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Development and Validation of HPLC Analytical Protocol for Quantification of Salicin from *Salix alba* L.

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Abstract: A reversed phase high performance liquid chromatography method (HPLC) coupled with a diode array; UV spectrometer was developed for the quantitative and qualitative standardization of salicin from *Salix alba* L. Salicin were separated on an phenomenox C18 column (250 mm × 4.6 mm I.D., 5µm), with a mobile phase composed of 0.1 % ortho-phosphoric acid (OPA) in water and acetonitrile, under gradient elution. The method was shown to be linear (r^2 > 0.99), repeatable with instrumental precision < 2.0 and intra-assay precision < 2.0 (% CV, percent coefficient of variation), precise with intra-day variation < 2.0 and inter-day variation < 2.0 (% CV, percent coefficient of variation) and sensitive over the calibration range for salicin. Salicin is fully recovered in the presence of normal condition (recovery rates > 95%). Statistical analysis proves that the proposed method was more accurate, selective, reproducible and repeatable for the estimation of the salicin from *Salix alba*.

INTRODUCTION

Single drug therapy has been mentioned since ancient time and now it has been largely accepted by the physician for a number of diseases. An herb–drug interaction has also been given a considerable attention in clinical practice. [1] The development of a marker profile as a standardization process is of great importance for quality control and scientific validation of the single drug. [2, 3] Salicin is the metabolic precursor of salicylic acid, a wonder compound. Salicin is an alcoholic beta-glycoside which contains D-glucose. Salicin is an anti-inflammatory which is produced from all Willow barks. *Salix alba* L commonly known as White Willow (specifically, the bark) is the original source of salicin, a weaker forerunner of aspirin. [4] The chemical constituents like Glycosides (1.5-11%) particularly salicylates (salicin, salicortin, populin, fragilin, tremulacin); Tannins (8-20%); Aromatic aldehydes and acids specifically salidroside, vanillin, syringin, salicylic acid, caffeic and ferulic acids; Salicyl alcohol (saligenin); Flavonoids have been isolated and identified from the plant. [5-9] Having antipyretic and analgesic effects, salicin can be used for the treatment of fever and diseases, like arthritis. [4] Salicin is closely related to aspirin and has a very similar action in the human body (Figure 1). When ingested, salicin, the active glycoside, is hydrolysed in the intestine and liver to saligenin, which in turn is absorbed and then oxidized to the therapeutically active compound salicylic acid. Because of this conversion process, white willow generally takes longer to act than aspirin, but the effects last for an extended period of time. Salicin provides a more sustained release of salicylic acid than acetylsalicylic acid (ASA - aspirin) itself, Table 1. [10]

High performance liquid chromatography (HPLC) is one of the most popular analytical techniques used to determine Salicin derivatives. However, most of these methods used the fluorescence detector [11-13] and polar columns which causes limitations in the quantitation of salicin. Therefore it is the need of the hour to develop faster, easier and more precise quantitative HPLC method to determine salicin from *salix alba* L. In this study, a novel gradient HPLC method with PDA and UV detection was developed and validated in light of International Conference on Harmonisation (ICH) guidelines using nonpolar column.

MATERIALS AND METHODS

Chemicals

HPLC-grade solvents such as acetonitrile, methanol and ortho-phosphoric acid were obtained from Merck Ltd. Bangalore India. Standard and *Salix alba* extract were purchased from Sigma Aldrich and Phyto concentrate India.

Preparation of Mixture of Standard Solution

About 6 mg of Salicin was dissolved into 25 ml volumetric flask and mixed well. This solution was used as standard stock solution. Further 3.5 ml of standard stock solution was diluted up to 5 ml with methanol. The solution was filtered through a 0.45-µm syringe filter and the resulting solution was used as standard working solution.

Preparation of the Test Solution

200 mg *Salix alba* extract was weighed and taken into 20 ml volumetric flask. About 15 ml of diluent was added and the solution was sonicated in ultrasonic water bath for 30 minutes. The solution was allowed to cool and the volume was made up to the mark with diluent. The resulting solution was filtered through Whatman No. 41 filter paper and further it was filtered through 0.45 µ syringe filter. The resulting solution was used as test solution.

