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Development and Validation of Simultaneous UV-Spectrophotometric Method for the Determination of Silybin and Resveratrol in Pharmaceutical (In-House) Gel Formulation

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ABSTRACT

UV-Spectrophotometric method was developed for the simultaneous determination of Silybin (SIL) and Resveratrol (RES) from pharmaceutical (In-house) gel formulation (SIL-RES hydrogel). The in-house SIL-RES hydrogel was formulated by using carbopol as gelling agent. The UV spectrophotometric method involves formation of Q-absorbance equation at 218nm (isoabsorptive point) and at 288 nm, using methanol as a solvent. The linearity for both Silybin and Resveratrol was in the range of 2-12 µg/ml and 1-6 µg/ml respectively. The % recovery was found to be 99-100% for both Silybin and Resveratrol indicating proposed method is accurate and precise for simultaneous estimation of Silybin and Resveratrol in gel.

Keywords: Silybin, Resveratrol, Q-Analysis, UV-Spectrophotometry, In-house gel, Isoabsorptive point

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INTRODUCTION

Silybin (SIL) Figure 1, a natural occurring polyphenolic flavnone, is the active constituent of silymarin, which consist a number of flavonolignans such assilibinin, isosilibinin, silicristin and silidianin. Silibinin, an effective anticancer and chemopreventive agent in various epithelial cancer models, has been reported to inhibit cancer cell growth through mitogenic signaling pathways, however, until date; exact mechanism is not well-elucidated[1]. The International Union of Pure and Applied Chemistry name for silybin is (2R, 3R)-3, 5, 7-trihydroxy-2-([2R,3R]-3-[4-hydroxy-3-methoxyphenyl]-2-[hydroxymethyl]-2,3dihydrobenzo [b] [1,4] dioxin-6-yl) chroman-4-one[1,2,3]. Resveratrol (RES) Figure 2 is a natural phenolic compound with various biological activities such as antioxidant[4], cancer preventive[5,6,7], cardioprotective[8], phytoestrogenic and neuroprotective[7,8]. Resveratrol is chemically 3,5,4′-trihydroxystilbene possesses antioxidant activities *in vitro*. It is obtained from roots of *polygonum cuspidatum* and leaves of *veratrugrandiflorum*[9].

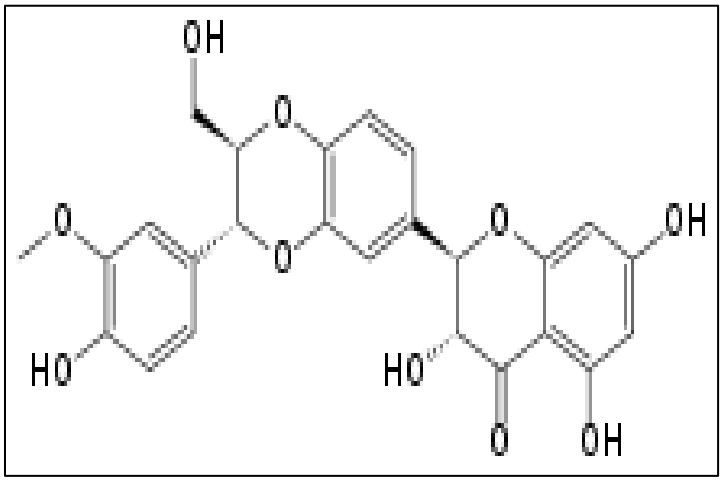

Figure 1: Chemical structure of Silybin

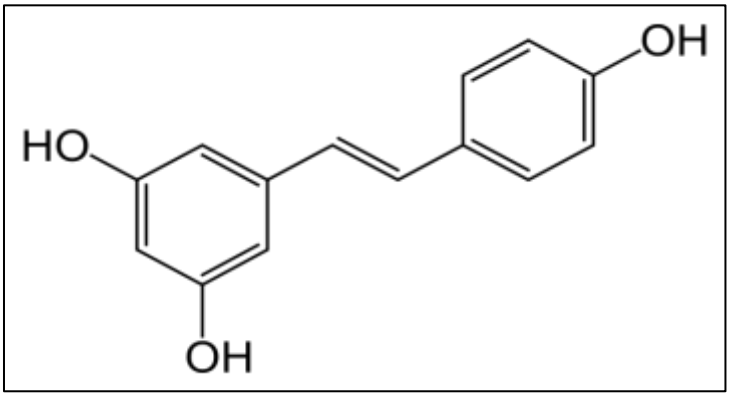

Figure 2: Chemical structure of Resveratrol

Literature survey reveals that it was found that many methods had been reported for determination of Silybin and Resveratrol individually but in combination not many methods have been reported

so far in pharmaceutical gel formulations. This work was aimed to investigate the utility of UV spectrophotometric method in the simultaneous determination of SIL and RES in pharmaceutical gel preparations. The method had sufficiently good accuracy, precision and permitted a simple and cost effective assay for these compounds in mixtures.

