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New RP-HPLC method development and validation for trioxsalen in tablet dosage form

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ABSTRACT

A new simple, accurate, rapid and precise Gradient High performance liquid chromatographic (HPLC) method was developed and validated for the determination of Trioxsalen C18 Column (4.6 x 150 mm and 3.5 µm) and flow rate of 1.2 ml/min with a load of 20µl. phosphate buffer (PH-3.9) was used as mobile phase A Methanol was used as Mobile Phase B and Diluent as Water: acetonitrile in the composition of 50:50. The Detection was carried out at 248nm.This newly developed method was successfully utilized for the Quantitative estimation of Trioxsalen in pharmaceutical dosage forms. This method was validated as per ICH guidelines.

Keywords: UV -Ultraviolet visible, µg -Micro gram, ml- Milliliter, nm- Nano meter, RP-HPLC-Reverse phase chromatography

INTRODUCTION

The goal of every chemical analysis is to obtain the required information within a period of time acceptable to the customer. This means that the analyst must know what information is needed to solve the current problem. Once the goals and limits of the analysis are defined and the literature search is completed, a more detailed plan of action must be developed. The analyst selects the method or combination of methods most likely to provide the desired information. A good analyst is always alert to the chemistry involved in the analysis as well as instrumental techniques. Thus, a combination of chemical and instrumental techniques may be used. Major components to be considered in planning an analysis are shown in fig each component is important in obtaining reliable information from the analysis. Field sampling and laboratory sub-sampling procedures must be designed to ensure integrity of results. Proper procedures must be used to store both sample and standard. All samples must be properly labeled and recorded. Laboratory operations are often performed on samples before measurement. Analytical chemistry is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts of chemistry.

Analytical method development

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.The number of drugs introduced into the market is increasing every year. These Drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopeias. This happens the possible uncertainty's in the continuous and wider usage of these

drugs, reports of new toxicities (Resulting in their withdrawal from the market).development of Patient resistance and introduction of better drugs by competitors, under these conditions, standards and analytical procedures for these drugs may not be available in the Pharmacopeia, it becomes necessary, Therefore to develop newer analytical methods for such drugs.

Analytical method validation

Analytical monitoring of a pharmaceutical productor of specific ingredients within the product is necessary to ensure its safety efficacy throughout all phases of its shelf life. Such monitoring is in accordance with the specification selaborated during product development. Analytical validation is the corner stone of process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new methods developed are validated.

Objective

The objective of validation of ananalytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH, typical analytical performance characteristics that should be considered in the validation of the types of methods are[7]:

- Accuracy
- Precision
- Specificity
- Detection limit
- Quantitation limit
- Linearity
- Range
- Ruggedness
- Robustness
- System suitability

MATERIAL AND METHODS

High Performance Liquid Chromatography (HPLC)

Reagents and Chemicals

- Trioxsalen working standard powder was gifted by Sankalp healthcare and Allied products Pvt.ltd. Mumbai and was used without purification.
- Trioxsalen tablets USP containing 25mg were purchased from local pharmacy (TROID-25, Manufactured by Resilient Cosmeceuticals Pvt.ltd)
- HPLC grade water, methanol, acetonitrile and monobasic potassium phosphate for the preparation of the buffer were purchased from Merck specialties pvt.ltd. (Mumbai, India)
- All solutions were filtered through 0.45 micron membrane filters purchased from Merck specialties Pvt.ltd, (Mumbai, India)
- All chemicals were of analytical grade unless stated otherwise and used as received. HPLC grade water was used to prepare all solutions.

Preparation of standard drug solution in methanol

Weighed accurately 10 mg of Trioxsalen RS and transferred into a 100ml standard flask. About 25ml of methanol was added and the solution was sonicated for 10 minutes, finally the volume was made up to 100ml with methanol. The resultant solution had a concentration of 100 mcg/ml (stock solution).

Optimization of HPLC conditions

Optimization was performed by injecting 20microliter standard drug solution (diluted to the UV linearity range) into the column, keeping a run time of 30 minutes.

