ABSTRACT

Objective: In the present study a novel stability-indicating high-performance thin-layer chromatography (HPTLC) method for quantitative determination of Swertiamarin (SW) in bulk drug and formulation has been developed and validated as per ICH guideline Q2 (R1) for global acceptance of standardized herbal formulations.

Methods: HPTLC method is developed and validated using solvent ethyl acetate: ethanol: chloroform (3:2.5:4 v/v/v) (R_f of SW 0.65±0.04) in the absorbance mode at 243 nm. Various forced degradation conditions were used to check degradation of drug.

Results: The method showed a good linear relationship (r² = 0.9990) in the concentration range 200-700 ng per spot. It was found to be linear, accurate, precise and specific.

Conclusion: It can be applied for quality control as well as for stability testing of different dosage forms containing swertiamarin. The developed method is validated as per ICH guideline Q2(R1) for global acceptance of standardized herbal formulations.

Keywords: Swertiamarin, Stability-indicating HPTLC method, ICH guidelines, Quality control

INTRODUCTION

Herbal medicinal technology is used for exploring medicinal plant materials as medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. The chromatographic techniques and marker compounds are used to standardize herbal formulations [1].

The stability indicating assays are important to determine the shelf life of the products. It also helps to determine the storage conditions by knowing the process of degradation. It is carried out by performing forced degradation studies. These types of studies using sophisticated techniques are important for global acceptance of herbal products [2].

It is revealed from the literature survey that aqueous extract of E. Littorale produces an anti-diabetic effect in type 2 diabetic rats. Based on activity guided phytopharmacological studies sw was reported to be one of the major active phytochemicals. Further studies showed that E. littorale significantly decreases both cholesterol and triglyceride level. Direct studies at molecular studies with sw revealed that up-regulates PPAR gene expression in 3T3-L1 cells. There are some reports on the application of spectrophotometry, spectrofluorimetry, thin layer chromatography, liquid chromatography-mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC) methods. ICH guidelines have given guidance for the estimation of degradation during storage. There is no stability indication method available for analysis for herbal formulations containing swertiamarin to detect degradation, hence, it was considered worthwhile to develop precise, cost-effective and stability indicating HPTLC method for determination of SW in bulk drug and dosage form. The HPTLC analytical methods are advantageous over other analytical methods for analysis of botanicals, plant-derived drugs and biomarkers. Several stability-indicating HPTLC methods have been published and being utilized for plant origin biomarkers like curcumin and forskolin. The method developed in the present investigation was validated as per the International conference on harmonization (ICH) guideline (ICH, 2005). The drug was analyzed under different stress conditions to explain the inherent stability characteristics of the active substance and to develop the validated stability-indicating HPTLC assay method (ICH, Q2 (R1) [3-10].

MATERIALS AND METHODS

Chemicals and reagents

Methanol, acetonitrile, chloroform, toluene, ethyl acetate, sodium hydroxide, hydrogen peroxide and hydrochloric acid of AR grade were purchased from astron, Ahmedabad. The reference standard of SW is purchased from sigma Aldrich.

Chromatographic conditions

Stationary phase was precoated silica gel G60 F 254 aluminum sheets 10×10 cm², layer thickness 0.2 mm. activated TLC plates by prewashing with methanol and activated in the oven at 50 °C for 5 minutes. The optimized mobile phase was ethyl acetate: chloroform (3:2.5:4.5 v/v/v). Chamber saturation time was 30 minute at ambient temperature and migration distance was 75 mm. The detection was done at 243 nm.

Preparation of solutions

Preparation of SW standard stock solution (1000 µg/ml)

Accurately weighed 10 mg of SW was transferred into 10 ml volumetric flask, dissolved and diluted up to the mark with methanol to get stock solution having concentration 1000 µg/ml.

Preparation of working standard solution (100µg/ml)

1 ml of a standard stock solution of SW was transferred to 10 ml volumetric flask and diluted to up to the mark with methanol to get working standard solution having 100 µg/ml.

Preparation of solution for calibration curve

To obtain a calibration curve, working standard solutions ranging from 2, 3.5, 5, 6.5, 8, 10 µl was applied by hamilton syringe with the help of linomat V applicator on TLC plate having concentration 200-1000 ng/µl.