Chromatographic Conditions

HPLC was performed using a Waters 2695 Alliance system with a 2996 photodiode array detector (PDA). The standard salicin was resolved on a reverse-phase 250 × 4.6 mm, 5-µm, Phenomenox SB C18 column. The mobile phase was prepared from 0.1% ortho-phosphoric acid in water of pH 2.5 (solvent-A) and Acetonitrile (100%

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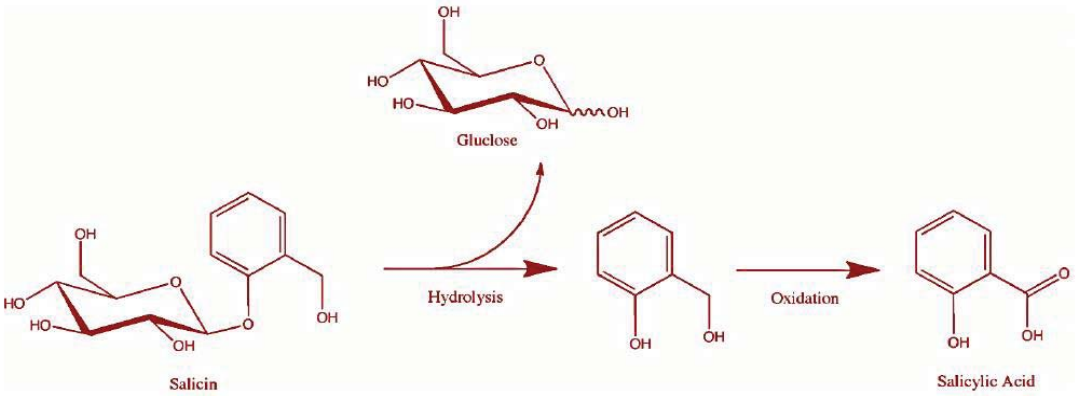


Figure 1: Conversion of Salicin→Saligenin→Salicylic Acid

Table 1: Chemical Properties of Salicin

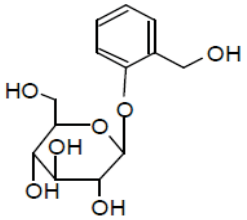
Particular	Details
CAS number	138-52-3
Chemical Formula	$C_{13}H_{18}O_7$
Chemical Name	2-(Hydroxymethyl)phenyl-β-D-glucopyranoside
Structural Formula	
Molecular Weight	286.27

Table 2: List of Standards

S. No.	Name of Standard	Batch No.	Potency (%)
1	Salicin	S0625	99
2	*Salix alba* L Extract	SA/PC1/JUNE14	1

Table 3: Details of Gradient Program

S. No.	Time	Flow	%A	%B
1	0.01	1.00	95.0	5.0
2	25.00	1.00	80.0	20.0
3	26.00	1.00	5.0	95.0
4	30.00	1.00	5.0	95.0
5	31.00	1.00	90.0	10.0
6	35.00	1.00	90.0	10.0

v/v) (solvent-B). The mobile phase was degassed and filtered through a 0.45-µm filter before use. The gradient program used is given in Table 3.

The mobile phase flow rate was kept at 1 ml/min. Before the first injection, the column was saturated for 30 min with the initial mobile phase. Column temperature was maintained at 30°C. Injection volume was decided to maintain at 20 µl. The PDA was set by optimizing the wavelength at 267 nm to acquire the chromatogram. The standard salicin was identified by comparing the retention time and spectra obtained from the sample and standard solutions. The present work was performed in an air-conditioned room maintained at 25°C.

Preparation of Calibration Graph

Seven different concentrations were prepared by diluting the standard stock solution. The calibration graph of each standard was constructed by plotting concentrations against peak area for the respective standards.

Validation of HPLC Method

The proposed HPLC method was validated in terms of specificity, precision, accuracy, standard solution stability, sample solution stability and robustness as per the International Conference on Harmonization (ICH) guidelines. [14-15]

1. Specificity

The specificity of the method was studied by assessment of peak purity of Salicin using the Waters empower software