Mobile phase selection

Selection of mobile phase was performed by trial and error method.
Different combinations of Acetonitrile, methanol and phosphate buffer were tested and the data's obtained are given below;

Table 1 : Solvent system selection trial and error data.

Methanol acetonitrile	90:10	-
Methanol : acetonitrile	80:20	-
Methanol :acetonitrile	70:30	-
Methanol acetonitrile	60:40	-
Methanol: acetonitrile	55:45	-
Water: acetonitrile	50:50	-
Phosphate buffer(5. 8): acetonitrile	50:50	Peak obtained but with least resolution, t_R more

pH of the buffer selected is a critical factor in sample retention as it keeps the sample substance in its fully ionized or neutral state.

- A rule of thumb is to choose a buffer with a Pk_a value ±2 unit of with that of the drug substance.
- The drug Trioxsalen has got a Pk_a value of 2.7 and considerably the pH of the mobile phase has been selected.

Preparation of buffer

- Phosphate buffer was used
- It was prepared as USP; A 5.04g concentration of the phosphate buffer was used. It was prepare by taking 0.84g of monobasic potassium phosphate in a 250ml standard flask and making up to the volume to get a phosphate solution. Further this solution was simply used as a part of organic solvent after adjusting the desired pH using orthophosphoric acid and/ NaOH.

Optimization of mobile phase

- Phosphate buffer (pH 5.4): Acetonitrile (ACN) system was optimized by changing the ratio of the solvents. Table shows the different ratio that has been tried.

Table 2 : optimization of mobile phase

Phosphate buffer pH (5.4):CAN 50:50
Phosphate buffer pH (5.4):CAN 60:40
Phosphate buffer pH (5.4):CAN 70:30
Phosphate buffer pH (5,4):CAN 80:20
Phosphate buffer pH (5,4):CAN 55:45

Mobile phase: Phosphate buffer pH (5.4): ACN (55:45) was chosen as themobile phase, which gives a chromatogram with good resolution for Trioxsalen. Preparation of Mobile phase: 500ml of the mobile phase was prepared instantly and utilized for the analytical study. It constituted 275ml of phosphate buffer (pH (5.4) and 225ml of ACN.

Optimization of other chromatographic parameters

- The t_R (Retention time) was found to be 6.18 min. and the pump pressure and the stop time was set as 400 Bar and 15 minutes respectively.
- Isocratic mode of elution was performed
- Column was used in an ambient temperature condition
- The detection wavelength was selected as 248nm, from the UV spectroscopic data of Trioxsalen.
- Peaks were analyzed at different flow rates (0.8-1.2ml/min.) and the optimum resolution with least retention time and peak tailing was obtained at a flow rate of 1.2ml/min.
- The tailing factor (<1.5) and theoretical plate count (>3000) was found to be optimum.

Preparation of calibration curve

Accurately pipette out 0.1, 0.2, 0.3, 0.4 and 0.5ml, respectively from the standard stock solution (100mcg/ml) into 5 and 10ml' volumetric flasks which were labeled appropriately and the volume was made up to 10 ml using HPLC grade methanol. Corresponding solutions had a concentration of 10, 20, 30, 40, and 50µg/ml respectively.

Development of chromatogram

1. Preparation of mobile phase

- Prepared by mixing 275ml of phosphate buffer with 225ml of acetonitrile.
- Then the mixture was filtered through 0.45 urn membrane filter under vacuum.
- The obtained mobile phase was further sonicated for 10 minutes for the removal of entrapped air.

2. Initial instrumental setup

- Fill the mobile phase in the mobile phase reservoir. Purging was carried out for the removal of any dissolved air bubbles. Then the mobile phase was allowed to run through the column at a flow rate of 1.2ml/min. Sample was injected only after the stabilization of the base line.

The detection wavelength was adjusted at 248nm.
Stop time was set as 15 minute.
Pump pressure was set as 400 bar

Table 3: Fixed instrumental parameters

Mobile phase flow rate	1.2ml/min.
Pump pressure	400 Bar
Stop time	15 min.
Detection wavelength	248 nm

3. Sample pretreatment

Each dilution of standard Trioxsalen solutions was passed through 0.22*mm* PVDF syringe filter for the removal of any minute particles.

