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ORIGINAL ARTICLE

A Validated RP-HPLC Method for Determination of Rose Bengal in Bulk and Surgical Strips

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All the authors contributed significantly to the research that resulted in the submitted manuscript.

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ABSTRACT

A simple validated RP-HPLC method for determination of Rose Bengal (RB) Surgical strips which are used for staining of damaged cells of eye. The successful separation of RB was achieved on a Cromosil 100-5µm column (4.6 x 250mm with 5µm diameter) and detector of UV 262nm 1.0ml/min as a flow rate, and 20µl as an injector volume. The mobile phase of proposed method was methanol and phosphate Buffer of pH 8.0 in 50:50 (v/v) ratios. The linearity of method was determined in the range of 8-40ppm. The regression coefficient; LOD and LOQ were respectively 0.9987, 1ppm and 3ppm. It was validated according to the guidelines of ICH and USP.

Keywords: RP-HPLC, Rose Bengal, validation.

INTRODUCTION

Rose Bengal is a pink colored dye, used for staining. Its eye drops and strips are mainly used as diagnostic agent, when conjunctival and corneal cells are suspected to damage [1, 2]. Different brands of RB strips are available in local pharmacy for ophthalmic uses. RB is not only used for staining of damaged cells but also used for treatment of certain cancers [2-4]. Its injectable formulation named PV-10 is under investigation of clinical trials.

RB is a halofluorescein dye which forms colored ion-pair complexes with basic nitrogen, which are easily extracted and estimated 550nm, this mechanism of RB has been used to estimate different drugs [1, 5-8], in literature review a method was also reported of direct estimation of Rb by spectrophotometer from water samples [9] but no direct estimation method of RB by HPLC was reported in literature.

The chemical name of RB is 4, 5, 6, 7-Tetrachloro-2, 4, 5, 7-tetraiodofluorescen and structure is shown in Figure **1**.

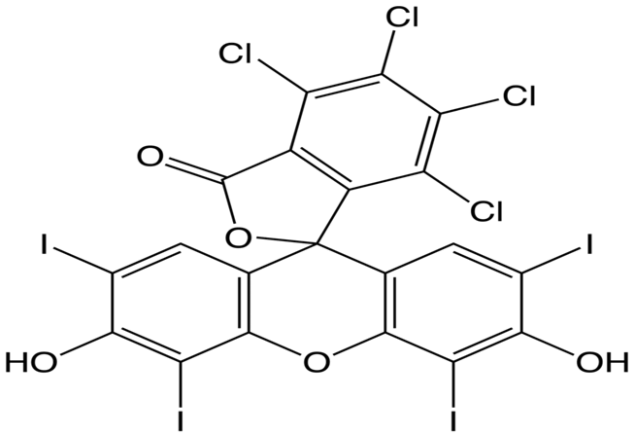


Figure 1. Structure of Rose Bengal.

MATERIALS AND METHODS

Apparatus

HPLC 10A series having UV-detector SPD-10Avp manual Rheodyne value with 20µl fixed loop, column C-18 Chromosil 250 x 4.6mm 100-5µm, Electronic balance Labrador (Shimadzu Japan) range 0-120g, Spectrophotometer UV-1601 (Shimadzu Japan) with 1cm quartz cell, and clarity lite Software for HPLC were used for development and validation of proposed method.

Materials and Reagents

Rose Bengal 95% dye content from Sigma Aldrich, potassium phosphate mono basic, potassium phosphate dibasic (99% assay) from J.K Enterprises and Methanol HPLC grade from Fischer scientific were purchased and used without further purification.

Reagent Preparations

Preparation of Mobile Phase:

50:50 (v/v) methanol, and phosphate buffer pH 8.0 were used as mobile phase after filtration from 0.45µm membrane filter.

Preparation of Buffer pH 8.0:

Accurately weighed potassium phosphate dibasic 70g was dissolved in 900ml of water, final pH adjusted with phosphoric acid/ NaOH to pH 8.0 and diluted with water to 1000ml.

Standard and Sample Preparations:

20mg of Rose Bengal was accurately weighed, transferred into 100ml volumetric flask, dissolved with buffer pH 8.0 and made the volume up to the mark. The final concentration of stock standard solution was 200ppm. The serial dilutions were prepared from the stock standard solution.