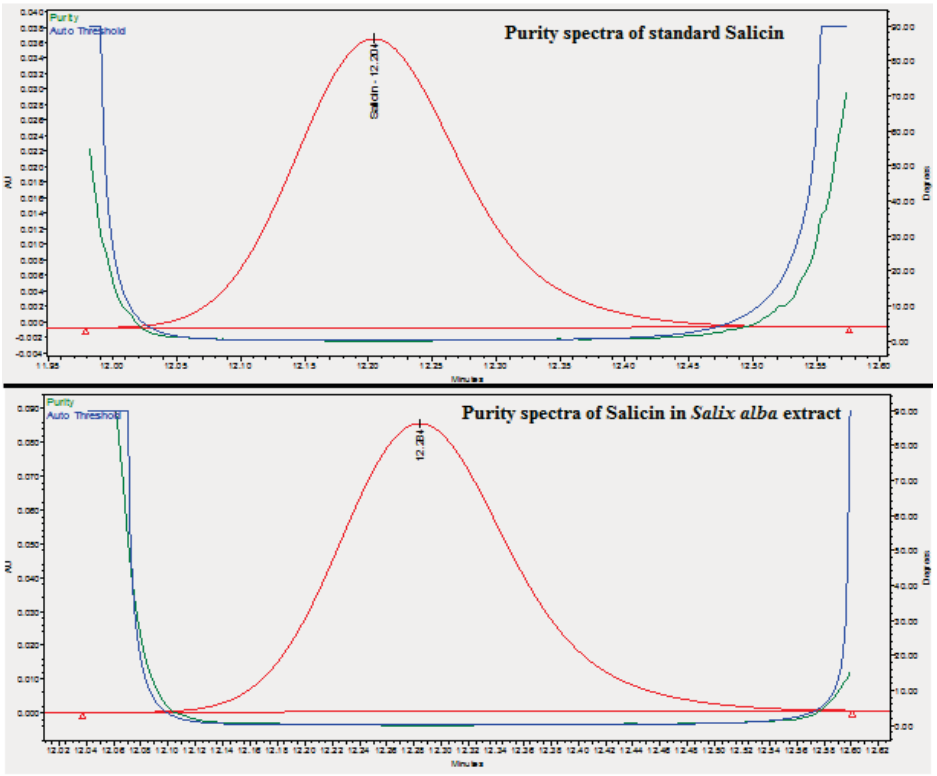


Figure 2: Purity spectra for specificity

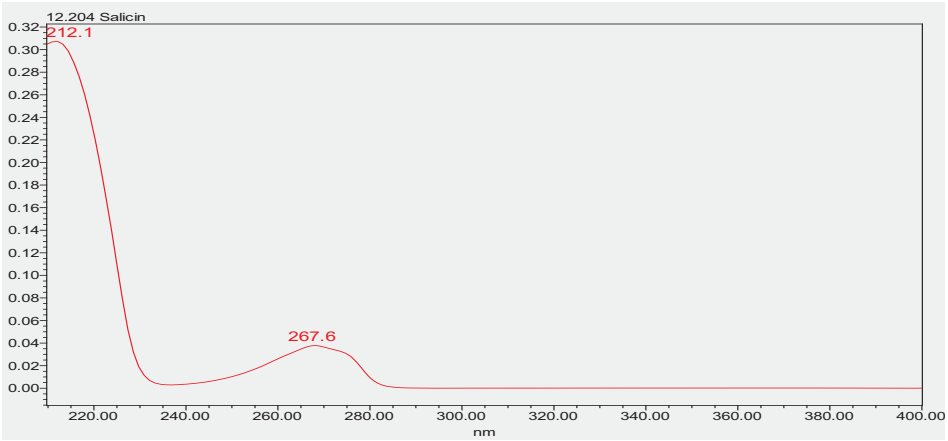


Figure 3: UV spectra for standard

and diode array detector and represented in terms of purity angle, purity threshold and purity flag.

2. Precision

Precision was studied in terms of system precision, method precision and intermediate precision.

3. System Precision

System precision was carried out by six replicate injections from the same vial of standard and was expressed in terms of percent relative standard deviation (% RSD) tailing, plate count and resolution.

4. Method Precision

The six separately prepared samples were analysed by proposed procedure. The % assay for each analyte was expressed in terms of % RSD.

5. Intermediate Precision

Intermediate precision was performed on different systems, one on Waters e2695 Alliance system with a 2996 PDA and the other on 2489 ultraviolet (UV) detector by different analysts by analysing six different samples of extract and was expressed in terms of % RSD.

6. Recovery Studies

The accuracy of the method was determined from recovery studies by adding a known amount of standard at the 80%, 100% and 120% level to the pre-analysed sample followed by replicate quantitative analyses by the proposed method.