4. Recording of chromatogram

First the mobile phase (Phosphate buffer: ACN (55:45) was filled in the mobile phase reservoir.

The mobile phase was purged to remove the entrapped air Then, the mobile phase was allowed to pass

through the column at a flow rate of 1.2ml/min. for a certain period of time until the base line becomes stable.

After the stabilization of base line, 20ml of each concentration of the standard analyte solutions were injected into the column and the chromatogram was recorded at 248nm for 15 min. The retention time and area under the curve were noted and given under the table, the HPLC chromatograms of standard dilutions as well as the calibration plot was also given below.

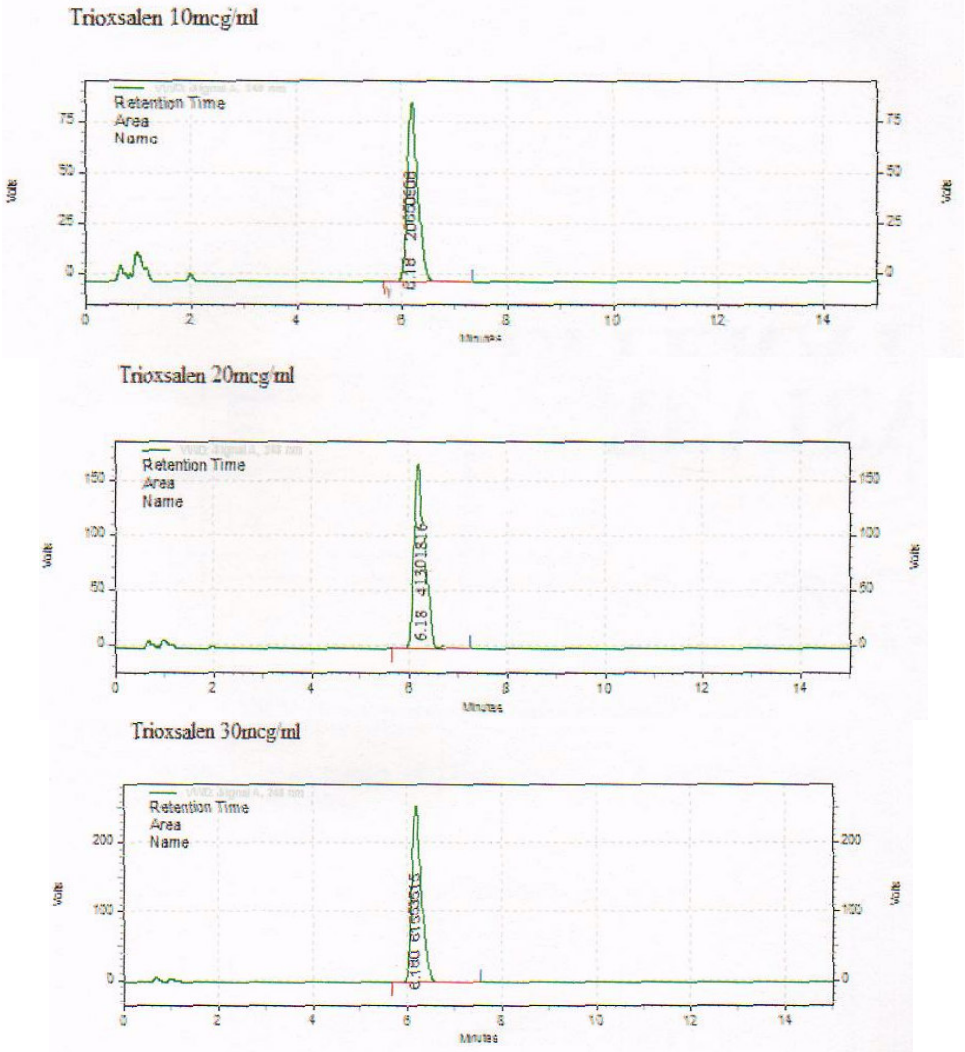

Fig 1: Chromatograms of Trioxsalen standard solutions

Table 4 : linearity data

S. No.	Concentration of Trioxsalen (mcg/ml)	Peak area	Retention time (min.)
1	10	2065090	6.18
2	20	4130181	6.18
3	30	6195272	6.18
4	40	8260363	6.18
5	50	10230925	6.17