MATERIALS AND METHOD

Instrumentation

A Shimadzu UV spectrophotometer UV-1800 with 1 cm matched quartz cells were used for the estimation.

Chemicals and reagents

Silybin and Resveratrol were kindly supplied by Prolab Pvt Ltd, New Delhi and Yucca Enterprises, Mumbai respectively. All reagents were of analytical grade used throughout the experiment.

Standard stock preparation

Accurately weighed quantities (10 mg each) of SIL and RES were dissolved separately in sufficient quantity of methanol in a 100 ml volumetric flask. The solutions were sonicated and the volume was adjusted up to the mark with methanol to obtain a stock solution of 100 µg/ml; each of SIL and RES. For the selection of analytical wavelength for the Q absorbance method, the stock solutions of SIL and RES were separately diluted in methanol, to get concentrations of 10 µg/ml each, and scanned in the wavelength range of 200- 400 nm. From the overlain spectra of both drugs, wavelengths 218 nm (isoabsorptive point) and 288 nm (λ_{max} of SIL) were selected for the formation of Q absorbance equation. For calibration curves, stock solutions of SIL and RES were appropriately diluted to obtain concentration range of 2-12 µg/ml and 1-6 µg/ml respectively. The absorbance of SIL was measured at 288 nm and 218 nm, and calibration curves were plotted. Similarly the absorbance of RES was measured at 218nm and 288 nm, and calibration curves were plotted. The absorptivities (A1%, 1 cm) of each drug at both the wavelengths were also determined.

Sample preparation

For the estimation of drugs from the in-house formulation, quantity of gel equivalent to 10mg of SIL and 10 mg of RES was transferred to 100 ml volumetric flask, dissolved in sufficient quantity of methanol, sonicated and the volume was adjusted up to the mark with methanol to obtain a stock solution of 100µg/ml of SIL and 100 µg/ml of RES. The solution was then filtered through whatman filter paper no. 41 and the filtrate was appropriately diluted to obtain final concentrations

10 µg/ml of SIL and 10 µg/ml of RES. Absorbance of this solution was measured at appropriate wavelengths, and values were substituted in the respective formulae to obtain concentrations.

Q-Analysis method[10,11]

The ratio of two absorbance determined on the two solutions at two different wavelengths is constant. This constant is termed as Q value. The Q value is independent of concentration and thickness of solution and therefore is used to access the purity of compounds. The absorbance ratio method is a modification of the simultaneous equation procedure. Graphical absorption ratio method uses the ratio of observed absorbance at two selected wavelengths, one of which is isoabsorptive point. It depends on property for that substance which obeys Beer's law at all wavelengths. The ratio of absorbance at any wavelength is constant value independent of concentration or path length. For Q analysis method, the overlain spectra of SIL and RES were recorded in the range of 200 to 400 nm. It showed that (Figure 6) the peaks were well resolved, satisfying the criteria for obtaining maximum precision, based on absorbance ratios. The criteria being the ratios, $(A_2/A_1)/(aX_2/aX_1)$ and $(aY_2/aY_1)/(A_2/A_1)$, should lie outside the range 0.1-2.0 for the precise determination of X (SIL) and Y (RES), respectively. Where A1, A2 represents the absorbance of the mixture at λ1 (wavelength at isoabsorptive point) and λ2 (λmax of SIL), aX_1 and aX_2 denote absorptivities of X at λ_1 an λ_2, and aY_1 and aY_2 denote absorptivities of Y at λ_1 and λ_2, respectively. In the present work, the above criteria was found to be satisfied for SIL (X) and RES (Y), where λ_1 was 218 nm and λ_2 288 nm for Q absorbance method. In the quantitative assay of SIL and RES in an admixture by absorbance ratio method, absorbances were measured at any two wavelengths, one being isoabsorptive point (λ_1) and the other being λ_{max} of one of the component i.e. SIL (λ_2). Two equations were constructed as described below (Eq. 1 and Eq. 2), using the relationship $aX_1 = aY_1$ at λ_1 and b = 1 cm. Equations are,