7. Solution Stability

The standard and sample solution were prepared as per the proposed method and subjected to stability study at 25°C for 24 h. The standard and test solution was analysed

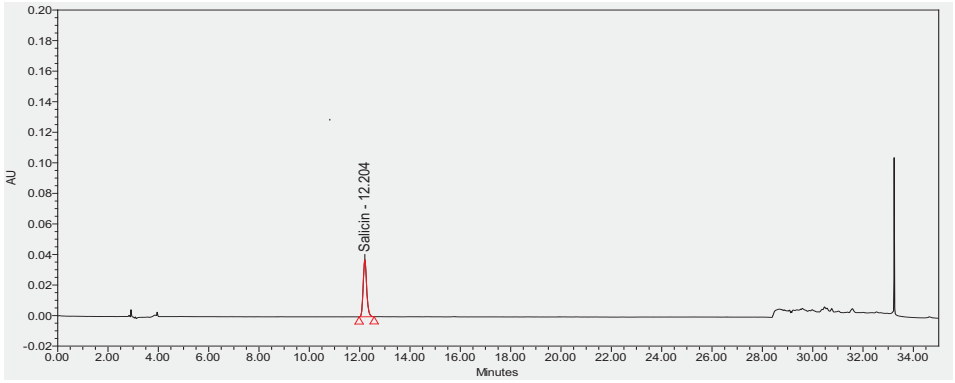


Figure 4: Chromatogram of standard salicin

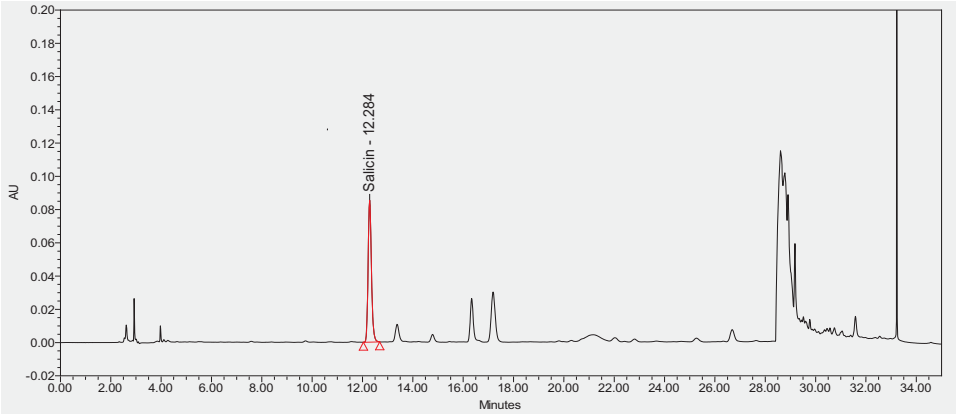


Figure 5: Chromatogram of *Salix alba* extract

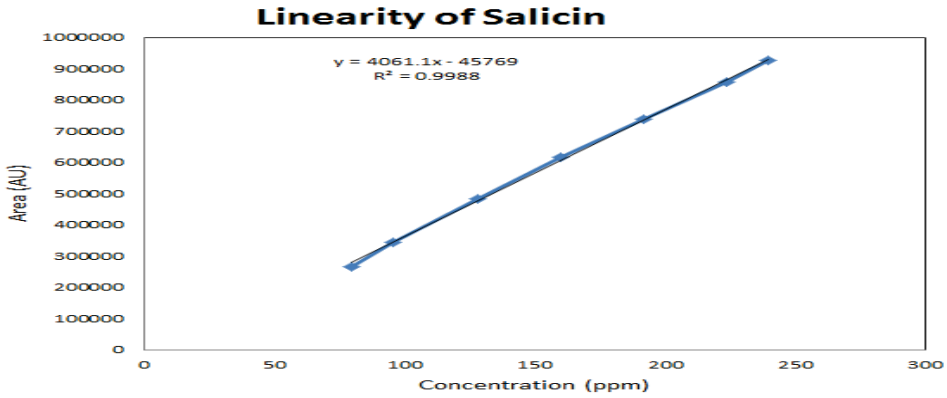


Figure 6: Linearity graphs for standard salicin

at initial and at different time intervals of 4 h up to 24 h. Change in the response of salicin in the test solution with respect to time was calculated as absolute percent difference against initial response.

8. Robustness

Robustness of the method was determined by small deviation in the method parameters. The parameters selected were deviation in wavelength, column temperature and flow rate. The retention time of Salicin and % RSD was determined using system suitability parameters.