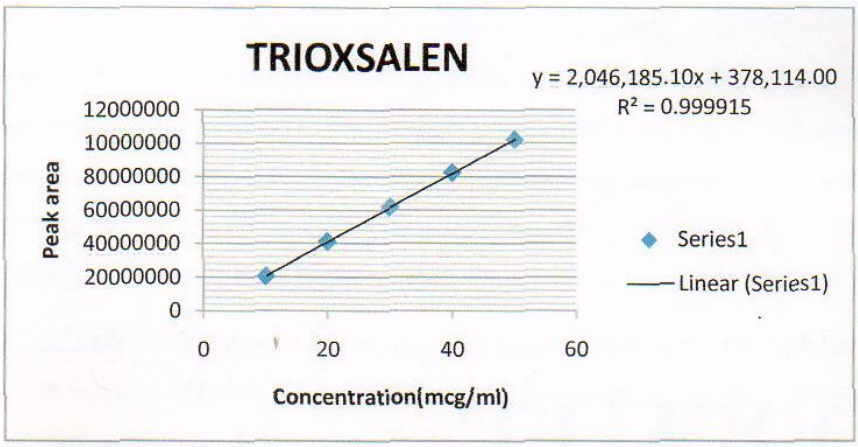

Fig 2: Calibration plot for Trioxsalen.

Statistical evaluation of the calibration plot

The calibration curve was plotted with peak area in the Y-axis and concentration (mcg/ml) in the X-axis. A linear plot was obtained within this concentration range. The correlation coefficient was obtained as 0.9999 and the regression equation was found to be Y=2,046,185.10x + 378,114.00.

5. Preparation of sample solution

Details of analyzed dosage Form:
Trioxsalen tablets USP in the brand name TRO1D-25
Label claim: Trioxsalen tablets USP
Batch No: STH1505
Mfg.Date:JAN.2016
Exp.Date:DEC.2018
Mfd. by: Resilient Cosmeceuticals PvLtd.

Accurately weighed 10 no's of TROID-25, Trioxsalen tablets and peeled off the outer film coating. Then, it was finely powdered and a weight equivalent to l0mg (0.1235) was taken. The weighed amount of drug was transferred into a stoppered flask, about 50rnl of methanol was added and sonicated for 10 minutes, and then the aggregate solution obtained was transferred into a 100ml standard flask by aiding filtration through whatmann filter paper (No.1). The stoppered flasks as well as the residue obtained were washed through the filter paper, using methanol and the volume was made up to 100ml with the same. The final solutions constituted a concentration of l00mcg/ml. Suitable dilutions for the drug analysis were prepared from the final 100 mcg/ml solution. After the preparation, sample solutions were allowed to run through the column as mentioned above (in preparation of calibration curve) to obtain the respective chromatograms.

Table 5: chromatographic data-sample solution

SI. No.	Conc, of Trioxsalen Sample solution (mcg/ml)	Peak area	Retention time (R)
1	**20**	**4101192**	**6.18**
2	**30**	**6118546**	**6.18**
3	**40**	**8230211**	**6.18**

Estimation of the amount of Trioxsalen in tablet dosage form

Quantitative estimation of drug was performed by comparing areas of the chromatograms obtained for the sample drug solution taken in three different concentrations with that of the standard.
Amount of drug present per table
Peak area for sample x . = Peak area of standard
dilution factor of sample dilution factor of standard
x weight of standard x average weight -, weight of sample
Each tablet contains {label claim) 25mg
Average weight of 10 tablets = 0.3089g
Weight of sample taken, equivalent to 10 mg = 0.1235g
Weight of standard taken = 0.0100g

Table 6: Assay results

Concentration	Peak area	Peak area	Amount	Label claim

(mcg/ml)	(sample)	(standard)	present per tablet (gm)	(%)
20	4101192	4130181	0.0248	99.2
30	6118546	6195272	0.0247	98.8
40	8230211	8260363	0.0249	99.6