Test Sample Preparation:

Accurately cut the colored part of 4 strips and transferred into 25ml volumetric flask. The final concentration of stock sample was 208ppm.

RESULTS

Determination of λ Max

The standard solution having concentration of 24ppm was scanned in spectrophotometer in the range of 190-400nm against buffer pH 8.0 as blank; the obtained wavelength 262nm was selected for detection of RB in RP-HPLC system. The spectrogram is represented in Figure **2**. Similarly, the standard and test chromatograms of RP-HPLC are presented in Figure **3**.

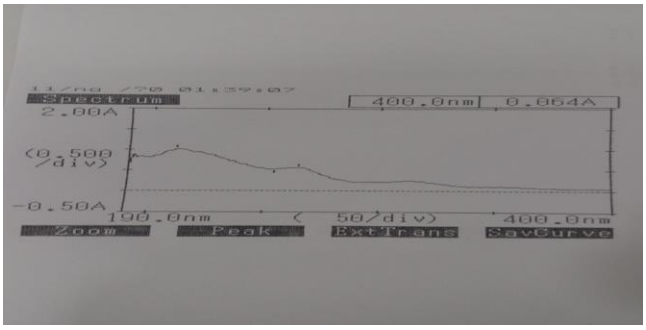
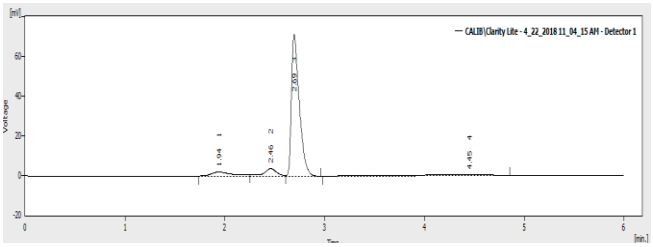


Figure 2. UV-Spectra (range 190 - 400nm).

A.



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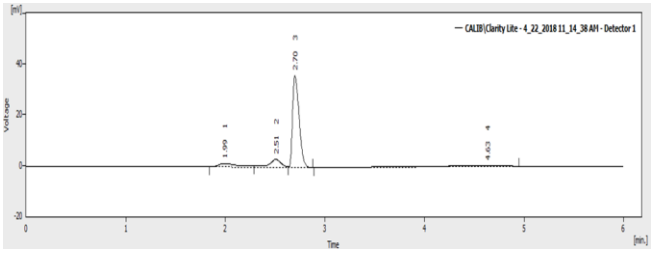

Figure 3. Standard chromatogram. **(A)** Peak of Rose Bengal (control). **(B)** Peak of Rose Bengal ophthalmic strips (sample).

DISCUSSION

Chromatographic Conditions and Equipment

The proposed method was developed and validated by using RP-HPLC 10A series, UV-detector (Shimadzu Japan) pump LC-10Avp in isocratic mode. Rheodyne injector manual of 20µl fixed loop with clarity lite software were used.

The composition of mobile phase was methanol and phosphate buffer 50:50 (v/v). The temperature of the column Chromosil 100-5µm C-18 (250mm x 4.6mm)

was ambient. The flow rate of mobile phase was 1ml/min and run time was 6 minutes. Detection of RB was performed at 262nm wavelength.

System Suitability

The system suitability tests, capacity factor, tailing factor and theoretical plates were applied to optimize the efficiency of chromatographic separation and quality. The system suitability criteria were set by keeping capacity factor ≤2.0, tailing factor ≤1.2% and theoretical plate's ≥ 2000.0. In all cases the %RSD was kept ≤ 2.0 for two consecutive injections. The parameters applied for system suitability are shown in Table **1**.

Table 1. System suitability parameter.

Conditions	Parameters
Mobile phase	Methanol and Buffer 50:50 (v/v)
Pump mode	Isocratic
Diluent	Buffer pH 8.0
Column	C-18 Chromosil 100-5µm (250 x 4.6 mm)
Column temperature	Ambient
Wavelength	262nm
Injection volume	20µl
Flow rate	1ml/min
Run time	10min
Retention time	2.69min
Area	177.23
T. plates	9670
Tailing factor	0.15%
Pump pressure	1430psi

METHOD VALIDATION

The proposed method was validated by monitoring accuracy, precision, robustness, linearity and ruggedness by using ICH and USP guidelines.