Preparation of sample solution of SW

20 mamejava pills were weighed and triturated. 5 gm of powdered sample was weighed and transferred to 100 ml volumetric flask containing 25 ml water, sonicated for 20 min and diluted to mark with ethanol to obtain 5 mg/ml extract solution. The resulting solution was filtered using whatman filter paper. From the above
solution, 1 ml was transferred into 10 ml volumetric flask and diluted to mark with the same solvent. So, resultant solution 12 µl was injected and amount of SW is calculated in % w/w.

Preparation of sample solution from extract
1 gm of powdered sample was weighed and transferred to 100 ml volumetric flask containing 25 ml ethanol, sonicated for 20 min and diluted to mark with ethanol to obtain 1 mg/ml extract solution. The resulting solution was filtered using Whatman filter paper. From the above solution, 1 ml was transferred into 12 ml volumetric flask and diluted to mark with the same solvent. So, resultant solution 8 µl was injected and amount of SW is calculated in % w/w.

Forced degradation studies
Stress studies were carried out under the acidic, basic, thermal and oxidative conditions as mentioned in ICH Q1A (R2).

Preparation of standard stock solution for forced degradation studies
Accurately weighed 10 mg of SW transferred to 10 ml volumetric flask and diluted up to the mark with methanol to produce 1000 µg/ml.

Preparation of control
2.5 ml aliquot of the standard stock solution was transferred to 25 ml volumetric flask and diluted up to the mark with methanol to produce a mixture of 100 µg/ml of SW which was used as a control. 4 µl of resultant solution of SW (400 ng/µl) was applied on TLC plate and developed and scanned as per optimized chromatographic conditions.

Degradation under acid catalyzed hydrolytic condition
2.5 ml of a standard stock solution of SW was mixed with 1 ml of 2 N HCl. The solution was diluted to 25 ml with methanol and the solution was refluxed for 1 hour. It was allowed to cool down. It was neutralized with 0.1 N sodium hydroxide. 4 µl of resultant solution of SW (400 ng/µl) was applied on TLC plate and developed and scanned as per optimized chromatographic conditions.

Degradation under alkali catalysed hydrolytic condition
2.5 ml of a standard stock solution of SW was mixed with 1 ml of 1 N NaOH. The solution was diluted to 10 ml with methanol and kept for refluxed for 30 min. It was neutralized using 0.1 N hydrochloric acid. It was allowed to cool down. 4 µl of resultant solution of SW (400 ng/µl) was applied to TLC plate and developed and scanned as per optimized chromatographic conditions.

Oxidative degradation
2.5 ml of a standard stock solution of SW was mixed with 5 ml of 5% H₂O₂. The solution was diluted to 25 ml with methanol and refluxed for 1 hour. 4 µl of resultant solution (400 ng/µl of SW) was applied to TLC plate and developed and scanned as per optimized chromatographic conditions.

Thermal degradation
2.5 ml aliquot of the standard stock solution was transferred to 25 ml volumetric flask and diluted up to the mark with methanol to produce 25 µg/ml. The resulting solution was subjected to 100 °C for 30 min. 4 µl of resultant solution (400 ng/µl of SW) was applied on TLC plate and developed and scanned as per optimized chromatographic conditions.

Method validation

Linearity
2 to 10 µl from the working standard solution having 100 µg/ml concentration of SW was injected 5 times. So, linearity responses for SW were assessed in the concentration range of 200-1000 ng/µl of working standard solution.

Precision
Method repeatability
The precision of the method was checked by repeated scanning and measuring the area of SW (200-1000 ng/µl) solutions (n=5) without changing the parameters.

Instrument repeatability
The precision of the instrument was checked by spotting 5 µl (500 ng/µl) of standard solution six times on a TLC plate, followed by the development of plate and recording the peak area for six spots.

A) Intra-day precision
The intra-day precision of the proposed method was determined by analysis of corresponding responses in triplicate on the same day for 3 different concentrations of a standard solution of SW (500, 650 and 800 ng/µl).

B) Inter-day precision
The inter-day precision of the proposed method were determined by analysis of corresponding responses in triplicate on 3 different days over a period of 1 w for 3 different concentrations of a standard solution of SW (500, 650 and 800 ng/µl). Results were reported in terms of % RSD.

Accuracy
The method was studied for accuracy by calculating recovery of SW by the standard addition method. Known amounts of standard solutions of SW (80,100 and 120%) were added to pre quantified separate sample solutions of SW. Each solution was determined in triplicates and recovery was calculated by calibration curve or regression equation.