Table 7: Assay results

Average content per tablet (in gm)	Average percentage label claim
0.0248	99.2%

6. Validation of the proposed method

a. Accuracy

Accuracy of the proposed method was determined by recovery study. The recovery studies were performed by standard addition method at three concentrations (80%, 100%, and 120%) and percentage recovery was calculated. 10 tablets of Troid-25 (containing 25mg of Trioxsalen) were taken, peeled off the outer film coat and weighed them accurately and finely powdered in a glass mortar, A weight equivalent to l0mg of Trioxsalen was weighed and transferred into a stoppered flask. To this, accurately weighed 8mg of Trioxsalen RS was added and extracted with 25ml of methanol initially by sonication for a period of 10 minutes.

The solution was transferred to 10ml standard flask, through a whatmann No: 1 filter paper. The residue was further extracted twice with 10ml each of methanol and passed through the same filter paper and the volume was finally made up with methanol (10mcg/ml).

About 1.5 ml of this solution was transferred into a 10ml standard flask and the volume was made up to 10ml using methanol (to obtain a concentration within the linearity range).20ul of this solution was injected into the column to obtain the corresponding chromatogram.

A standard solution of the same concentration (10mcg/ml) was prepared and chromatogram was developed in order to determine the peak area.

In a similar way recovery studies for 100% and 120% were conducted and peak heights and peak areas for each were measured in triplicates for each level. The results are statistically evaluated and the data obtained are given below.

Table 8 : Results of recovery study

Conc. (mcg/ml)	Peak area of sample spiked with standard	Peak area of standard	% recovery
27	5568925	5575745	99.87
	5546093	5546830	99.98
	5532617	5566523	99.39
30	6107470	6125272	99.70
	6109557	6117406	99.87
	6106469	6122348	99.74
33	6803681	6814799	99.84
	6804329	6814784	99.84
	6803582	6813911	99.85

Table 9: statistical results of recovery studies

Conc. (mcg/ml)	% Recovery	Standard deviation	% RSD
27	99.75	0.3137	0.31
30	99.77	0.0888	0.09
33	99.84	0.0057	0.01

b. Precision

Precision was determined in two levels: Repeatability and Intermediate precision.

Repeatability (System suitability study)

The repeatability of the method was studied using 100% test concentration of Trioxsalen prepared separately, and the peak area and retention time was determined six times at 248nm.The data is tabularized as given below:

Table 10: Results of repeatability study

Sl. No.	Conc. of Trioxsalenmcg/ml)	Peak area	Retention time
1	30	6107470	6.18
2	30	6104329	6.18
3	30	6107911	6.18
4	30	6108321	6.18
5	30	6107164	6.18
6	30	6107353	6.18

Table 11: Statistical results of repeatability study

Mean area	Standard deviation	% Relative standard deviation (%RSD)
61070918.17	14163.34	0.02

Intermediate/inter-day precision

The inter-day precision study of Trioxsalen was carried out by estimating the corresponding responses in triplicate for three days.

Table 11: Results of inter-day precision study

SI. No.	Cone, of Trioxsalen (mcg/ml)	Peak area		
		1st day	2nd day	3rd day
1	20	4130881	4108592	4099647
	20	4131727	4105236	4098747
	20	4132826	4106192	4099791
2	30	6112234	6125724	6108557
	30	6113256	6124681	6106693
	30	6114325	6126742	6107896
3	40	8252605	8304321	8356493
	40	8254978	8305409	8357346
	40	8253097	8306360	8358283

Table 12: Results of inter-day precision study

Concentration (mcg/ml)	Mean		
	1st day	2nd day	3rd day
20	41318117.66	41066736.66	40993952.00
30	61132718.66	61257161.00	61077158.66
40	82535601.66	83053635.33	83573747.66

Table 13: Results of inter-day precision study

Conc(mcg/ml)	Standard deviation			%RSD		
	1st day	2nd day	3rd day	1st day	2nd day	3ra day
20	9748.35	17291.09	5657.64	0.02	0.04	0.01
30	10458.9	9448.84	10305.75	0.02	0.02	0.02
40	12521.5	10206.06	8951.73	0.02	0.01	0.01

c) Linearity and range

The linearity study was conducted to evaluate the linear relationship across the range of analytical procedure. Linearity was determined by using five different concentrations of each drug. Chromatogram was developed and peak area by scanning at 248nm. Calibration graph (concentration v/s peak area) was plotted for the drug and from this, the linearity was determined.