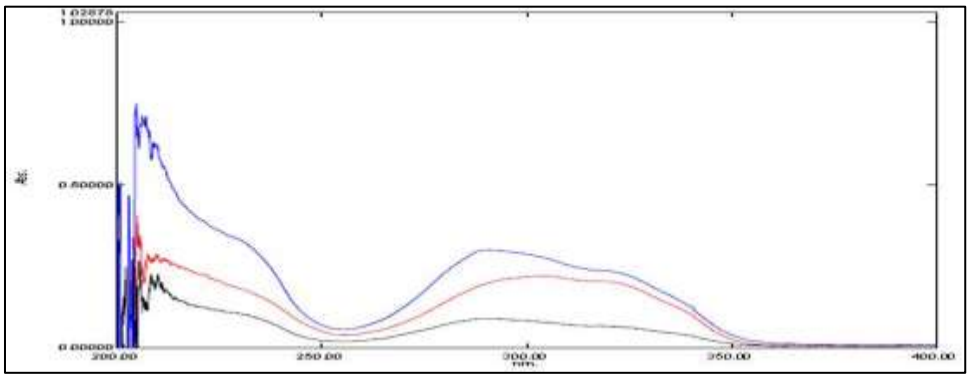

Figure 6: Overlain spectra of Mixture (Silybin + Resveratrol).

$$A_1 = aX_1C_X - aX_1C_Y \text{ at } \lambda 1 > aX_1 = aY_1 \text{ at } \lambda 1 \text{ ..... (1)}$$

And

$$A_2 = aX_2C_X - aY_2C_Y \;\; at\; \lambda 2 \; \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; (2)$$

Dividing Eq. 2 by Eq. 1

$$\frac{A_2}{A_1} = \frac{F_{XaX2} - F_{XaY2} + ay2}{aX1}$$

But $F_Y = 1 - F_X$

$$\frac{A_2}{A_1} = \frac{F_{XaX2}}{aX_1} - \frac{F_{XaY2}}{aY_1} + \frac{aY2}{ay_1} > aX_1 = aY_1 \; at\; \lambda 1$$

Let

$$Q_x = \frac{aX_2}{aX_1} \quad , \qquad Q_y = \frac{aY_2}{aY_1} \; , \qquad Q_M = \frac{A_2}{A_1}$$

$$Q_M = F_X(Q_X - Q_Y) + Q_Y$$

$$F_x = \frac{Q_M - Q_Y}{Q_X - Q_Y} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; (3)$$

Eq. (3) gives the fraction of X in the mixture of SIL and RES. For the determination of absolute concentration of X and Y the equation 5 was rearranged. A1 = $aX_1(C_X + C_Y)$

$$C_X + C_Y = \frac{A_1}{aX_1} \; \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; (4)$$

From Eq. 3

$$\frac{C_X}{C_X + C_Y} = \frac{Q_M - Q_Y}{Q_X - Q_Y} > F_x = C_X \;\; ?\, (C_X + C_Y)$$

$$\frac{C_X}{A_1/aX_1} = \frac{Q_M - Q_Y}{Q_X - Q_Y}$$

$$C_X = \frac{Q_M - Q_Y}{Q_X - Q_Y} \times \frac{A_1}{aX_1} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; (5)$$

Similarly,

$$C_Y = \frac{Q_M - Q_X}{Q_Y - Q_X} \times \frac{A_1}{aY_1} \; \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; (6)$$

Where, C_X and C_Y are concentrations of SIL and RES, respectively.

Method Development

SIL and RES, both are freely soluble in methanol, hence methanol was chosen as a solvent for their determination in semi-solid dosage form. The UV spectra of standard solutions of SIL and RES (10 µg/mL each) were determined separately in methanol (Figure 3 and 4). The λ_{max} of SIL was found to be 288 nm whereas the λ_{max} of RES was recorded at 306 nm. Absorbance ratio (Q analysis) method was applied for the analysis of both the drugs in gel formulation. The developed method for the simultaneous analysis of SIL and RES was validated with respect to stability,

linearity, sensitivity, precision, accuracy, specificity, robustness and ruggedness. The stability of both the drugs in methanol was checked by recording their UV spectra at an appropriate time interval. They were compared with freshly prepared solutions and not any difference was found between them. This indicated that both these drugs were highly stable in solution phase. Further, UV spectrum of standard solution containing SIL and RES (mixture) was also recorded to check any chemical interaction between these drugs. The λ_{max} of both the drugs in a mixture was found to be similar as compared to individual drugs indicating no chemical interference with each other (Figure 5).