Sensitivity
The sensitivity of measurement of SW by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were calculated by the standard formula.

Specificity
The specificity of an analytical method is the ability to measure accurately an analyte in presence of interferences like synthetic precursor, excipients, degradants or matrix component. The purity of spectra was determined at three different levels, at starting, middle and end. Correlation between the spectra of standard and spectra of the drug in sample track was considered for determination of peak purity. The specificity was also determined by checking whether the sample matrix, solvent or mobile phase interfered in the analysis.

RESULTS AND DISCUSSION
Chawla R et al. showed rapid schematic representation for the holistic approach needed for standardized herbal medicines for the management of diabetes [2]. The use of stability indicating HPTLC analytical method is one of the thoughtful approaches to achieve globally standardized herbal medicines.

Selection of detection wavelength
The sensitivity of HPTLC method with UV detection depends upon the proper selection of detection wavelength. An ideal wavelength was the one that gives good response for the drug to be detected. The TLC plate was scanned between 200-400 nm. SW showed maximum absorption at 243 nm. Thus it was selected as detection wavelength for HPTLC analysis. Fig. 1 showed the UV spectra for SW in methanol.

Mobile phase optimization
The solvent system was developed and optimized using trial and error method. Vishwakarma SL et al. has developed the HPTLC method for analysis of commercial formulation containing Swertiamarin which suggested the use of ethyl acetate, water, methanol can be used for separation of Swertiamarin. [3] Various proportions of different solvents were tried to get a resolution of the compound. The optimized mobile phase was ethyl acetate: ethanol: chloroform (3:2.5:4.5 v/v/v). The optimized mobile phase could resolve the compound and the band obtained was compact too. Various mobile phases were tried which are listed in table 1 below:
Fig. 1: Wavelength selection for SW

Table 1: Optimization of mobile phase

| S. No. | Mobile phase                   | Ratio (v/v/v) | Remark                                      |
|--------|--------------------------------|---------------|---------------------------------------------|
| 1      | ethyl acetate: methanol: water | 8: 1.5: 0.5   | Unresolved peak                             |
| 2      | ethyl acetate: methanol: chloroform | 5: 1: 4     | Unresolved peak in acid degradation         |
| 3      | ethyl acetate: ethanol: toluene | 5: 1.5: 3.5   | Peak tailing was observed                   |
| 4      | ethyl acetate: ethanol: chloroform | 5:1:4       | Good shape peak was obtained with peak purity |

\( v/v/v = \text{ratio in volume} \)

Fig. 2: Standard chromatograph of SW (450 ng/µl)

Results from various stress condition for standard SW are shown in table 2 below: Forced degradation shows that SW is most degraded by alkali conditions. It is quite stable with thermal stress.

Table 2: Results of degradation

| Stress condition     | % degradants formed |
|----------------------|---------------------|
| Alkali               | 34.55%              |
| Acidic               | 23.45%              |
| Oxidative            | 31.18%              |
| Thermal degradation  | 17.99%              |

Method validation

Linearity

Linearity responses for SW were assessed in the concentration range of 200-1000ng/µl of working standard solutions. Table 3 shows the data of calibration curve and fig. 3 shows the calibration curve (200-700ng/µl). The linear equation for the calibration plot was \( y = 5.573x + 1619 \) with correlation coefficient \( r^2 = 0.9990 \). Fig. no 4 shows 3D densitogram for SW. The value of correlation coefficient \( r^2 = 0.9990 \) suggests good linearity.

For all concentration levels of the calibration curve, % RSD is less than 2 which is an acceptable limit as per ICH guideline Q2 (R1).

Table 3: Result of calibration curve for SW

| Concentration (ng/µl) | Area (AU)         | % RSD |
|-----------------------|-------------------|-------|
| 200                   | 2689±14.93        | 1.56  |
| 350                   | 3601.83±43.43     | 1.21  |
| 500                   | 4455.50±82.79     | 1.86  |
| 650                   | 5201.17±71.19     | 1.37  |
| 800                   | 6105.50±79.59     | 1.30  |
| 1000                  | 7171.67±84.09     | 1.17  |

*= (n=5), Area are presented as mean±SD % RSD = % relative standard deviation
Precision

**Method repeatability**

The % RSD for peak area value of SW was found to be ranging between 0.87 to 1.49%, as given in table 4.

**Instrument repeatability**

The % RSD for peak area value of SW was found to be 1.47, as given in table 4.

