitrile, 50:50 v/v at flow rate of 1.0 ml/min, resulted in delayed elution and a broad peak.

Mobile phase, flow rate and injection volume were optimized to get an optimum elution time and a symmetrical sharp peak. The run time should be 2.5 times the retention time of peak of analyte for optimum analysis of degradation products [13], so the run time of 20 min was selected for the study. The mode of elution was isocratic and separation was carried out at ambient temperature.

Calibration curve
The stock solution of β-acetyldigoxin (1000 µg/ml) was serially diluted with methanol: acetonitrile (50:50 v/v) to prepare solutions with concentration of 31.25, 62.5, 125, 250, 500 µg/ml respectively. Each solution was injected in triplicates to obtain the chromatograms. A calibration curve was constructed by plotting average peak area versus concentration.

Method validation and system suitability testing
The method for analytical determination of β-acetyldigoxin was validated and tested for system suitability according to International Conference on Harmonization (ICH), United States Food and Drug Administration (FDA) and United States Pharmacopeia (USP) guidelines [18-20].

Linearity
Linearity was determined by computing correlation coefficient from calibration curve, in the range of 31.25-500 µg/ml.

Precision
For interday precision studies, analysis was carried out for three consecutive days at three different concentrations, 125, 250, 500 µg/ml respectively, in triplicate. To study intraday precision, analysis was carried out at three different times in a day at three different concentrations, 125, 250, 500 µg/ml respectively, in triplicate. % RSD (relative standard deviation) of peak areas were calculated for both the studies.

Accuract
Standard addition method was employed for accuracy studies. Spiking was done at levels of 80, 100 and 120% in previously analyzed sample of 250 µg/ml to measure percent recovery by the assay of known added amounts of analyte.

Robustness
Robustness was measured by making small but deliberate changes in detection wavelength (±5 nm), flow rate (±0.1 ml/min.) and organic content in mobile phase (±2 units). % RSD of peak areas was calculated for each of the evaluations.

Limit of detection (LOD) and limit of quantification (LOQ)
The specificity of the method was determined by forced degradation studies. LOD and LOQ were determined using the formulas, LOD = 3.3×σ/S and LOQ = 10×σ/S, where, ‘σ’ is the standard deviation of the response and ‘S’ is the slope of calibration curve.

System suitability testing
System suitability was evaluated by analyzing six replicates (500 µg/ml) of β-acetyldigoxin. Suitability for intended use was tested in terms of % RSD of retention time, % RSD of peak area, tailing factor (T), asymmetry factor (A), number of theoretical plates (N) and height equivalent to theoretical plates (HETP).

Specificity-forced degradation studies
β-acetyldigoxin (1 mg/ml) was subjected to acidic stress (0.1 N HCl, 40 °C, 30 min.), alkaline stress (0.1 N NaOH, 40 °C, 30 min.), oxidative stress (30 % H2O2, RT, 24 h) and photo stress (UV, 365 nm, 96 h). The stressed samples were appropriately diluted before injection. For each stress condition, three chromatograms were recorded, namely; control (analyte not subjected to stress), blank (stressor and solvent) and stressed (analyte subjected to stress).

RESULTS AND DISCUSSION
Chromatographic conditions and calibration curve
The chromatographic conditions were optimized, after multiple trials, to get a symmetric optimum eluting peak. The average retention time for β-acetyldigoxin was 9.207±0.031 min. (n=3) (fig. 2). Thus, the developed method provides faster elution time than the previously reported methods [15, 16].

The method is isocratic and is thus simple as compared to European Pharmacopoeia method [1], and also overcomes the limitations of a gradient method [17]. The method is rapid and is thus cost effective. The method does not use buffer and therefore, various additional steps like pH adjustment [21] are eliminated.

Also, buffers are aggressive towards column packing and longer periods of column washing are needed [22].