Table 14: Results of linearity study

Method parameters	Trioxsalen
	Peak area wise
Linearity area	10-50 µg/ml
Slope	2,046,165.10
Intercept	378,115.00
Correlation coefficient (r)	0.9999

d) Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were estimated from the set of 5 calibration curves used to determine the linearity of the developed method. Five calibration curves were drawn for the drugs that come across within its linearity range. From each calibration curve y-intercept and slope were determined and are substituted in the corresponding equation for finding the LOD and LOQ.

LOD = 3.3a/s •/ LOQ=100/s

Where, a = S.D of Y intercepts of regression lines S — slope of the calibration curve

Table 15: data for LOD and LOQ determination.

Standard deviation of Y-intercept	Mean of slope
174382.19	2048422.61

Table 16. 1imit of detection and limit of quantitation

Limit of Detection (LOD)	0.27mcg/ml
Limit of Quantification (LOQ)	0.84mcg/ml

RESULTS AND DISCUSSION

This research work was done to develop simple, accurate, and economic methods for the estimation of Trioxsalen in commercial tablet dosage forms. By fulfilling this objective, four individual methods were developed. Proper selection of the method depends on the nature of the sample, molecular weight and solubility. The selected drug Trioxsalen for present study was polar. Polar compounds can be separated by Reverse phase chromatography and hence RP-HPLC method was selected for separation of the drug in tablet dosage form. C18 column was chosen as stationary phase and a mixture of buffer and acetonitrile was used as mobile phase. Then it was proceeded with different ratios for the achievement of suitable resolution. During selection and optimization of mobile phase, it was observed that the retention time of Trioxsalen is decreased with increase in the proportion of organic modifiers like acetonitrile in the mobile phase. The sharpness of the peak is achieved by increasing the proportion of acetonitrile whereas an increase in the proportion of aqueous phase resulted in broadening of the peak. The pH of the mobile phase was chosen to be at least two units away from the pKa of the drug substance. The drug has got a pKa of 2.7 and consequently the pH of the buffer for the composition of mobile phase was selected as 5.4. The selection of wavelength was based on the Xmax obtained by the scanning of standard drug solution within a range of 200-400nm in a UV spectrometer and it was taken as 248nm. Phosphate buffer (pH 5.4): Acetonitrile in the ratio 55:45, v/v, was chosen as the mobile phase. The flow rate was set as 1.2ml/min., pump pressure 400 Barr and the stop time 15 minutes. The established system gave good resolution and optimum retention time with appropriate tailing factor (<2) number of theoretical plates (>2000).

After setting up the method in the laboratory drug solutions were prepared and analyzed. The Rt was found to be 6.18.

Calibration curve for the drug was plotted by using the peak area in the ordinate and concentration in the abscissa. A linearity range of 10-50mcg/ml was obtained with a good correlation of 0.9999.

TROID-25, the marketed product was analyzed by the developed method and gave good results. Evaluating the peak area, percentage label claim of Trioxsalen was found to be 99.2% and the amount present per tablet, 0.0248gm.

- Accuracy was determined by recovery study employing the standard addition method at three levels (80%, 100%, 120%). The percentage recovery was found to be more than 99%,
- The precision of the method was studied by two methods; repeatability and intermediate precision. The percentage RSD was found to be<2.

- The LOD and LOQ of the method were obtained respectively as 0.28mcg/ml
- The developed HPLC method ensued good, accurate, reproducible and reliable analytical results and therefore can be extended for the estimation of Trioxsalen in its tablet dosage formulation.

CONCLUSION

More rapid, precise, specific, sensitive, economic, and reproducible, gradient reverse phase HPLC method was developed and validated for simultaneous determination of Trioxsalen tablets. The method was validated for specificity, linearity, and precision as per ICH guidelines.

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