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### Table 4: Intra-day, Inter-day method and instrument repeatability study of SW

| Concentration (ng/µl) | Intraday precision * % RSD | Interday precision * % RSD | Concentration (ng/µl) | % RSD ** |
|-----------------------|----------------------------|----------------------------|-----------------------|----------|
| 500                   | 1.60                       | 1.71                       | 200                   | 1.49     |
| 650                   | 0.89                       | 1.84                       | 350                   | 1.59     |
| 800                   | 0.97                       | 1.22                       | 500                   | 0.47     |
|                       |                            |                            | 650                   | 0.87     |
|                       |                            |                            | 800                   | 0.99     |
|                       |                            |                            | 1000                  | 1.09     |

* = (n=3), ** = (n=5), % RSD = %Relative standard deviation

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### Table 5: Determination of accuracy for SW

| Level               | Concentration of sample taken ng/µl | Concentration of standard added ng/µl | Total standard ng/µl | Mean total concentration obtained* ng/µl | Average assay (%) recovery | Assay % RSD |
|---------------------|-------------------------------------|--------------------------------------|----------------------|------------------------------------------|---------------------------|-------------|
| Level 0             | 450                                 | 0                                    | 450                  | 445±1                                    | 98.89±0.22                | 0.22        |
| Level I (80%)       | 450                                 | 360                                  | 810                  | 798.67±2.51                             | 98.60±0.31                | 0.31        |
| Level II (100%)     | 450                                 | 450                                  | 900                  | 889.33±6.11                             | 98.81±0.67                | 0.68        |
| Level III (120%)    | 450                                 | 540                                  | 990                  | 982.67±2.51                             | 99.26±0.25                | 0.25        |

* = (n=3), Results are presented as mean±SD % RSD = % relative standard deviation
**Accuracy**
The amount of drug was calculated by employing corresponding calibration curve equations. The recovery was found to be between 98.60 % to 99.26 % (table 5). The closeness of the result nearly to 100 % assured the accuracy of the developed method.

**Sensitivity**
Based on the standard deviation of the response and the slope, the values obtained are 32.22 ng/µl and 134.11 ng/µl respectively for LOD and LOQ.

**Specificity**
The peak purity of SW was assessed by comparing their respective spectra at peak start, apex and peak end positions of the spot i.e., r (s, m) = 0.9877 and r (m, e) =0.9990. A good match was obtained between standard and sample spectra of SW which suggest that the developed method is specific.

### Analysis of SW in marketed formulation
Table 6 shows the % w/w of SW in marketed preparation which suggests the quality of the products.

| Formulation           | Avg. amt. recovered* % w/w |
|-----------------------|-----------------------------|
| Mamejava pill 250 mg   | 9.94 %                      |
| Marketed extract      | 11.12 %                     |

* = (n=3), Avg. Amt. = Average amount, % w/w = % weight by weight

**Summary**
The HPTLC method for determination of SW was developed and validated. The results for each validation parameters confirmed linearity, accuracy, precision and selectivity of the developed analytical method as per ICH guidelines. The method showed good linearity over the selected linearity range. The summary validation parameters for the developed analytical method are shown in table 7.

| Parameters              | HPTLC method                          |
|-------------------------|---------------------------------------|
| Linearity (ng/µl)*      | 200-1000                              |
| LOD (ng/µl)             | 32.23                                 |
| LOQ (ng/µl)             | 134.11                                |
| % Recovery              | 99.26 % -98.60 %                      |
| Repeatability (%RSD) *  | 0.87 %                                |
| Precision (%RSD)**      | 0.89 to 1.60 %                        |
| Intra-day               | 1.22 to 1.84 %                        |

% RSD = % relative standard deviation, *= (n=5), **= (n=3)

**CONCLUSION**
The developed method is validated as per ICH guideline Q2 (R1) for global acceptance of standardized herbal formulations. The method showed linearity in the concentration range of 200-1000 ng/µl with a coefficient of correlation, r²= 0.9990 at 243 nm. The result of the analysis by the proposed method was found to be highly reproducible and reliable. So, the developed HPTLC method is simple, precise and accurate and can be used for determination of SW in pharmaceutical dosage forms. Degradation study is a requirement of ICH guidelines. Here developed method can be used to detect the degradation of swertiamarin during storage. The method can be applicable for routine analysis of SW as per ICH guidelines in quality control lab.

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**CONFLICT OF INTERESTS**
Declared none

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