Optimized chromatographic conditions are summed up in table 1. An injection of blank (only solvent, no analyte) was also run (fig. 3). A calibration curve was (fig. 4) plotted to compute regression equation and correlation coefficient.

| S. No. | Parameter | Condition |
|--------|-----------|-----------|
| 1      | Stationary phase | Innovonal C-18 column (4.6x250 mm, 5µ) |
| 2      | Mobile phase | Water: acetonitrile (65:35 v/v) |
| 3      | Flow rate | 1.5 ml/min |
| 4      | Detector | Photo diode array |
| 5      | Injection volume | 5 µl |
| 6      | Detection wavelength | 225 nm |
| 7      | Retention Time | 9.207 min |
| 8      | Diluent/solvent | Methanol: acetonitrile (50:50 v/v) |
| 9      | Run time | 20 min |
Fig. 2: Chromatogram for β-acetyl digoxin showing elution time of 9.207 min

Fig. 3: Blank (Methanol: Acetonitrile (50:50 v/v)) does not show any peak around elution time of β-acetyl digoxin

Fig. 4: Calibration curve for β-acetyl digoxin for concentration range from 31.25 to 500 µg/ml
Method validation and system suitability testing

The developed method was validated according to regulatory guidelines [18-20]. Results of validation and system suitability testing are summed up in table 2. The results complied with the acceptance criteria laid down under regulatory guidelines [20, 23] with % RSD values, tailing factor, asymmetry factor and height equivalent to theoretical plates being well below the acceptable limits and correlation coefficient, and number of theoretical plates were above the minimum acceptance criterion.

| Parameter | Observation | Acceptance criterion [20, 23] |
|-----------|-------------|--------------------------------|
| Regression equation | $y=1035.4x-16859$ | - |
| Correlation coefficient | 0.9995 | $\geq 0.995$ |
| Range | 31.25-500 µg/ml | - |
| Average %RSD (Peak area, n=5) (Linearity) | 0.20 | $\leq 2.0\%$ |
| Average %RSD (Peak area, n=3) (Intraday precision) | 0.13 | $\leq 2.0\%$ |
| Average %RSD (Peak area, n=3) (Interday precision) | 0.45 | $\leq 2.0\%$ |
| Average %Recovery (n=3) (Accuracy) | 101.02% | 100±3% |
| Average %RSD (n=6) Peak area (Robustness) | 0.34 | $\leq 2.0\%$ |
| Limit of detection | 0.289 µg/ml | Low |
| Limit of quantitation | 0.965 µg/ml | Low |
| Average tailing factor | 1.45 | $\leq 2.0$ |
| Average asymmetry factor | 1.63 | $\leq 2.0$ |
| %RSD (Peak area, n=6) | 0.37 | $\leq 1.0\%$ |
| %RSD (Retention time, n=6) | 0.36 | $\leq 1.0\%$ |
| Average number of theoretical plates | 8876.0 | $>2000$ |
| Average height equivalent to theoretical plates | 0.028 mm | <0.1 cm |

Forced degradation studies-specificity

β-Acetyldigoxin was subjected to various stress conditions. The results of forced degradation studies are summed up in table 3. β-Acetyldigoxin was found to be resistant to degradation under oxidative stress (percent recovery lying in 100±5% [24]). Whereas, significant degradation occurred under all other stress conditions tested. The chromatograms of stressed samples are shown in fig. 5-8.

The peak of β-acetyldigoxin was completely separated from the degradation products generated under tested stress conditions. There was no interference of any of the degradation products from the stress conditions tested in current study with the retention time of β-acetyldigoxin. Thus, the developed method for analytical determination of β-acetyldigoxin was established to be specific and stability-indicating [13].

| Stress condition | Degradation (%) |
|------------------|-----------------|
| 0.1 N HCl, 40 °C, 30 min. | 44.19 |
| 0.1 N NaOH, 40 °C, 30 min. | 56.13 |
| 30% H$_2$O$_2$, RT, 24 h | 3.0 |
| UV, 365 nm, 96 h | 36.18 |

![Fig. 5: Chromatogram for β-acetyldigoxin subject to acidic degradation](image-url)
Fig. 6: Chromatogram for β-acetyldigoxin subject to alkaline degradation

Fig. 7: Chromatogram for β-acetyldigoxin subject to oxidative degradation

Fig. 8: Chromatogram for β-acetyldigoxin subject to photo degradation
CONCLUSION

A novel RP-HPLC method for analytical determination of β-acetyldigoxin was developed. It was found to be simple, linear, accurate, precise, specific, robust, rapid and suitable for routine quality control and determination as API, and in pharmaceutical formulations and stability study samples. The peak of β-acetyldigoxin was distinguishable and quantifiable among the peaks of degradation products under tested stress conditions, thus the method is stability indicating. This method can be employed for determination of β-acetyldigoxin as API, and in pharmaceutical dosage forms and stability study samples. The rapidity and simplicity of this method overcomes limitations of previously reported methods, and make it suitable for routine quality control of β-acetyldigoxin.

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CONFLICT OF INTERESTS

Declared none

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