Range of Linearity

Linearity curves were constructed after taking n=5 replicate results of 8, 16, 24, 32, and 40ppm concentration levels (Figure **4**). The resultant area of all levels of concentration and other necessary parameters of method calibration are tabulated in Table **2**.

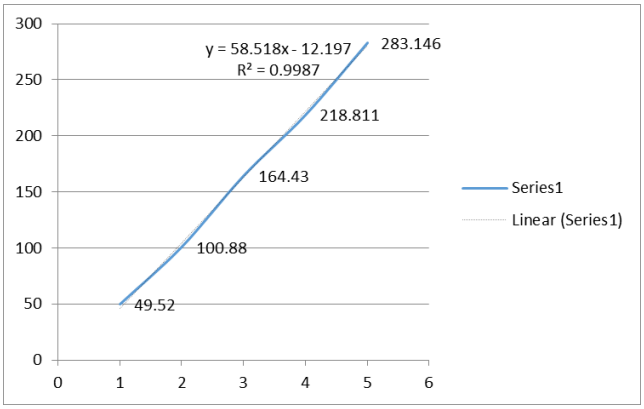

Figure 4. Linearity curve.

Limit of Detection and Limit of Quantification

The limit of detection was determined by continuous dilution of standard solution by buffer pH 8.0 and injected until the peak was disappeared. The peak was disappeared at concentration 1.0ppm. Hence the LOD and LOQ are respectively 1.0ppm and 3.0ppm. It is represented in Table **3**.

Table 2. Calibration parameters.

Concentration	8ppm	16ppm	24ppm	32ppm	40ppm
Peak area	49.52	99.822	179.89	218.87	283.146
Range	8 --- 40 ppm				
Regression equation	Y=106.7X + 41.59				
Correlation coefficient	0.9991				
Slop	6.7975				
R2	0.9990				

Table 3. LOD and LOQ.

S. No.	Parameters	Measured Values
1.	LOD	1.0ppm
2.	LOQ	3.0ppm

Precision

Six replicate samples of RB (24ppm) were prepared and analyzed by using the proposed method. The % RSD (Relative Standard Deviation) was calculated for peak responses and it was found 0.14% for intraday and 0.08% for interday precision complies the standard criteria NMT 2.0% RSD. The results are tabulated in Table **4**.

Robustness

To evaluate the robustness of the proposed method, some typical variations were made in chromatographic conditions, such as mobile phase, pH and variations in wave length. These variations did not show reasonable changes in capacity factor, tailing factor and theoretical plates. The variations made in proposed method were shown in Table **5**.

Ruggedness

To evaluate the rigidity of method, the temperature of standard solution was varied from 15, 25, to 40°C by keeping other chromatographic conditions the peak area variation % RSD was 0.17, an indication of the good rigidity of proposed method. The results are shown in Table **6**.

Recovery

Recovery of RB was estimated by applying the proposed method at 40%, 80% and 100% concentration levels. The obtained results are tabulated in Table **7**.

Table 4. Precision.

Precision Type	Concentration (ppm)	Injection No.	Peak Area	%RSD
Intraday Precision	24	1	179.88	0.14%
		2	179.46	
		3	180.16	
		4	179.79	
		5	179.86	
Interday Precision	24	1	179.96	0.08%
		2	179.84	
		3	180.24	
		4	180.01	
		5	179.98	

Table 5. Robustness.

S. No.	Parameters	Conditions	Peak Area of Std	Peak Area After Variation
1.	Methanol: Water 50:50 (v/v)	Methanol: Phosphate Buffer 40:60	179.97	180.21
		60:40		179.52
2.	Mobile phase pH	8.0	179.97	178.68
		6.4		179.146
3.	Wavelength	262nm	179.97	178.32
		260nm		177.98

Table 6. Ruggedness.

S. No.	Temperature Variation	Peak Area	%RSD
1	15°C	283.521	0.17
2	25°C	283.146	
3	40°C	282.574	

Table 7. Recovery.

S. No.	Concentration Level %	Peak Area	%Recovery
1.	40%	100.88	38.2
2.	80%	218.811	77.8
3.	100%	283.146	100.02

CONCLUSION

The validated proposed method for assay of RB in eye drops and strips is very simple accurate, precise and rapid, therefore it can be used for routine quality control testing.

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