9. % Assay of *Salix alba* Extract

Salix alba extract was analysed to determine the content of Salicin as per method described under chromatographic conditions by HPLC. All analysis was repeated three times and results were expressed in mean±SD.

RESULTS AND DISCUSSION

The composition of the mobile phase in the HPLC method was optimized by testing different solvent compositions of varying polarity, column chemistry, column temperature and pH of mobile phase and the best results were obtained by using the present method, which produces highly symmetrical peaks showing good resolution between salicin standard and other peaks (Figure 2 and 3). The scanning wavelength selected was 267 nm to provide comparable results and at this wavelength all analyte showed optimum response (Table 4). Salicin was satisfactorily resolved with retention time about 12 min (Figure 4 and 5). The calibration graph was linear in the

Table 4: Specificity Parameters

S. No.	Standard	Purity Angle	Purity Threshold	Purity Flag
1	Salicin	0.182	0.263	No flag found

Table 5: System Precision Parameters

S. N0.	Name of Analyte	% RSD	USP Tailing	USP Plate Count
1	Salicin	0.43	1.21	37996

Table 6: Method Precision Parameters

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Weight (mg)	201.9	201.8	202.1	201.5	201.3	201.7
Area of Test	592134	618990	616853	616165	621445	628144
	637958	626951	617621	620618	616180	617815
Average	615046	622970.5	617237	618391.5	618812.5	622979.5
Assay	0.01601	0.01622	0.01605	0.01613	0.01615	0.01623
% Assay	1.601	1.622	1.605	1.613	1.615	1.623
Average Assay	1.613		SD	0.0090136	**% RSD**	**0.56**

Table 7: Intermediate Precision Parameters

S. No.	Name of Analyte	% RSD for System-1	% RSD for System-2	Overall % RSD
1	Salicin	0.56	0.81	1.72

Table 8: Robustness Parameter

S. No.	Robustness Parameter	% RSD	Peak Tailing	Theoretical Plates	Remark
1	Wavelength: - 5nm	1.05	1.01	11173	Pass
2	Wavelength: +5nm	0.78	1.02	11286	Pass
3	Temperature: - 5°C	0.56	0.95	12492	Pass
4	Temperature: + 5°C	0.37	0.88	10276	Pass
5	Flow: - 1%	0.79	0.91	13377	Pass
6	Flow: +1%	0.61	0.99	11249	Pass

Table 9: Recovery Studies

Analyte	Recovery Level	% Recovery	Average % Recovery
Salicin	80% - 1	102.59	101.40
	80% - 2	103.31	
	80% - 3	98.32	
	100% - 1	98.99	99.67
	100% - 2	99.75	
	100% - 3	100.28	
	120% - 1	97.28	97.50
	120% - 2	97.46	
	120% - 3	97.75	

Table 10: % Assay of Salicin from *Salix alba* Extract

S. No.	Name of Analyte	Average Assay
1	Salicin	1.92±0.020

working range of 50-150 µg/ml, with acceptable correlation coefficients 0.9988 for Salicin. The graph for standard salicin is given in Figure 6. The values of system precision, method precision and intermediate precision are given against sample application and scanning of peak area and are expressed in terms of % RSD. For system precision % RSD values were found to be 0.43 % for salicin (Table 5). Method precision was done and %RSD value was found to be 0.56 % for salicin (Table 6). For intermediate precision, % RSD values between the two analysts were found to be 1.72 % for salicin (Table 7). For the values of system precision, method precision and intermediate precision, the %RSD values showed that the proposed method provides an acceptable level of system precision, method precision and intermediate precision.

The peak purity for each analyte was assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot from standard and extracts (Figures 1 and 2). The purity angle and purity threshold values are given in Table 4. The given method

was optimized by doing robustness. The peak area for each analyte was calculated for each parameter and % RSD was found to be less than 2%. The values of % RSD as shown in Table 8 indicate better robustness of the method.

The recovery study was carried out by spiking known amount of *salix alba* into pre-analysed solution at 80%, 100% and 120% of working concentration. The overall recovery percent were found to be for 99.53% for salicin (Table 9). Analytical solution stability was done on standard solution and sample solution. The standard and test solutions were analysed at initial and at different time intervals for 24 hrs. Both the solutions were found to be stable up to 24 hrs.

The average assay of salicin was expressed as mean ±SD and it was found to be 1.92±0.022 from *Salix alba* extract (Table 10).

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