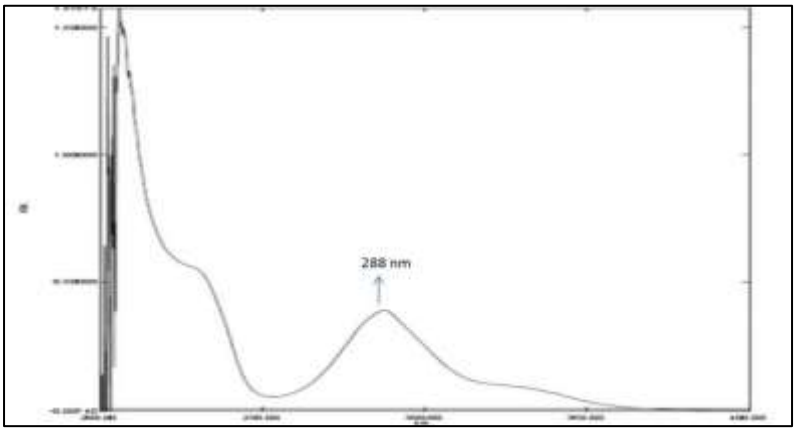

Figure 3: UV Spectra of Silybin

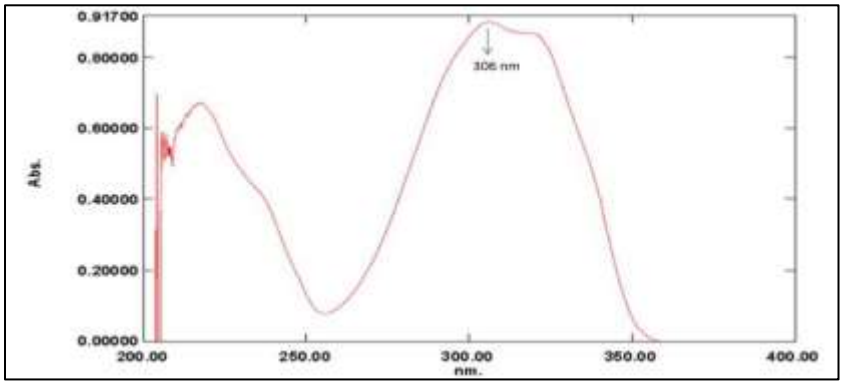

Figure 4: UV Spectra of Resveratrol.

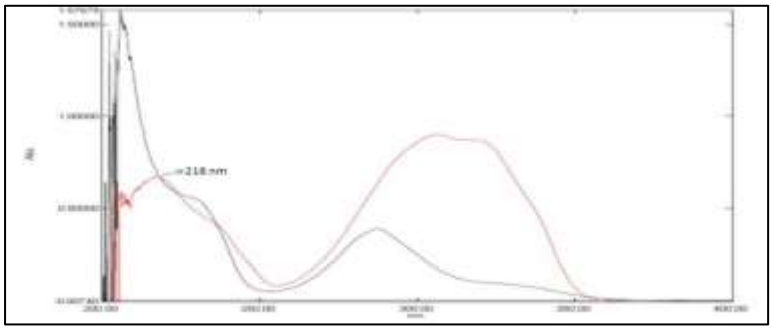

Figure 5: UV Spectra of Mixture (Silybin + Resveratrol).

Method validation[12]

Linearity and precision

In quantitative analysis the calibration curve was constructed for both SIL and RES after analysis of consecutively increased concentrations. To check the precision and reproducibility of the method, six samples of the same concentration (n=6) of SIL and RES were prepared and analysed.

Analysis in (in-house) hydrogel formulation

For the determination of SIL and RES from in house gel formulations by Q analysis method, the absorbance of sample solutions and absorptivity values at the particular wavelengths were calculated and substituted in the following equation (equations 4 and 5) to obtain the concentrations of two components.

$C_{SIL} = (Q_M-Q_Y)\times A_1/(Q_X-Q_Y)\times aX_1$, $C_{RES} = (Q_M-Q_X) \times A_1/(Q_Y-Q_X)\times aY_1$

where, C_{SIL} and C_{RES} are concentrations of SIL and RES, respectively, A_1 is the absorbance of sample at 218 nm, aX_1 is the absorptivity of SIL at 218 nm, aX_2 is the absorptivity of SIL at 288 nm, aY_1 is absorptivity of at 218 nm, aY_2 is absorptivity of RES at 288 nm, Q_x was obtained by using the equation, (absorptivity of SIL at 288 nm aX_2)/(absorptivity of SIL at 218 nm aX_1). Similarly, Q_Y was obtained from (absorptivity of RES at 288 nm aY_2) / (absorptivity of RES at 218 nm aY_1) and Q_M from, (absorbance of sample at 288 nm A2) / (absorbance of sample at 218 nm A_1). The respective absorptivity values for SIL and RES at λ1 and λ2 are represented in Table 2. The results obtained from analysis of dosage forms are given in Table 3.

Table 2: Absorptivity values at 218 nm and 288 nm (λmax of SIL).

Absorptivity at 218 nm* (Mean ± S.D.)		Absorptivity at 288nm* (Mean ± S.D.)	
SIL	**RES**	**SIL**	**RES**
aX_1	aY_1	aX_2	aY_2
792 ± 0.29	865.35 ± 0.18	491.50 ± 0.35	901.14 ± .28

SIL: Silybin; RES: Resveratrol;*Indicates mean of six determinations (n=6).

Table 3: Analysis of dosage forms and recovery studies

Product	Drug	Label Claim	%Estimated	* % RSD	% Recovery
In-house gel formulation	SIL	50 mg.	98.62	0.48	99.1
	RES	50 mg.	98.42	0.96	99.38

SIL: Silybin; RES: Resveratrol; * Indicates mean of six determinations (n=6).

Reproducibility

The accuracy and specificity of the proposed method was tested by recovery experiments. Recovery studies were carried out at 100 % level by adding a known quantity of pure drug to the preanalyzed formulation and the proposed method was followed. From the amount of drug found, percentage recovery was calculated (Table 4).

Table 4: % RSD values for repeatability, LOD, LOQ, Precision intra-day, inter-day variation robustness and ruggedness (n=3).

Parameter	SIL	RES
Repeatability	0.2864	0.5690
LOD	12.34	6.17
LOQ	37.4	18.72
Precision		
Intra-day	0.2986	0.5757
Inter-day	0.2743	0.5623
Robustness	0.4758	0.573
Ruggedness		
Analyst1	0.2743	0.5623
Analyst 2	0.2741	0.5622

SIL: Silybin; RES: Resveratrol; LOD: Limit of detection; LOQ: Limit of quantification; n: No. of experiments.

RESULTS AND DISCUSSIONS

The low % RSD values obtained for SIL (0.48) and RES (0.96) indicated that the method had high precision and reproducibility. The regression equation, slope, intercept, correlation coefficient, precision and linearity range are given in Table 1.

Table 1: Validation parameters for standard SIL and RES

Parameter	SIL	RES
Linearity range (µg/ml)	2-12	1-6
Correlation coefficient (r^2)	0.9998 a	0.9994 a
	0.9997 b	0.999 b
Intercept	0.0045 a	0.026 a
	0.0027 b	0.0334 b
Slope	0.0501 a	0.1006 a
	0.08 b	0.1009 b
Regression equation	y = 0.0501x - 0.0045 a	y = 0.1006x - 0.026 a
	y = 0.08x - 0.0027 b	y = 0.1009x - 0.0334 b
Precision (% RSD)*	0.48	0.96

SIL: Silybin; RES: Resveratrol; a: at 218 nm; b: at 288 nm; *Indicates mean of six determinations (n=6).

The % recovery for SIL and RES were found to be in the range of 99.1-100% (% RSD ± 0.48) and 99.38-100% (% RSD ± 0.96) respectively for both the formulations tested. The high recovery rate with low % RSD values indicated that the method had a good accuracy and specificity, as there was no interference from the excipients present in formulations. Intra-day precision and accuracy were evaluated by analyzing three samples of two different concentrations, prepared on same day. Inter-day variability was assessed by analyzing two concentrations on three different days, over a

period of one week. No significant difference was found in these experiments, indicating accuracy and reproducibility of the assays. The % RSD values reported in Table 4 shows that proposed method provides acceptable intra-day and inter-day variation of SIL and RES. Ruggedness of the proposed methods was determined by analyzing SIL and RES by different analysts, using similar operational and environmental conditions; the % RSD values are reported in Table 4 and found to be less than 2 %.

CONCLUSION

The proposed method was successfully applied to the simultaneous determination of SIL and RES from bulk and pharmaceutical (In-house) gel formulation. The presented method was found to be simple, accurate, precise and rugged. It can be directly and easily applied to the analysis of the combined pharmaceutical gel formulation of SIL and RES. Moreover, the present method is quick and cost effective as compared to chromatographic techniques. Therefore, it can be concluded that the proposed method provides an alternative procedure for the quality control of SIL and RES in pharmaceutical gel formulations.

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